Supplementary Information

Tracking Iron-associated Proteome in Pathogens by a Fluorescent Approach

Nan Jiang,[a‡] Tianfan Cheng,[b‡] Minji Wang,[a‡] Godfrey Chi-Fung Chan,[c] Lijian Jin,[b] Hongyan Li,[a] Hongzhe Sun*[a]

[a] Department of Chemistry, the University of Hong Kong, Pokfulam Road, Hong Kong
[b] Faculty of Dentistry, the University of Hong Kong, 34 Hospital Road, Sai Ying Pun, Hong Kong
[c] Department of Paediatrics and Adolescent Medicine, Li Ka Shing Faculty of Medicine, the University of Hong Kong, 21 Sassoon Road, Hong Kong
‡ These authors contributed equally to this work.
* Correspondence to: hsun@hku.hk

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]$^{-}\cdot 2H_2O$, 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 $FeCl_3$ containing different amounts of *TRACER* and Fe^{3+} 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 Fe_2-hTF, respectively. To investigate the selectivity of **Fe-*TRACER*** towards proteins, 1 μM **Fe-*TRACER*** was mixed with 8 μM of either apo-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 Fe^{2+} The iron standard solution (1 mM $Fe(NH_4)_2(SO_4)_2$, 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 Fe^{2+} 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 Fe^{2+} 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 Fe_2-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 $NaHCO_3$ (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 Fe_C-hTF protein was prepared from apo-hTF, buffered in 15 mM HEPES-KOH (pH 7.5) and 5 mM $NaHCO_3$, 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.

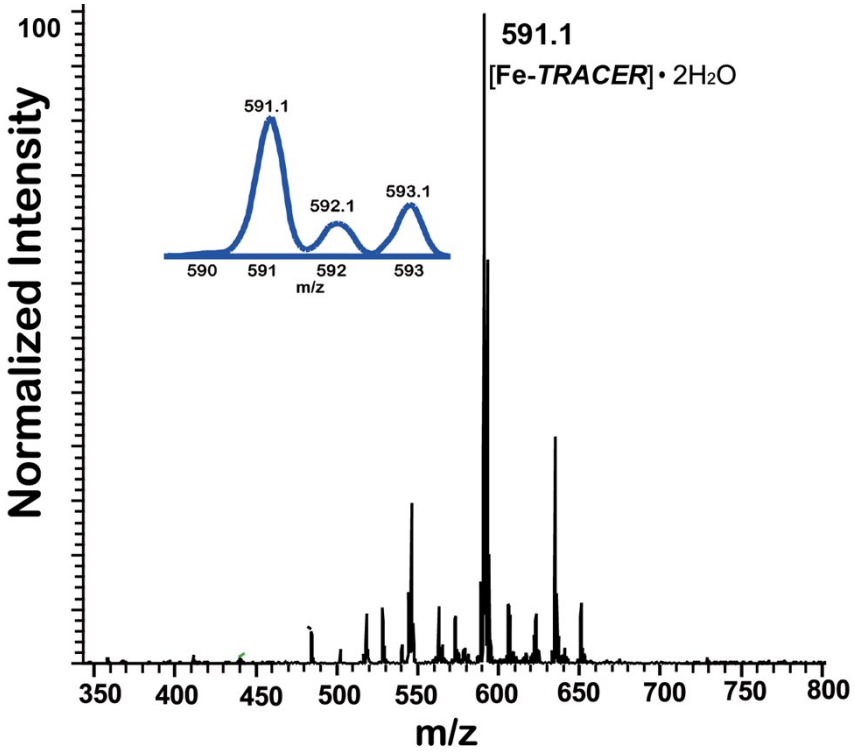

Fig. S1 ESI spectrum of **Fe-*TRACER***. The peak at m/z 591.1 is assignable to [**Fe-*TRACER***- 3H]$^{-}$ ·$2H_2O$ (calcd. 592.3). The pattern of isotopic distribution is shown in the inset.

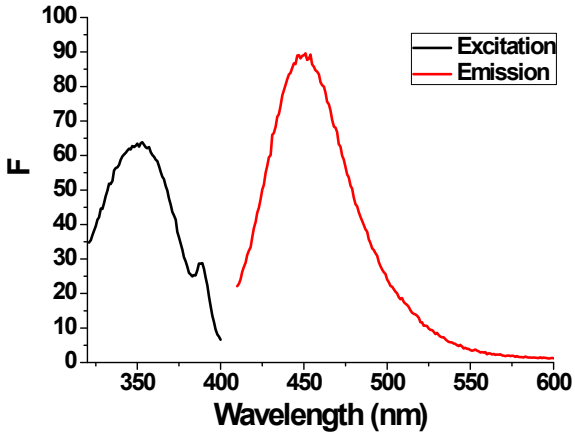

Fig. S2 The excitation (λ_{ex}= 350 nm) and emission (λ_{em}= 450 nm) spectra of 1 μM **Fe-*TRACER***.

