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Electronic Supporting Information

for

Revealing the elevation of Zn^{2+} in the brain of depressive mice by a ratiometric fluorescence probe with dual near-infrared emissions

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Contents

1. Experimental Section

1.1 Chemicals

1,1,2-trimethylbenz[e]indole, 1,3-propanesultone, 2,6-bis(chloromethyl)pyridine, potassium phthalimide, bis(2-picolyl)amine, and N,N-diisopropylethylamine were received from Sigma-Aldrich. Chloral hydrate, N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and 2-chloro-3-(hydroxymethylene)cyclohex-1-enecarbaldehyde were bought from Macklin Biochemical Co. Ltd. Hydrazine hydrate, sodium acetate trihydrate, $CuCl_2$, NaCl, KCl, $CaCl_2$, $MgCl_2$, $ZnSO_4$, $NiCl_2$, $CdCl_2$, $CoCl_2$,$FeCl_3$, $AlCl_3$, $Pb(NO_3)_2$, $BaCl_2$, $CrCl_3$, $FeCl_2$, and K_2CO_3 were supplied by Sinopharm Chemical Reagent Co. Ltd. MitoTracker Green was purchased from AAT Bioquest. Annexin V-FITC/PI apoptosis detection kit was obtained from Beyotime Biotechnology. High glucose Dulbecco's modified Eagle's media (DMEM) were received from KeyGEN Biotech. Co. Ltd. All the mentioned reagents were of analytical grade and used as received.

1.2 Characterizations

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker 500 MHz spectrometer (Bruker, Germany). MALDI-TOF mass spectroscopy was performed with an AutoFlex TOF/TOF mass spectrometer (Bruker, Germany). UV-Vis absorption spectra were acquired on a UH5300 spectrophotometer (Hitachi, Japan). Fluorescence spectra were measured with a F-4700 fluorescence spectrophotometer (Hitachi, Japan). Flow cytometry experiments were conducted on a BD Accuri C6 Plus Flow Cytometer (BD Biosciences, USA). Confocal fluorescence

and bright field images (512 × 512 pixels) were captured with a TCS-SP8 confocal laser scanning microscope (Leica, Germany). Experimental cells were imaged using a 63× objective lens.

1.3 Synthesis of Zn^{2+} probe (Mito-Zn)

The identity and purity of the product was confirmed by nuclear magnetic resonance (NMR) and mass spectroscopy analysis.

Mito-Zn was synthesized according to the route depicted in Scheme 1A.

Compound **1**. To a round bottomed flask was added 2,6-bis(chloromethyl)pyridine (1.75 g, 10 mmol), potassium phthalimide (1.85 g, 10 mmol), and acetonitrile (50 mL). The resulting mixture was stirred at room temperature overnight. After drying under vacuum, the residue was collected, purified on a silica gel column using petroleum ether/ethyl acetate (1:1) as eluent to afford compound **1** as white powder (2.33 g, yield: 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.59 (s, 2H), 5.01 (s, 2H), 7.17 (d, 1H), 7.37 (d, 1H), 7.65 (t, 1H), 7.74 (m, 2H), 7.89 (dd, 2H).

Compound **2**. To a 100 mL round-bottomed flask was charged with compound **1** (2.36 g, 8 mmol), bis(2-picolyl)amine (2.02 g, 10 mmol), K_2CO_3 (2.21 g, 16 mmol) and acetonitrile (40 mL). The mixture was refluxed under rapid stirring for 12 h. After cooling down to room temperature naturally, acetonitrile was removed on a rotary evaporator and the solid residue was further purified on a silica gel column with dichloromethane/methanol (10:1) eluent to give white compound **2** (2.16 g, yield: 60%). ^{1}H NMR (400 MHz, CD_3OD): δ = 4.32 (s, 2H), 4.48 (s, 4H), 5.36 (s, 2H), 7.68 (d, 1H), 7.86 (m, 5H), 8.01 (ddd, 2H), 8.17 (d, 2H), 8.21 (t, 1H), 8.56 (td, 2H), 8.89

(dd, 2H).

Compound **3**. At room temperature, to a solution of compound **2** (2.24 g, 4.8 mmol) in ethanol (30 mL) was added hydrazine hydrate (1.0 mL) dropwise. After being heated at 60 °C for 0.5 h, the solution was concentrated to 10 mL under reduced pressure, filtrated to collect the filtrate. The solvent was then removed, and the obtained residue was purified by silica gel column chromatography (dichloromethane/methanol/ammonium hydroxide, 10/1/0.1) to give compound **3** as light-yellow oil (1.27 g, yield: 80%). ^{1}H NMR (400 MHz, CD_3OD): δ = 4.31 (s, 2H), 4.35 (s, 2H), 4.53 (s, 4H), 7.37 (dd, 2H), 7.70 (ddd, 2H), 7.79 (m, 3H), 8.20 (td, 2H), 8.76 (m, 2H).

