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Supporting Information

A novel Genetically Encoded Fluorescent Protein for Cu(I) indicator

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Expression and purification of PMtb-FRET.The copper(I)-binding domain of Mycobacterium tuberculosis (MTB) CDC 1551(residues 1-162) was subcloned between CFP and YFP using SphI and SacI in the plasmid system (Figure S1). The PMtb-FRET plasmid was transformed into BL21star(DE3). A 10 mL overnight pre-culture grown from a single colony was inoculated into 1 L autoclaved LB medium containing 50 mg ampicilin. The cells were grown at 37^0C, 250 rpm to OD_{600} = 0.6 and then the temperature was reduced to 16 ºC. Protein expression was induced with 0.5 mM IPTG 30 min later, 1.6 mM CuSO4 was added to the growth medium and cells were grown overnight. Cells were harvested at 4^0C by centrifugation at 6,000 rpm for 10 min. All subsequent steps were performed at 4^0C. The pellet was suspended in 20 mL buffer A, (10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 4 mM DTT) and 10 mM PMSF. The cells were lysed by sonication and centrifuged at 12,000 rpm for 25 min. The supernatant was filtered through a 0.45 μm filter and applied to a Ni-NTA column. The column was washed with 5% buffer B (10 mM Tris-HCl [pH 7.4], 500 mM imidazole, 300 mM NaCl,4 mM DTT) and eluted with a linear gradient

from 5% to 100% buffer B over 40 ml. Peak fractions were pooled and kept at 4 ºC. The purity of PMtb-FRET was verified by SDS-PAGE gel (Figure S2).

To obtain PMtb-FRET without copper(I), 10 mM NaCN was added to the purified protein and the protein was run through a desalting column using Buffer A containing 8 mM DTT.

Fluorescent measurements. All fluorescence measurements were carried out at room temperature on a Varian Cary Eclipse Fluorescence Spectrophotometer. The samples were excited at 433 nm, the excitation and emission slit widths were set to 5 nm and the emission spectrum was scanned from 453 nm to 650 nm at 120 nm / min. The titration and selectivity measurements were performed in a buffer containing 10 mM Tris-HCl (pH 7.4), 300 mM NaCl, and 6 mM DTT. Tetrakis(acetonitrile) copper(I) hexafluorophosphate dissolved in acetonitrile at 100μM concentration was used as the copper(I) source in all measurements. For the other metals 100 μM stock solutions were prepared from the following salts with purity >99 %: $ZnCl_2$, $MgCl_2$, $CaCl_2$, $Cr(NO_3)_3 \cdot 9H_2O$, $MnCl_2$, $Fe(NH_4)_2SO_4$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $CdCl_2$, $Hg(NO_3)_2$ and $Pb(NO_3)_2$, $AuCl_3$, $La(NO_3)_3 \cdot 6H_2O$, $Eu(NO_3)_3 \cdot 6H_2O$. For the titration curves of PMtb-FRET, 0μM to 6μM of each metal was added to a solution of 1μM PMtb-FRET protein and the intensity ratio at the peaks was taken as a measure of FRET change ($R = I_{527} / I_{477}$) (Figure 4). The % FRET change in response to the other metals was calculated by setting the PMtb-FRET proteins ratio at 0% change and the fully copper-loaded PMtb-FRET protein at 100% change in the FRET using the following formula.

$$\% \text{ FRET Change} = (R - R_{min}) / (R_{max} - R_{min}) \times 100$$

where R is the intensity ratio for a given sample, Rmin the intensity ratio for PMtb-FRET and Rmax the intensity ratio for PMtb-FRET –Cu(I). For the selectivity measurement of PMtb-FRET, 5μM of each metal was added to 1μM PMtb-FRET, and the response to the metal was measured. 5μM Cu(I) was added to the same solution and the spectrum was recorded again (Figure 5).

$H^+ + CN^- \leftrightarrow HCN$	logKa = 9.04
$Cu^+ + 2\ CN^- \leftrightarrow Cu(CN)^{2-}$	$\log\beta_2$ Cu = 21.7
$Cu^+ + 3\ CN^- \leftrightarrow Cu(CN)_3^{-2}$	$\log\beta_3$ Cu = 26.8
$Cu^+ + 4\ CN^- \leftrightarrow Cu(CN)_4^{3-}$	$\log\beta_4$ Cu = 27.9
$Zn^{2+} + 2\ CN^- \leftrightarrow Zn(CN)_2$	$\log\beta_2$ Zn = 11.07
$Zn^{2+} + 3\ CN^- \leftrightarrow Zn(CN)^{3-}$	$\log\beta_3$ Zn = 16.05
$Zn^{2+} + 4\ CN^- \leftrightarrow Zn(CN)_4^{2-}$	$\log\beta_4$ Zn = 19.62.