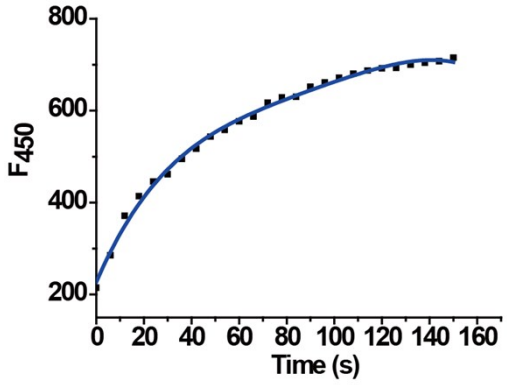

Fig. S3 Dissociation kinetics (K_{off}) of Fe^{3+} to *TRACER* was determined by fitting the plot of time-dependent increase in fluoresce intensity at 450 nm owing to displacement of Fe^{3+} from **Fe-*TRACER*** (5 μM) by excess amounts of NTA (200 μM).

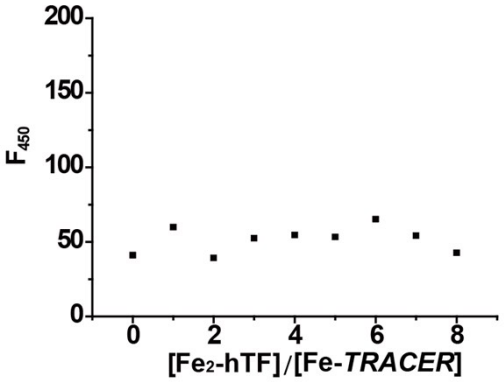

Fig. S4 The fluorescence responses of 1 μM **Fe-*TRACER*** at 450 nm towards 0–8 μM Fe_2-hTF. No significant fluorescence changes were observed after addition of different amounts of Fe_2-hTF.

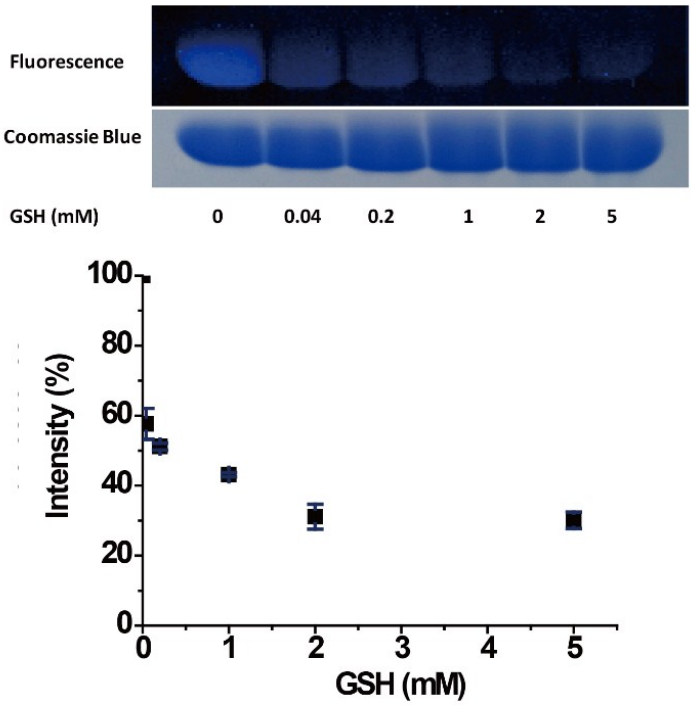

Fig. S5 The labelling of apo-hTF (10 μM) by **Fe-*TRACER*** (50 μM) in the presence of GSH (0–5 mM), which was monitored by Coomassie Blue and fluorescence staining.