Compound **4**. To a 50 mL round-bottomed flask was introduced 1,1,2-trimethyl-1H-benzo[e]indole (2.14 g, 10 mmol), 1,3-propanesultone (1.25 g, 10 mmol), and acetonitrile (20 mL). The mixture was refluxed under stirring for 40 h. Afterward, the reaction mixture was cooled down to ambient temperature, followed by the addition of anhydrous diethyl ether (500 mL), which obtained compound **4** without purification (3.33 g, yield: 99%). ^{1}H NMR [400 MHz, $(CD_3)_2SO$]: δ = 1.76 (s, 6H), 2.25 (m, 2H), 2.69 (t, 2H), 2.94 (s, 3H), 4.79 (t, 2H), 7.74 (t, 1H), 7.80 (t, 1H), 8.25 (t, 2H), 8.30 (d, 1H), 8.37 (d, 1H).

Compound **5**. To a 100 mL round-bottomed flask was added compound **4** (3.18 g, 10 mmol), 2-chloro-3-(hydroxymethylene)cyclohex-1-enecarbaldehyde (0.97 g, 5 mmol), and ethanol (50 mL). The reaction solution was refluxed for 12 h, followed by concentrating its volume to 20 mL. Then, the concentrated solution was poured into

ethyl acetate (500 mL) to afford compound **5** with a blue color (2.45 g, 60 %). ^{1}H NMR [400 MHz, $(CD_3)_2SO$]: δ = 1.89 (m, 2H), 1.96 (s, 12H), 2.13 (m, 4H), 2.66 (t, 4H), 2.81 (t, 4H), 4.53 (t, 4H), 6.58 (d, 2H), 7.53 (t, 2H), 7.68 (t, 2H), 7.88 (d, 2H), 8.11 (q, 4H), 8.31 (d, 2H), 8.40 (d, 2H).

Mito-Zn. Under nitrogen atmosphere, a mixture of compound **3** (0.42 g, 1.3 mmol), compound **5** (0.81 g, 1.0 mmol), and N,N-diisopropylethylamine (0.13 g, 1.0 mmol) was dissolved in DMF (10 mL) and stirred at room temperature for 24 h in the dark. Then, the generated mixture was transferred into ethyl acetate (100 mL), and blue solid residue was achieved, which was further purified by HPLC system using a gradient program (mobile phase: methanol to water, from 10% to 80%, with 0.1% TFA) at a flow rate of 2.5 mL/min to afford desired product **Mito-Zn** as blue-green powder (0.17 g, yield: 16%). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.66 (s, 12H), 1.83 (s, 2H), 2.26 (s, 4H), 2.62 (s, 4H), 2.98 (s, 4H), 4.23 (s, 2H), 4.32 (br, 4H), 4.47 (s, 4H), 5.12 (s, 2H), 5.97 (d, 2H), 7.35 (t, 3H), 7.48 (m, 6H), 7.64 (t, 2H), 7.77 (d, 2H), 7.89 (m, 9H), 8.16 (t, 2H), 8.71 (d, 2H). ^{13}C NMR (400 MHz, CD_3OD): δ = 21.82, 23.38, 25.64, 27.67, 48.62, 57.24, 111.38, 122.00, 123.59, 123.98, 124.80, 125.48, 127.75, 128.33, 130.35, 130.83, 139.10, 140.95, 146.91, 153.68, 157.80. High-resolution mass (m/z): calcd. for $C_{63}H_{68}N_7O_6S_2^+$ $[M]^+$: 1082.4667, found: 1082.4673.

1.4 Computational procedures

Density functional theory (DFT) calculations were carried out with Gaussian 16 program. The absorption spectra of **Mito-Zn** and its complex with Zn^{2+} were calculated using the B3LYP geometries and 6-31G (d, p)/LanL2DZ (for complex of

Mito-Zn with Zn^{2+}) basis set, utilizing the SMD model of water, during the DFT calculations.