For the Kd measurement of PMtb-FRET with Cu(I), Cu(I)/CN^- buffers were used to obtain free Cu(I) concentrations ranging from 9.73×10^{-25} M to 6.79×10^{-16} M using Cu(I) binding constant to cyanide and the proton association constant (Ka) for CN^- obtained from the NIST Critical Stability Constants of Metal Complexes the equilibrium constants given above. Free copper concentrations were calculated using the program HySS 2006 for total Cu(I) concentrations in the buffers from 1.0×10^{-7} M to 3.0×10^{-4} M and CN^- concentrations from 1.5×10^{-3} M to 6.0×10^{-2} M. PMtb-FRET concentration used in this measurement is 3.0μM in 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl and the copper(I) loading was measured for each copper(I) buffer prepared (Figure 2). The % FRET response which is also equal to the copper occupancy of PMtb-FRET versus the free copper(I) concentration in the buffer was plotted and the binding curve was fitted for the Hill equation using Origin 8.

For the Kd measurement of PMTB-FRET with Zn^{2+}, similar Zn(II)/CN- buffers were used in the Zn(II) complex formation constants with cyanide given above. Free Zn(II) concentrations ranging from 3.21 × 10-11 M to 4.71 × 10-5 M were prepared by using total Zn(II) concentrations ranging from1.00 × 10-3 M to 8.0 × 10-2 M and CN- concentrations ranging from 0.1 M to 0.03 M. PMTB-FRET used for these measurement was at 3.5μM in a buffer containing 100 mM Tris-HCl (pH 7.4), 100 mM NaCl and 100μM Tris-HCl (Figure 3).

Purification of PMTB-FRET for cytotoxicity study with PMTB-FRET protein with HEK293T cells and HepG-2 cells. Millipore Amicon was used for change PMTB-FRET protein buffer from the desalting buffer of the protein to PBS. Endotoxin of the protein was removed using a PMB-agarose column (Sigma) according to the method suggested by the manufacturer.

Cell cultures

HEK293Tcells and HepG-2 cells were maintained in DMEM (Sigma–Aldrich), containing 10% Fetal Bovine Serum (FBS) at 37℃ in a humidified 6% CO2 environment.

MTT assay

Cells were preincubated in 96-well plates at a density of 5000 cells/well. After the cells adhered the wall , PMTB-FRET was added in each well to a final concentration of 0μM 0.2μM, 1μM and 5μM. Medium was renew every 24h, and at the same time, PMTB-FRET was feeded into the wells to maintain the concentrations. Three times were replicated for each concentration using medium as a blank. The cell viability was assessed by the MTT assay. Briefly, MTT was diluted in PBS to a final concentration of 5 mg/ml, and sterile filtered through a 0.22 μm sterile filter. At a defined time point , the culture media were removed and replaced with 100 μl fresh medium, then 10 μl of the MTT solution was added to each well. The cells were further incubated at 37 ℃ in a humidified 6% CO_2 environment for additional 4h. To stop the MTT reaction, 100 μl of 10% SDS in 0.01M HCl was directly added into the medium containing MTT and the plate was incubate overnight in the dark The optical density of formazan was detected at 570 nm with 630 nm as reference wavelength.

Statistical analysis

Values are expressed as standard deviation (SD). Comparison between two groups were performed by two-tailed Student's t-test. A value of $P<0.05$ was accepted as different , while value of $P<0.01$ was accepted as significantly different.

Table S1 ICP-MS results for the PMtb-FRET

Sample	Cu Concentration (μ g/g)	Cu Concentration (μ M)	Protein Concentration (μ M)	Cu: PMtb-FRET
PMtb-FRET	0.11 ± 0.03	1.73 ± 0.43	12.0	0.14
PMtb-FRET-Cu	2.84 ± 0.021	44.68± 3.36	12.0	3.72
Cu Reference	2.12 ± 0.024	33.36 ± 3.16	- -	
5% HNO_3 blank	0.00 ± 0.03	0.00 ± 0.038	- -	

MRVIRMTNYEAGTLLTCSHEGCGCRVRIEVPCHCAGAGDAYRCTCGDELAPVK

Figure S1. Sequence of MTB ,which was inserted between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP).