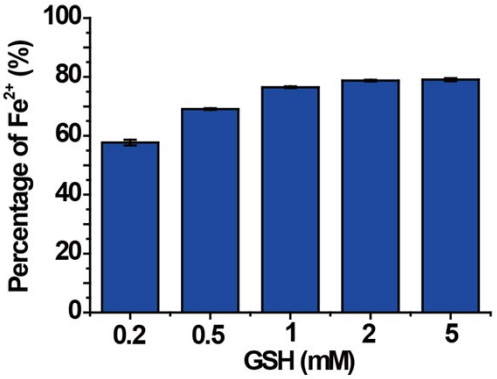

Fig. S6 The percentage of Fe^{2+} in 50 μM **Fe-*TRACER*** in the presence of GSH (0–5 mM), which was determined by measuring the OD450 of Fe^{2+}-1,10-phenanthroline complex.

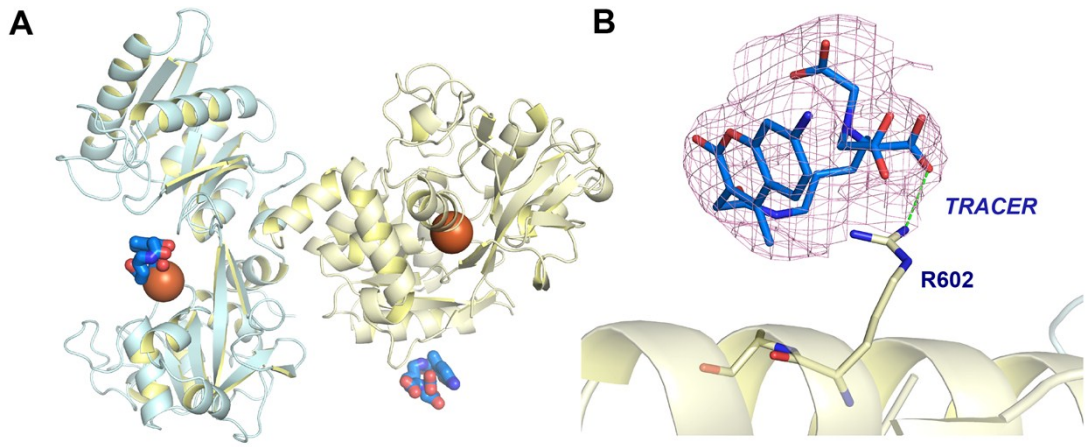

Fig. S7 (A) Cartoon representation showing the *TRACER* binding sites at the N-lobe and C-lobe respectively. (B) The *TRACER* molecule that bound to Arg602 at the surface of the C-lobe. The 2m*F*o-D*F*c mesh for *TRACER* is contoured at 1.0 σ.

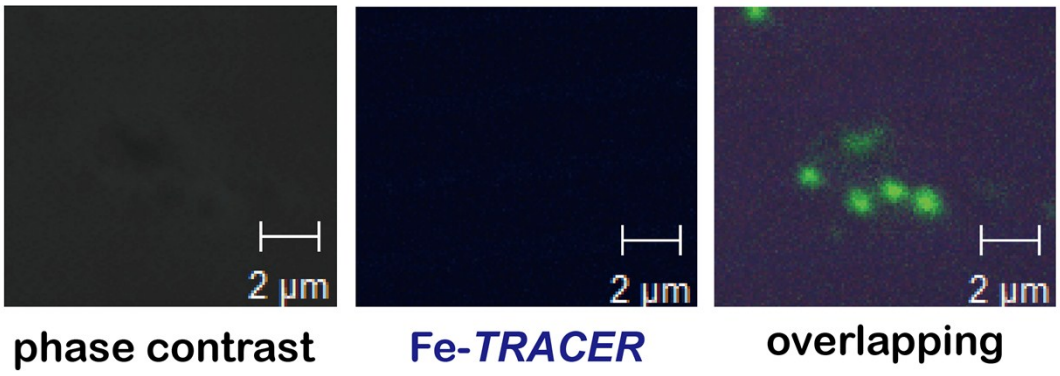

Fig. S8 The confocal imaging for the control group of *P. gingivalis* bacterial cells. LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Scientific) was applied to stain live cells in green (by SYTO 9) and dead cells in red (by PI). The scale bar was set as 2 μm.