1.5 Cell cytotoxicity assay

HeLa cells were seeded at a density of 1×10^5 cells per well in 96 multi-well plates and cultivated in DMEM supplemented with 10% fetal bovine serum, 80 $\mu g \cdot mL^{-1}$ streptomycin, and 80 $U \cdot mL^{-1}$ penicillin in an atmosphere of 5% CO_2 and 95% air at 37 °C for 12 h. Then, the culture medium was replaced by fresh DMEM with **Mito-Zn** of different amounts (0, 5, 10, 20, 30, 40, 50 and 60 μM). Five replicate experiments were performed for each concentration. After 48 h culture, 40 μL of MTT solution (0.5 mg mL^{-1}) was added to each cell for 4 h to allow the formation of formazan crystals. Finally, 150 μL of DMSO was injected into the wells. Absorption intensity (A) of the resulting mixture was measured. Cell viability rates were then estimated by the following equation: cell viability (%) = $A_{test}/A_{control} \times 100\%$, where $A_{control}$ stands for the absorbance values of the untreated cells, i.e. the control group, and A_{test} stands for the absorbance obtained from the experimental groups, i.e., in the presence of **Mito-Zn**.

1.6 Cell Apoptosis Assay

The apoptosis assay was conducted with an Annexin V-FITC Apoptosis Detection Kit based on the manufacturer's protocol. Typically, HeLa cells were incubated with the probe **Mito-Zn** at dosages of 0, 20, 40, and 60 μM for 48 h. Following the treatment, floating cells, in the culture medium, and adhered cells, detached using EDTA-free trypsin, were combined, and collected by centrifugation (1000 g, 5 min). The cell

pellets were dispersed again in 195 μL of Annexin V-FITC binding buffer, then stained with 5 μL of Annexin V-FITC, and 10 μL of a propidium idiode solution (PI) for 20 min in the dark. The cells were then immediately analyzed on a flow cytometer.

1.7 Fluorescence imaging of Zn^{2+} in living cells

The subcellular distribution of **Mito-Zn** was investigated by colocalization imaging experiments, in which **Mito-Zn** (5.0 μM) labeled cells (labeling time: 0.5 h) were further stained with MitoTracker green (100 nM) for 0.5 h. After the staining experiment, cells were washed thrice with DMEM gently and subjected to confocal fluorescence imaging. MitoTracker green was excited with a 488 nm laser and its emission signal was collected in the wavelength range of 500-570 nm, while for **Mito-Zn**, the excitation wavelength was set at 552 nm and dual-color fluorescence images were simultaneously collected in the wavelength range of 610-690 nm and 760-840 nm. For bioimaging of mitochondrial Zn^{2+}, HeLa cells were pretreated with various concentrations of Zn^{2+} (0, 1.2, 2.4, 3.6, and 4.8 μM) for 0.5 h, and further labeled with the probe (5.0 μM) for 0.5 h. As for imaging endogenous Zn^{2+}, cells were stimulated by H_2O_2 (50, 100, 150 μM) for 6 h with or without further treatment with TPEN (50 μM) for 0.5 h, and then labeled with **Mito-Zn** (5.0 μM) for 0.5 h.

1.8 Fluorescence imaging of Zn^{2+} in brain tissues and living mice

All experiments involving mice were carried out in accordance with the principles and guidelines approved by the Animal Care and Use Committee of Jiaxing University. Six-week-old adult male C57BL/6J mice (body weight: 20 ± 2 g) were received

from the Laboratory Animal Center of the Chinese Academy of Science. Upon arrival, all mice were housed in propylene cages under a 12:12 light/dark cycle and had free access to tap water and commercial mouse food. The mice were allowed to adapt to local environmental conditions for 1 week and then randomly divided into 3 groups, i.e., one control group and two depression groups. The depression models were established according to previous reported procedure,[S1] in which the mice were feed with water containing corticosterone, while for the control group, pure water was provided. 21 days later, the depression model was successfully established.

After then, three groups of the mice were anesthetized using chloral hydrate (4%) and the brains were carefully dissected out from the skull. The obtained brains were fixed in an iron chamber filled with cold artificial cerebrospinal fluid (aCSF) and cut into slices with a thickness of 300 μm, with a VT300 vibrating-blade microtome (Leica). The aCSF was composed of 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 3 mM KCl, 124 mM NaCl, 2.4 mM $CaCl_2$, and 10 mM glucose. Then, the slices were cultured in aCSF containing **Mito-Zn** (5.0 μM) with or without TPEN (50 μM) for 0.5 h. After washing thrice with aCSF, the slices were subjected to confocal fluorescence imaging under an excitation of 552 nm.