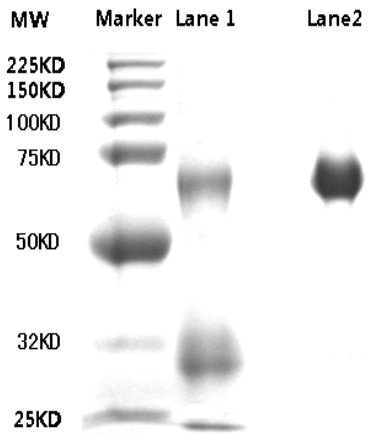

Figure S2. SDS-protein gel of the purified PMTB-FRET-Cu(I). The most concentrated Fractions (lane 2) were used in experiments.

(1) Zhang, J.; Ma, Y.; Taylor, S. S.; Tsien, R. Y. Proc Natl Acad Sci U S A 2001, 98,14997-15002.

(2) Martell, A. E.; Smith, R. M. Standard Reference Database 46 2001.

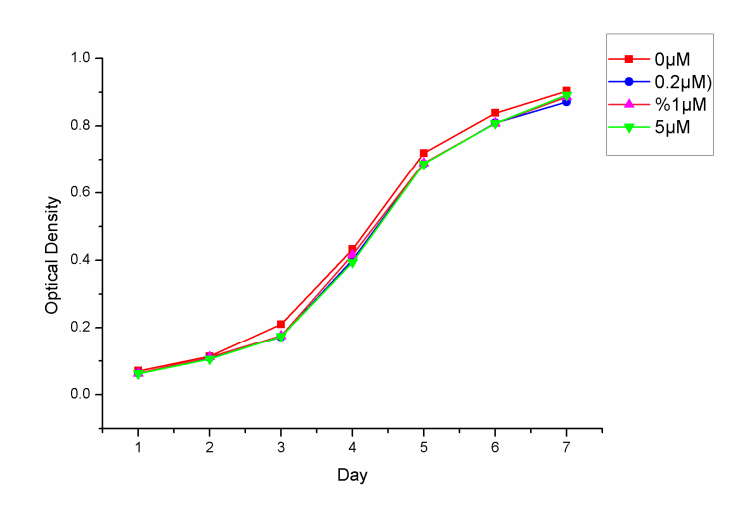

Figure S3. HEK293T cells were respectively treated with various concentrations (0μM, 0.2μM, 1μM, 5μM)of PMTB-FRET for 7 days. Cell lysates were prepared and subjected to MTT analysis as described in MTT assay, P>0.05. There were no growth differences between the experimental group cells and the control group cells. The result showed that PMTB-FRET protein was no cytotoxicity for HEK293T cells in vitro in the range of the experiment concentration .

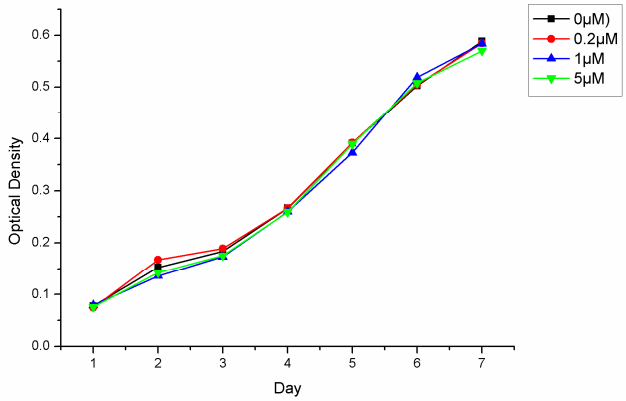

Figure S4. HepG-2 cells were respectively treated with various concentrations (0μM, 0.2μM, 1μM, 5μM)of PMTB-FRET for 7 days. Cell lysates were prepared and subjected to MTT analysis as described in MTT assay, P>0.05. There were no growth differences between the experimental group cells and the control group cells. The result showed that PMTB-FRET protein was no cytotoxicity for HepG-2 cells in vitro in the range of the experiment concentration .