**Supplementary Information**

**Tracking Iron-associated Proteome in Pathogens by a Fluorescent Approach**

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**Experimental Section**

1. Materials

**Synthesis of Fe-TRACER:** TRACER was synthesized based on our previous report\(^1\). The stock solution of TRACER (2 mM) was prepared in water whilst the FeCl\(_3\) (10 mM) was prepared by dissolving appropriate amounts of FeCl\(_3\) in aqueous solution containing 10 mM HCl to prevent hydrolysis. Equal molar amounts of TRACER and Fe\(^{3+}\) stock solutions were mixed and vortexed for 1.5 hrs at room temperature. Prior to use of Fe-TRACER, appropriate amounts of Tris-base were added to neutralize HCl. Formation of Fe-TRACER was confirmed by ESI-MS. Fe-TRACER of ESI-MS (m/z): [Fe-TRACER - 3H] - 2H\(_2\)O, calcd. 592.3, obsd. 591.1.

**Proteins:** Apo-hTF and lysozyme were subjected to centrifugation using Amicon Ultra-15 (Millipore) to remove low molecular mass impurity. Ubiquitin (with His\(_6\)-tag at the C terminus) was expressed in *E. coli* and purified by His Trap Ni-NTA column (GE Healthcare) and FPLC (GE Healthcare, using HiLoad™ 16/60 Superdex™ 75 prep grade column in 20 mM HEPES containing 300 mM NaCl, pH 7.4). Apo-hTF was mixed with 2 molar equivalents of ferrous ammonium sulphate (Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) and Fe(NTA)\(_2\)) in the presence of sodium bicarbonate to obtain the Fe\(_2\)-hTF\(^2\). After incubation on ice for 1 hrs, excess ferric ions were removed by centrifugation using Amicon Ultra-15.

2. Spectroscopic measurements and 1-D SDS-PAGE

**Fluorescence spectra** The binding affinity of TRACER to Fe\(^{3+}\) was determined by monitoring the decrease in fluorescence intensities at 450 nm with addition of 0 to 10 µM of FeCl\(_3\), followed by fitting the data with Ryan–Weber equation to give rise to the dissociation constant. The binding stoichiometry of TRACER to Fe\(^{3+}\) was measured by a Job’s plot. A series of 10 µM (total
concentration) of TRACER and FeCl₃ containing different amounts of TRACER and Fe³⁺ were prepared, incubated at room temperature for 1.5 hrs and fluorescence spectra were then recorded to construct a Job’s plot. The dissociation kinetics of Fe-TRACER was determined by monitoring fluorescence changes at 450 nm upon addition of 200 µM NTA into 5 µM Fe-TRACER, followed by fitting the data using a single exponential model.

Time dependent fluorescence spectra of Fe-TRACER (1 µM) in the presence of 8 µM proteins were recorded using kinetic mode, whilst the intensity at 450 nm was monitored at different time intervals. For the binding stoichiometry of Fe-TRACER to apo-hTF, 1 µM Fe-TRACER was mixed with 0–2 µM apo-hTF and the fluorescence spectra were recorded and the intensity at 450 nm changed with the addition of apo-hTF. For the fluorescence responses of Fe-TRACER or TRACER towards apo-hTF, 1 µM TRACER or Fe-TRACER was mixed with gradient amounts (0–8 µM) of apo-hTF or Fe₂⁻hTF, respectively. To investigate the selectivity of Fe-TRACER towards proteins, 1 µM Fe-TRACER was mixed with 8 µM of eitherapo-hTF, lysozyme or ubiquitin for 1.5 hrs. These samples were subsequently divided into two parts. One of them was subjected to UV-irradiation at 365 nm for 20 mins whilst the other was not treated prior to recording fluorescence spectra. The binding constant of Fe-TRACER to apo-hTF was determined by monitoring gradual decreases of the fluorescence intensities of Trp of apo-hTF (0.4 µM) upon addition of Fe-TRACER (0–3.2 µM), the data was then fitted similarly as in Ref 3. All protein containing samples were incubated with Fe-TRACER in ice-bath for 1.5 hrs unless otherwise specified.