1.9 In vivo imaging Zn^{2+} in living mice

Female Kunming mice were purchased from the Experimental Center of Weifang Medical College and kept in the SPF Animal Room of Pharmacology of Weifang Medical College. The mice were fluorescently imaged after subcutaneous injection of the probe (20 μL, 500 μM) and different concentrations of zinc ions (0 μM, 20 μM, 50 μM) with a PerkinElmer IVIS Spectrum in vivo fluorescence imaging system, and fluorescence from the two channels was collected separately. The excitation

wavelength was set at 522 nm and the emission wavelengths were 653 nm and 807 nm.

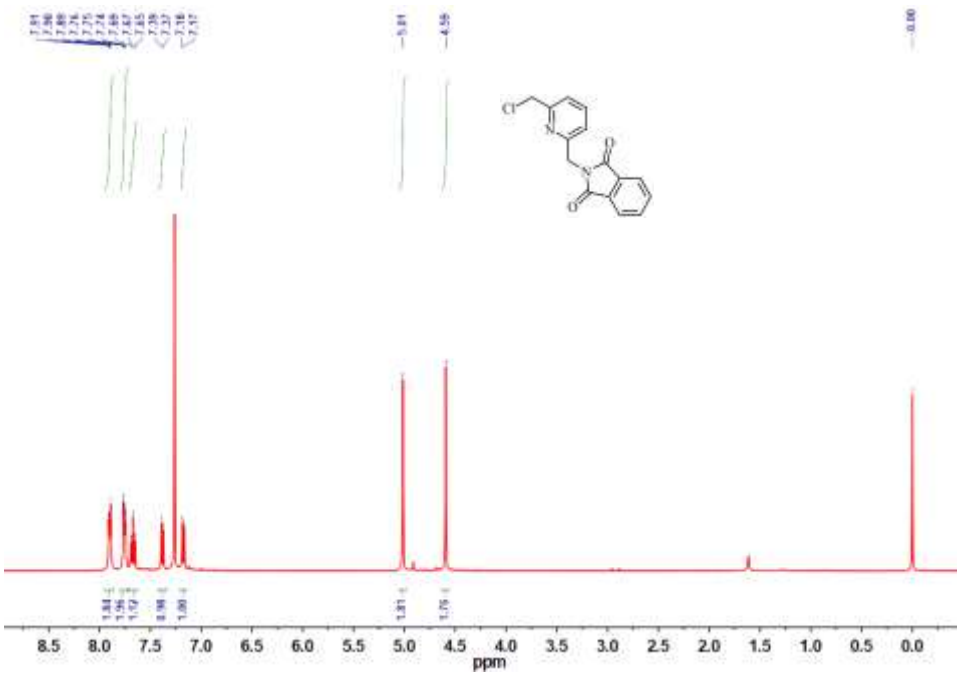


Fig. S1 ^{1}H NMR spectrum of compound **1** in $CDCl_3$.