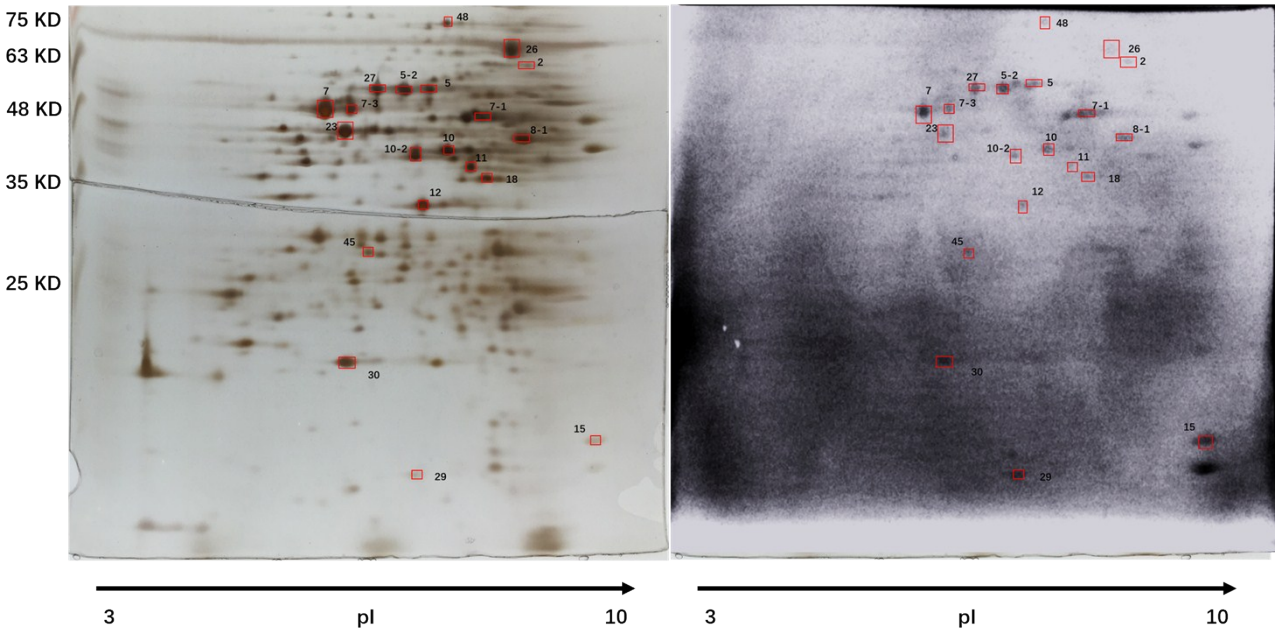

Fig. S9 The 2-D gel of *P. gingivalis* lysate. The color of fluorescence gel was inverted by ImageJ whilst the silver staining was captured for compassion. The protein spots appeared in both fluorescence image and silver stained gel, which are labelled here, were excised and identified by MALDI-TOF-MS.

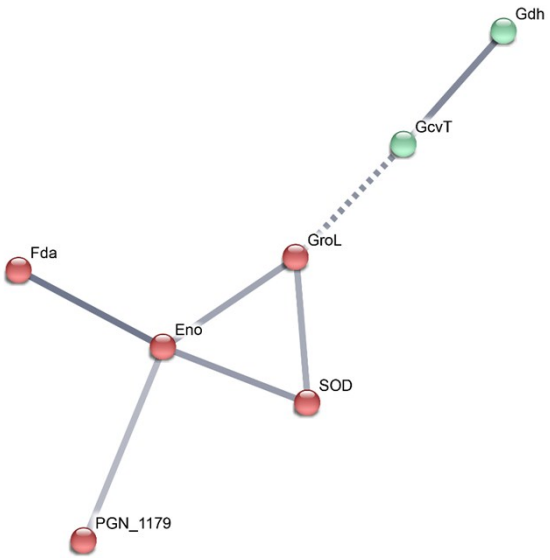

Fig. S10 The MCL clustering analysis of the identified iron-associated proteome. The dash line indicates potential interactions between the proteins that are not in one cluster.

Table S1 Summary of crystallographic data for **Fe-*TRACER*-**hTF.

Protein	**Fe-*TRACER*-**hTF
Data collection	
X-ray wavelength (Å)	0.93001
Space group	$C222_1$
a, b, c (Å)	138.01, 155.75, 107.55
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	100.00–2.86 (2.90–2.86)
R_{sym}	0.06 (0.73)
I / σ	24.6 (2.5)
Completeness	98.2% (90.0%)
Redundancy	6.6 (6.6)
Refinement	
Resolution(Å)	100.00–2.86
Reflections	25317
R_{work}, R_{free}	0.1545, 0.2289
No. of atoms	5075
Average B factor (Å^2)	65.7
RMS bond (Å)	0.01
RMS Angle (°)	1.36
Ramachadran	
Preferred (%)	94.7
Allowed (%)	4.8
Outlier (%)	0.5

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.