**Measurement of Fe²⁺** The iron standard solution (1 mM Fe(NH₄)₂(SO₄)₂, 5 mM GSH) and 0.1% 1,10-phenanthroline solution were prepared with water. Fe-TRACER (50 µM) was incubated with GSH ranging from 0 to 5 mM whilst 5 mM GSH was added to a series of Fe²⁺ solutions (0–100 µM). The above samples were incubated with 0.02% 1,10-phenanthroline for 1.5 hrs at room temperature and Multiskan EX Microplate Reader (Thermo Scientific) was applied to measure the OD₄₅₀ of each sample. The percentage of Fe²⁺ in Fe-TRACER was determined similarly as in Ref 4.

**Imaging of proteins in 1-D SDS-PAGE** To compare the labelling efficiency of Fe-TRACER towards apo-hTF and Fe₂⁻hTF, proteins (10 µM) were pre-incubated with equimolar probe for 1.5 hrs in ice-bath. For the effect of GSH on protein labelling, apo-hTF (10 µM) was incubated with GSH ranging from 0 to 5 mM in ice-bath for 1.5 hrs following by addition of Fe-TRACER (50 µM) and further incubation for another 1.5 hrs in ice-bath. For the labeling efficiency of Fe-TRACER to apo-hTF, apo-hTF (10 µM) in 10 mM HEPES, 100 mM NaCl and 5 mM NaHCO₃ (pH 7.4) was incubated with 0, 0.2, 0.5, 1, 2, 5, 10, 12, 15 molar equivalents of Fe-TRACER. All samples were subjected to UV-irradiation at 365 nm using a UVP UVGL-25 Mineralight® UV lamp for 20 mins prior to SDS-PAGE. All SDS-PAGE analysis was performed using 4% stacking gel and 12% separation gel unless otherwise stated. All the fluorescence images were captured by MY ECL Imager (Thermo Scientific) and the gels were also stained by Coomassie blue for comparison. The intensities of protein bands on SDS-PAGE or fluorescence images were quantified and analyzed by the ImageJ software.

3. **Crystallization of Fe-TRACER-hTF**

The mono-ferric Fe₃⁻hTF protein was prepared from apo-hTF, buffered in 15 mM HEPES-KOH (pH 7.5) and 5 mM NaHCO₃, and concentrated to 1–1.3 mM. Sitting-drop method was used for crystallization. The precipitant contains 0.14 M PIPES-NaOH (pH 6.4), 8 mM disodium malonate, 16–18% PEG3350 and 18% glycerol. Pale-red protein crystals appeared after 2–7 days. The volumes for both protein and precipitant are 0.7 µL in each drop.
**Fe-TRACER** was prepared via the addition of equal molar of FeCl$_3$ (0.5 M) with 0.1 M HCl into TRACER solution, followed by addition of appropriate amounts of Tris-base, shake until dissolved. The final concentration of stock **Fe-TRACER** was 10 mM.

7 days after the crystals stopped growing, 9 µL of cryo-protectant containing 0.1 M PIPES-NaOH (pH 6.4), 8 mM disodium malonate, 27% PEG3350 and 31% glycerol were added into the crystallization drop and followed by 2 µL of **Fe-TRACER** solution. The entire crystallization plate was then wrapped in aluminum foil.

After soaking in cryo-protectant for 14 days, the crystals were exposed to UV light at 365 nm for 15 mins. Selected crystals were picked up and cryo-cooled in liquid nitrogen for X-ray diffraction experiment.

Diffraction data were collected at beam line BL17U1, Shanghai Synchrotron Radiation Facility using 0.93001Å radiation. HKL2000$^5$ was used for data reduction and scaling. CCP4 suite$^6$-$^8$ was used for molecular replacement (model PDB: 4X1B), model refinement and calculating the opening angle of N-lobe. Sketcher (CCP4 suite) was used for generating the CIF of ligand “NAC” (TRACER-NH$_2$).

4. Tracking iron-associated proteome in *P. gingivalis*

*P. gingivalis* cell culture *Porphyromonas gingivalis* strain ATCC 33277 was used for the study. *P. gingivalis* bacterial cells were maintained on the blood agar plate (40 g/L of tryptic soy agar (Difco), 5.0 g/L of yeast extract, 0.5 g/L of L-cysteine hydrochloride, 5.0 µg/mL of hemin and 1.0 µg/mL of vitamin K1). *P. gingivalis* was cultured according to the standard culture protocol$^9$ in supplemented tryptic soy broth (TSB), which contains 30 g/L of tryptic soy broth (Difco), 5.0 g/L of yeast extract, 0.5 g/L of L-cysteine hydrochloride, 5.0 µg/mL of hemin and 1.0 µg/mL of vitamin K1.