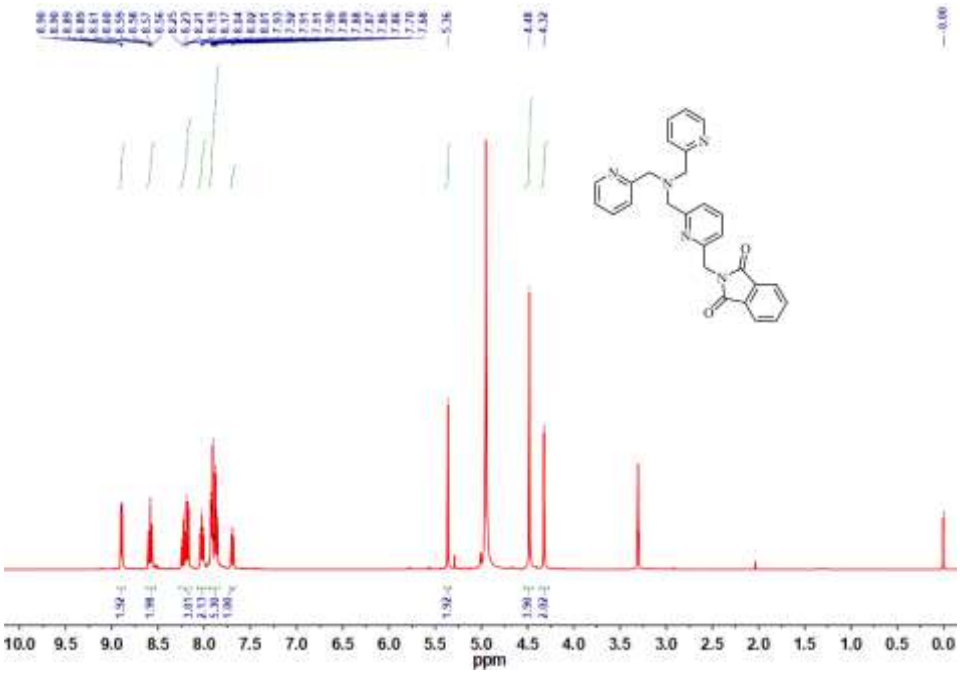


Fig. S2 ^{1}H NMR spectrum of compound **2** in CD_3OD.

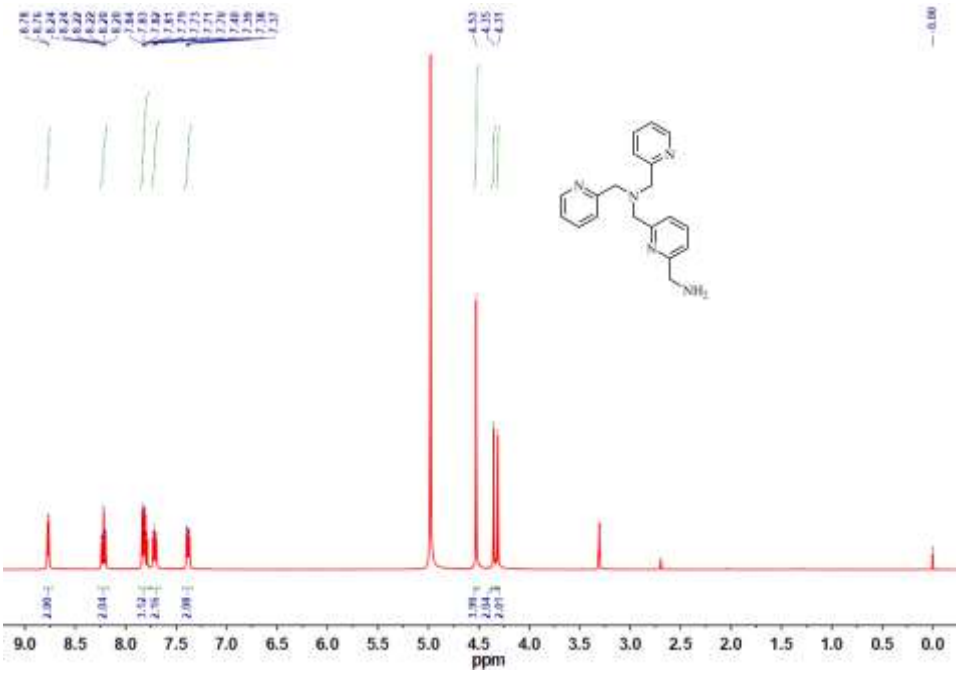


Fig. S3 ^{1}H NMR spectrum of compound **3** in CD_3OD.