Peak	m*F*o-D*F*c (σ)	Anomalous (σ)	closest residue
1	20.46	16.11	Tyr426
2	14.20	16.72	Tyr188

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)

Bond no.	Atom1	Atom2	bond length (Å)
	Coordination bond		
1	Tyr188/Oη	Fe(N)	2.1
2	*TRACER*/O5	Fe(N)	2.2
3	*TRACER*/O13	Fe(N)	2.2
4	*TRACER* /N1	Fe(N)	2.0
	Hydrogen bonds between probe and protein		
5	Asp63/Oδ2	*TRACER* /O26	3.2
6	Lys206/Nζ	*TRACER* /O4	2.6
7	H_2O/O	*TRACER* /O13	2.8
8	H_2O/O	*TRACER* /O29	3.1
9	H_2O/O	Tyr95/Oη	3.1

Table S4 Proteins identified by **Fe-*TRACER*** in *P. gingivalis.*

Protein name	**Spot number**	**STRING identifier**	**Reference**
Aminomethyltransferase	10-2	PGN_0550, gcvT	**10**
Glutamate dehydrogenase	7, 7-3	PGN_1367, gdh	**11**
UDP-glucose 4-epimerase	18	PGN_1370	
GroEL	26	PGN_1452, groL	**12**
Phosphate acetyltransferase	11	PGN_1179	
Superoxide dismutase [Mn/Fe]	30	PGN_0564, sod	**13**
Enolase	7-1	PGN_1743, eno	**14**
Class I fructose-bisphosphate aldolase	12	PGN_1695, fda	
NAD(P) transhydrogenase subunit alph	23	PGN_1120	
Fimbrilin	10	PGN_0180, fimA	**14**
Gingipain R1	15	PGN_1970, rgpA	**15**
Lys-gingipain OS	5, 5-2,27	PGN_1728, kgp	**15**
Receptor antigen A	2	PGN_0293, ragA	**15**
Peptidoglycan domain protein	29	PGN_1670	
OmpA family protein	45	PGN_0728	**16**
Hypothetical protein	8-1	PGN_0876	
Mfa1 fimbrilin	48	PGN_0287, mfa1	**14**

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.

Pathway ID	GO term	p-value	STRING identifier
GO:0009405	pathogenesis	0.0054	PGN_0180, fimA; PGN_1970, rgpA PGN_1728, kgp
GO:0008150	biological_process	0.0147	PGN_0550, gcvT; PGN_1367, gdh PGN_1452, groL; PGN_1743, eno PGN_1695, fda; PGN_0180, fimA; PGN_1970, rgpA; PGN_1728, kgp
GO:0044712	single-organism catabolic process	0.0147	PGN_0550, gcvT; PGN_1743, eno PGN_1695, fda
GO:0006096	glycolytic process	0.0166	PGN_1743, eno; PGN_1695, fda
GO:0019752	carboxylic acid metabolic process	0.0166	PGN_0550, gcvT; PGN_1367, gdh PGN_1743, eno; PGN_1695, fda
GO:0044238	primary metabolic process	0.0166	PGN_0550, gcvT; PGN_1367, gdh PGN_1452, groL; PGN_1743, eno PGN_1695, fda; PGN_1970, rgpA PGN_1728, kgp
GO:0071704	organic substance metabolic process	0.0166	PGN_0550, gcvT; PGN_1367, gdh PGN_1452, groL; PGN_1743, eno PGN_1695, fda; PGN_1970, rgpA PGN_1728, kgp
GO:1901575	organic substance catabolic process	0.0166	PGN_0550, gcvT; PGN_1743, eno PGN_1695, fda
GO:0006508	proteolysis	0.034	PGN_1970, rgpA; PGN_1728, kgp
GO:0003674	molecular_function	0.0257	PGN_0550, gcvT; PGN_1367, gdh PGN_1452, groL; PGN_1743, eno PGN_1695, fda; PGN_0180, fimA; PGN_1970, rgpA; PGN_1728, kgp
GO:0004197	cysteine-type endopeptidase activity	0.0342	PGN_1970, rgpA; PGN_1728, kgp
GO:0003824	catalytic activity	0.0362	PGN_0550, gcvT; PGN_1367, gdh PGN_1743, eno; PGN_1695, fda PGN_1970, rgpA; PGN_1728, kgp
GO:0009986	cell surface	0.00299	PGN_1367, gdh; PGN_1743, eno
GO:0005576	extracellular region	0.00446	PGN_1743, eno; PGN_1728, kgp
00680	Methane metabolism	0.00986	PGN_1179; PGN_1743, eno PGN_1695, fda
01200	Carbon metabolism	0.0278	PGN_0550, gcvT; PGN_1179 PGN_1743, eno; PGN_1695, fda