Confocal imaging of *P. gingivalis* Appropriate amount of *P. gingivalis* (OD600=0.6) cultured at the mid-log phase was washed with PBS for three times, re-suspended in PBS and the suspension was divided into two parts. One was incubated with 50 µM of **Fe-TRACER** for protein labeling and the other was treated as the control. Reagents of LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Scientific) were mixed and added into both parts of suspension at the same time and incubated for 15 mins at 37 °C in the dark anaerobically. The bacterial suspension treated with the probe was then subjected to confocal imaging.

2-D SDS-PAGE of *P. gingivalis* labelled by **Fe-TRACER** *P. gingivalis* was grown in 25 mL liquid medium to mid-log phase. Cells were collected and washed by PBS buffer for three times. Then the cells were resuspended in 1mL PBS and divided into two tubes equally. One tube of cells was incubated with 100 µM **Fe-TRACER** in darkness for 30 mins (sample A) while the other tube of cells was used as control (sample B). Cells were then centrifuged at 3,500 g for 5 mins at 4 °C and washed with PBS buffer (pH 7.4) for three times. Pellets of sample A were then resuspended in buffer and radiated by UV at 365 nm for 15 mins to activate the azide in **Fe-TRACER**. Both samples were resuspended in 1 mL of lysis buffer and proteins were recovered by centrifugation at 14,000 g for 30 mins at 4 °C. The total protein concentration was determined by BCA Protein Assay Kit (Thermo) and cell lysate was cleaned up by the ReadyPrep 2-D Cleanup Kit (BioRad). 150 µg total proteins were loaded to the nonlinear pH (3–10) gradient Immobiline Dry Strip (13 cm, GE Healthcare) and then isoelectrically focused (IEF). The strip was then applied onto the 13.5% PAGE gel for the
second-dimension separation.

**Fig. S1** ESI spectrum of Fe-TRACER. The peak at m/z 591.1 is assignable to [Fe-TRACER- 3H]·2H₂O (calcd. 592.3). The pattern of isotopic distribution is shown in the inset.

**Fig. S2** The excitation (λ<sub>ex</sub> = 350 nm) and emission (λ<sub>em</sub> = 450 nm) spectra of 1 µM Fe-TRACER.
**Fig. S3** Dissociation kinetics ($K_{\text{off}}$) of Fe$^{3+}$ to TRACER was determined by fitting the plot of time-dependent increase in fluorescence intensity at 450 nm owing to displacement of Fe$^{3+}$ from Fe-TRACER (5 μM) by excess amounts of NTA (200 μM).

**Fig. S4** The fluorescence responses of 1 μM Fe-TRACER at 450 nm towards 0–8 μM Fe$_2$-hTF. No significant fluorescence changes were observed after addition of different amounts of Fe$_2$-hTF.
**Fig. S5** The labelling of apo-hTF (10 μM) by Fe-*TRACER* (50 μM) in the presence of GSH (0–5 mM), which was monitored by Coomassie Blue and fluorescence staining.

**Fig. S6** The percentage of Fe$^{2+}$ in 50 μM Fe-*TRACER* in the presence of GSH (0–5 mM), which was determined by measuring the OD450 of Fe$^{2+}$-1,10-phenanthroline complex.
Fig. S7 (A) Cartoon representation showing the TRACER binding sites at the N-lobe and C-lobe respectively. (B) The TRACER molecule that bound to Arg602 at the surface of the C-lobe. The 2mFo-DFc mesh for TRACER is contoured at 1.0 σ.

Fig. S8 The confocal imaging for the control group of *P. gingivalis* bacterial cells. LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Scientific) was applied to stain live cells in green (by SYTO 9) and dead cells in red (by PI). The scale bar was set as 2 μm.
Fig. S9 The 2-D gel of *P. gingivalis* lysate. The color of fluorescence gel was inverted by ImageJ whilst the silver staining was captured for compassion. The protein spots appeared in both fluorescence image and silver stained gel, which are labelled here, were excised and identified by MALDI-TOF-MS.