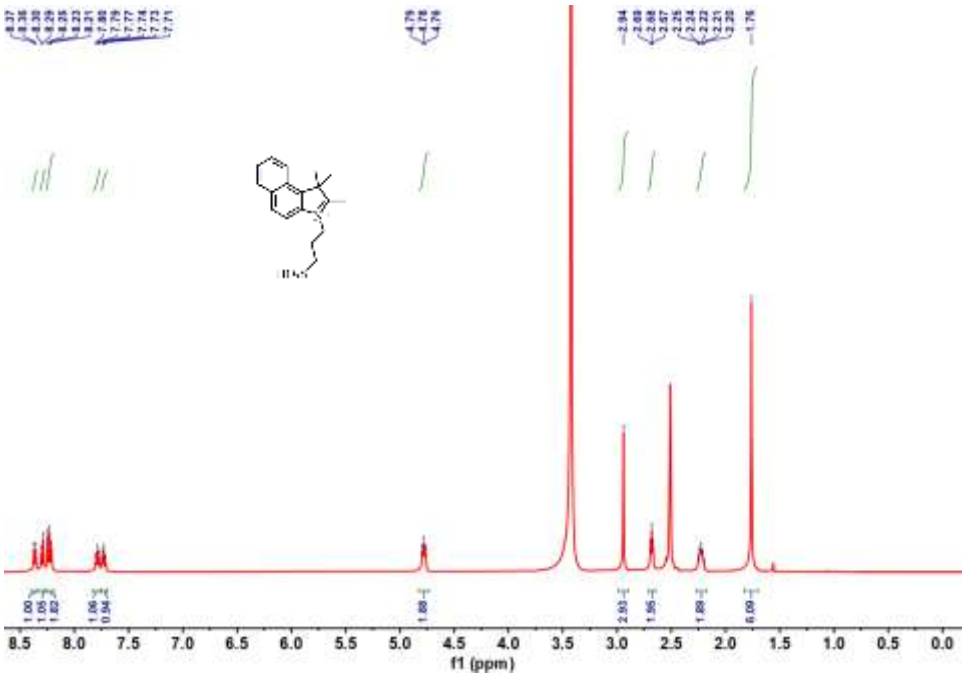


Fig. S4 ^{1}H NMR spectrum of compound **4** in $(CD_3)_2SO$.

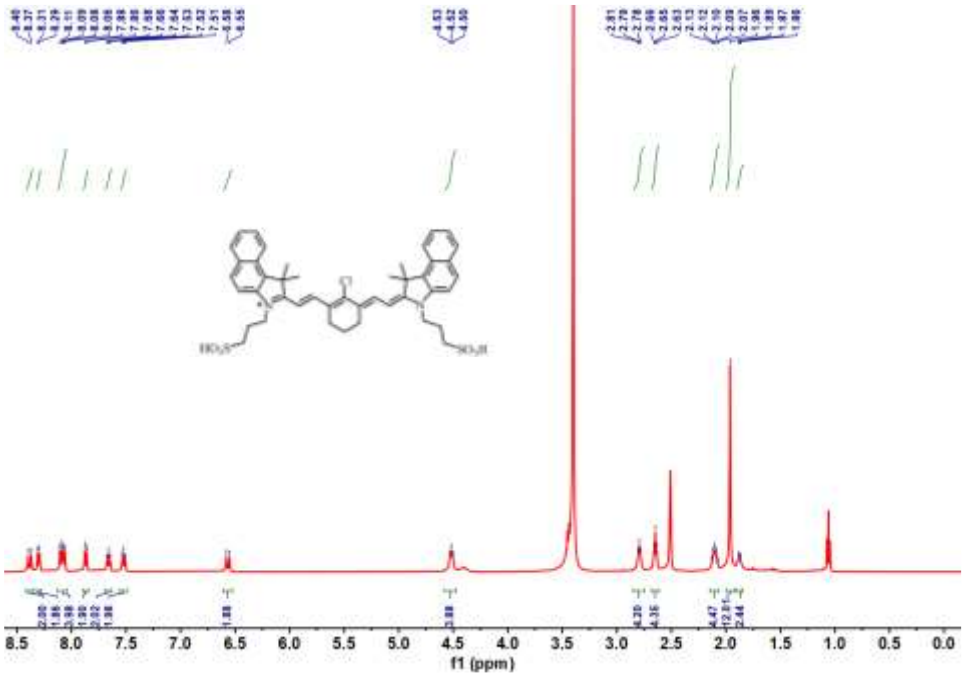


Fig. S5 ^{1}H NMR spectrum of compound **5** in $(CD_3)_2SO$.