References

1 Y. T. Lai, Y. Y. Chang, L. G. Hu, Y. Yang, A. L. Chao, Z. Y. Du, J. A. Tanner, M. L. Chye, C. M. Qian, K. M. Ng, H. Li and H. Sun, Rapid labeling of intracellular His-tagged proteins in living cells, *Proc. Natl. Acad. Sci. USA*, 2015, **112**, 2948.

2 H. Sun, M. C. Cox, H. Li, A. B. Mason, R. C. Woodworth and P. J. Sadler, [^{1}H,^{13}C] NMR determination of the order of lobe loading of human transferrin with iron: comparison with other metal ions, *FEBS Lett*., 1998, **422**, 315.

3 D. E. Epps, T. J. Raub, V. Caiolfa, A. Chiari and M. Zamai, Determination of the affinity of drugs toward serum albumin by measurement of the quenching of the intrinsic tryptophan fluorescence of the protein, *J. Pharm. Pharmacol.*, 1999, **51**, 41.

4 A. E. Harvey Jr, J. A. Smart and E. Amis, Simultaneous spectrophotometric determination of iron(II) and total iron with 1,10-phenanthroline, *Anal. Chem*., 1955, **27**, 26.

5 Z. Otwinowski and W. Minor, Processing of X-ray diffraction data collected in oscillation mode, *Method. Enzymol.*, 1997, **276**, 307.

6 M. D. Winn, C. C. Ballard, K. D. Cowtan, E. J. Dodson, P. Emsley, P. R. Evans, R. M. Keegan, E. B. Krissinel, A. G. Leslie and A. McCoy, Overview of the CCP4 suite and current developments, *Acta Cryst. Sect. D*, 2011, **67**, 235.

7 G. N. Murshudov, A. A. Vagin and E. J. Dodson, Refinement of macromolecular structures by the maximum-likelihood method, *Acta Cryst. Sect. D*, 1997, **53**, 240.

8 A. J. McCoy, R. W. Grosse-Kunstleve, P. D. Adams, M. D. Winn, L. C. Storoni and R. J. Read, Phaser crystallographic software, *J. Appl. Cryst.*, 2007, **40**, 658.

9 M. Bélanger, P. Rodrigues and A. Progulske-Fox, Genetic manipulation of *Porphyromonas gingivalis, Curr. Protoc. Microbiol.*, 2007, **5**, 13C.2.1.

10 T.-C. Chao, J. Buhrmester, N. Hansmeier, A. Pühler and S. Weidner, Role of the regulatory gene *rirA* in the transcriptional response of *Sinorhizobium meliloti* to iron limitation, *Appl. Environ. Microb.*, 2005, **71**, 5969.

11 V. Taneja, K. Mishra and K. Agarwal, Effect of maternal iron deficiency on GABA shunt pathway of developing rat brain, *Indian J. Exp. Biol.*, 1990, **28**, 466.

12 M. A. González-López, N. Velázquez-Guadarrama, M. E. Romero-Espejel and J. de Jesús Olivares-Trejo, *Helicobacter pylori* secretes the chaperonin GroEL (HSP60), which binds iron, *FEBS Lett.*, 2013, **587**, 1823.

13 A.-F. Miller, Superoxide dismutases: ancient enzymes and new insights, *FEBS Lett.*, 2012, **586**, 585.

14 K. Maeda, H. Nagata, M. Ojima and A. Amano, Proteomic and transcriptional analysis of interaction between oral microbiota *Porphyromonas gingivalis* and *Streptococcus oralis*, *J. Proteome Res.*, 2014, **14**, 82.

15 T. Olczak, W. Simpson, X. Liu and C. A. Genco, Iron and heme utilization in *Porphyromonas gingivalis, FEMS Microbiol. Rev.*, 2005, **29**, 119.

16 K. L. Naylor, M. Widziolek, S. Hunt, M. Conolly, M. Hicks, P. Stafford, J. Potempa, C. Murdoch, C. Douglas and G. P. Stafford, Role of OmpA2 surface regions of *Porphyromonas gingivalis* in host–pathogen interactions with oral epithelial cells, *MicrobiologyOpen*, 2017, **6**, e00401.