Fig. S10 The MCL clustering analysis of the identified iron-associated proteome. The dash line indicates potential interactions between the proteins that are not in one cluster.
Table S1 Summary of crystallographic data for Fe-TRACER-hTF.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fe-TRACER-hTF</th>
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<tbody>
<tr>
<td>Data collection</td>
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<tr>
<td>X-ray wavelength (Å)</td>
<td>0.93001</td>
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<tr>
<td>Space group</td>
<td>C222₁</td>
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<tr>
<td>a, b, c (Å)</td>
<td>138.01, 155.75, 107.55</td>
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<tr>
<td>α, β, γ (°)</td>
<td>90.00, 90.00, 90.00</td>
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<tr>
<td>Resolution (Å)</td>
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<tr>
<td>R_{sym}</td>
<td>0.06 (0.73)</td>
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<td>I / σ</td>
<td>24.6 (2.5)</td>
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<tr>
<td>Completeness</td>
<td>98.2% (90.0%)</td>
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<tr>
<td>Redundancy</td>
<td>6.6 (6.6)</td>
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<tr>
<td>Refinement</td>
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<tr>
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<td>R_{work}, R_{free}</td>
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<td>RMS Angle (°)</td>
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<td>Ramachadran</td>
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<td>Preferred (%)</td>
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<td>Allowed (%)</td>
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<tr>
<td>Outlier (%)</td>
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Table S2 The highest peaks on the difference Fourier map and the anomalous map, when the final model (Fe removed) was used for peak-searching. The resolution cut-off was 2.9 Å for this search.

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<tr>
<th>Peak</th>
<th>mF₀-DFc (σ)</th>
<th>Anomalous (σ)</th>
<th>closest residue</th>
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<tr>
<td>1</td>
<td>20.46</td>
<td>16.11</td>
<td>Tyr426</td>
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<tr>
<td>2</td>
<td>14.20</td>
<td>16.72</td>
<td>Tyr188</td>
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**Table S3** List of coordination and hydrogen bonds that link TRACER, Fe$^{3+}$ and the N-lobe of hTF. (Bonds 7 & 8 connect the N-lobe via bond 9)

<table>
<thead>
<tr>
<th>Bond no.</th>
<th>Atom1</th>
<th>Atom2</th>
<th>bond length (Å)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Tyr188/Oη</td>
<td>Fe(N)</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>TRACER/O5</td>
<td>Fe(N)</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>TRACER/O13</td>
<td>Fe(N)</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>TRACER /N1</td>
<td>Fe(N)</td>
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**Coordination bonds**

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<th>Bond no.</th>
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<th>Atom2</th>
<th>bond length (Å)</th>
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<td>5</td>
<td>Asp63/Oδ2</td>
<td>TRACER /O26</td>
<td>3.2</td>
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<tr>
<td>6</td>
<td>Lys206/Nζ</td>
<td>TRACER /O4</td>
<td>2.6</td>
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<tr>
<td>7</td>
<td>H$_2$O/O</td>
<td>TRACER /O13</td>
<td>2.8</td>
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<tr>
<td>8</td>
<td>H$_2$O/O</td>
<td>TRACER /O29</td>
<td>3.1</td>
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<tr>
<td>9</td>
<td>H$_2$O/O</td>
<td>Tyr95/Oη</td>
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**Hydrogen bonds between probe and protein**

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<td>Aminomethyltransferase</td>
<td>10-2</td>
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<td>UDP-glucose 4-epimerase</td>
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<td>GroEL</td>
<td>26</td>
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<td>Phosphate acetyltransferase</td>
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<td>Superoxide dismutase [Mn/Fe]</td>
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<td>Enolase</td>
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<td>Class I fructose-bisphosphate aldolase</td>
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<td>NAD(P) transhydrogenase subunit alph</td>
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<td>Fimbrilin</td>
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<td>2</td>
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<td>Peptidoglycan domain protein</td>
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<td>OmpA family protein</td>
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<td>Mfa1 fimbrilin</td>
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Table S5 The analysis results of the identified iron-associated proteome in *P. gingivalis* by STRING. Those proteins that have been reported previously are highlighted in yellow.

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<th>Pathway ID</th>
<th>GO term</th>
<th>p-value</th>
<th>STRING identifier</th>
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<td>GO:0006096</td>
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References