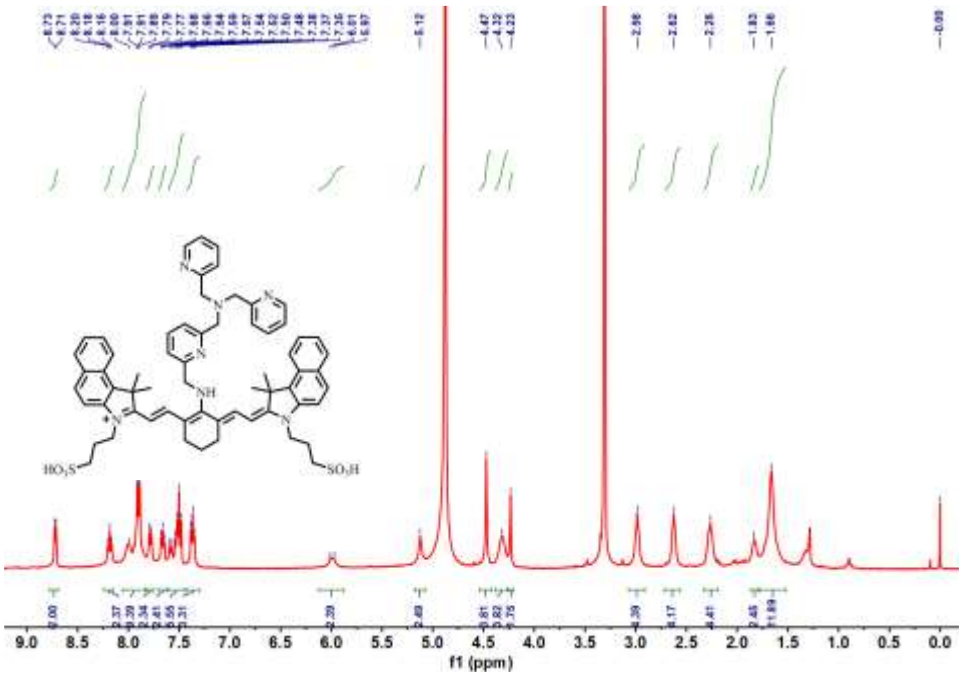


Fig. S6 ^{1}H NMR spectrum of **Mito-Zn** in CD_3OD.

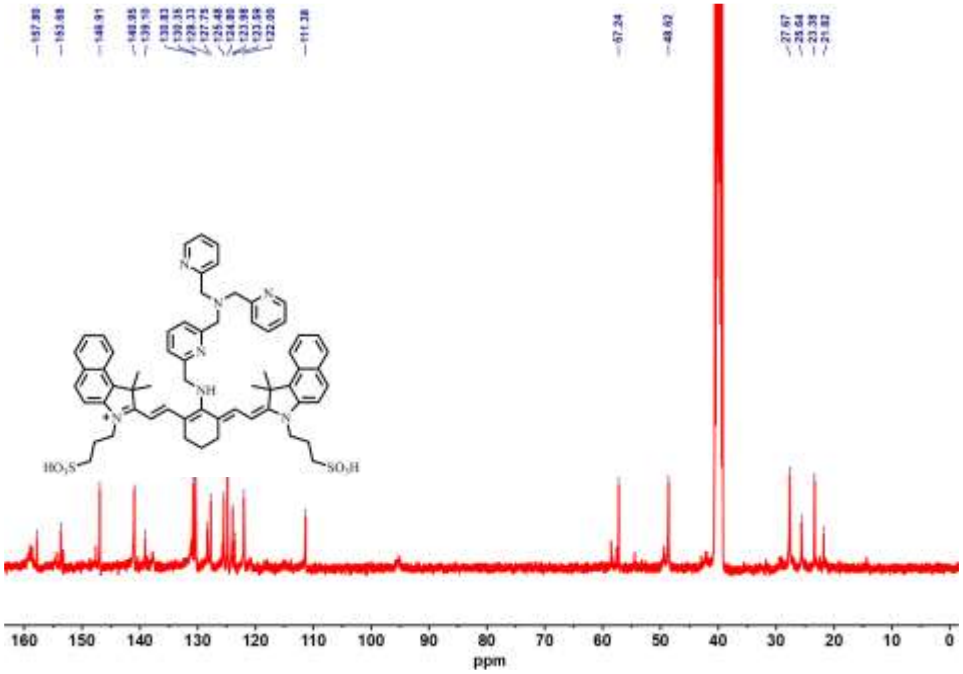


Fig. S7 ^{13}C NMR spectrum of **Mito-Zn** in CD_3OD.

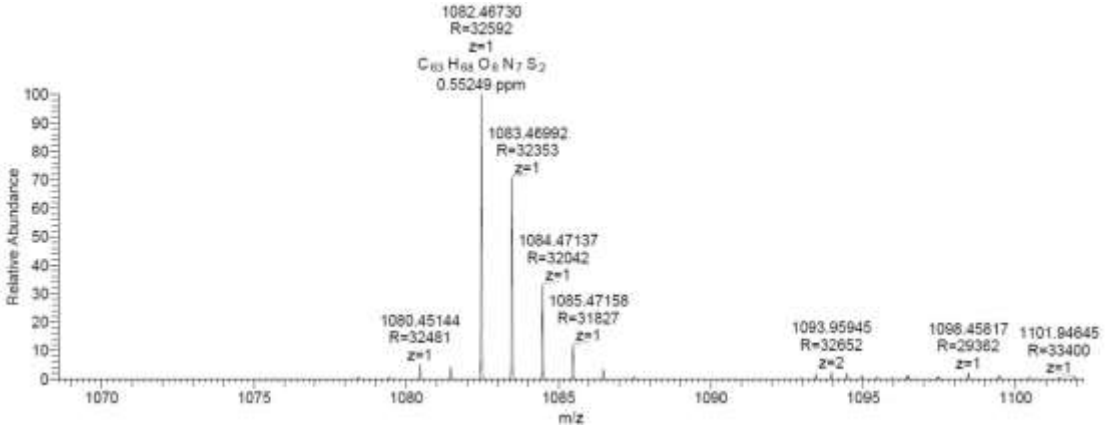


Fig. S8 Mass data of **Mito-Zn**.

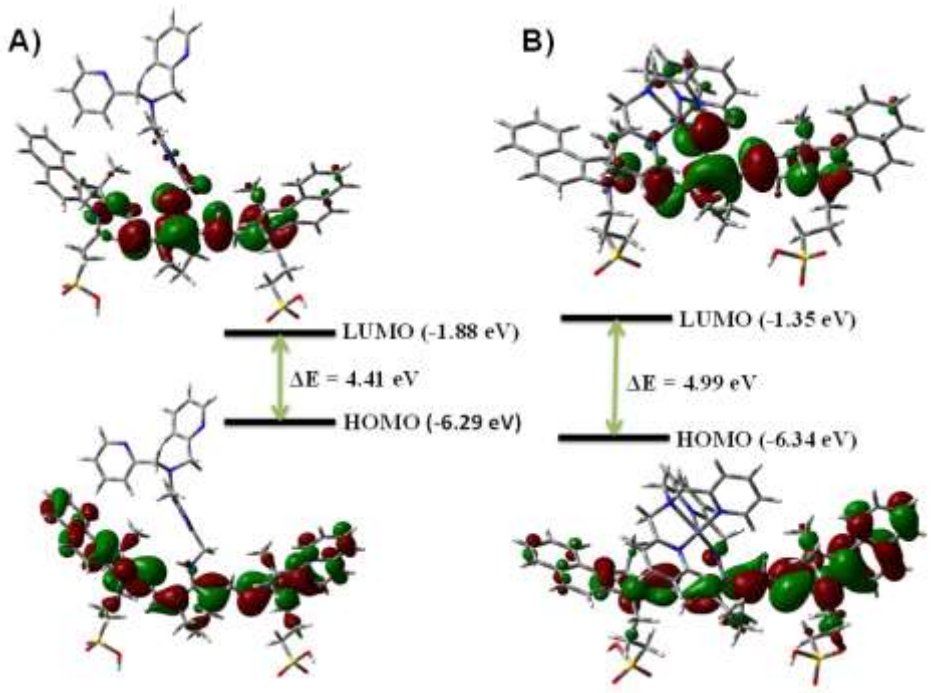


Fig. S9 Frontier orbitals (HOMO and LUMO) of (A) **Mito-Zn** and (B) the complexes of **Mito-Zn** with Zn^{2+}.

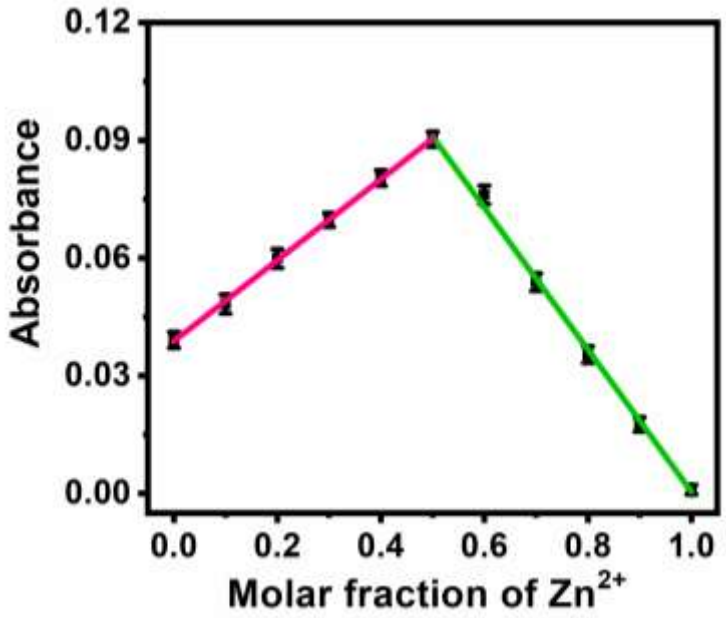


Fig. S10 Job's plot for the determination of stoichiometry of **Mito-Zn** and Zn^{2+}. The total concentration of **Mito-Zn** and Zn^{2+} were kept at 10 μM for absorption determination and the absorbance at 502 nm was collected.

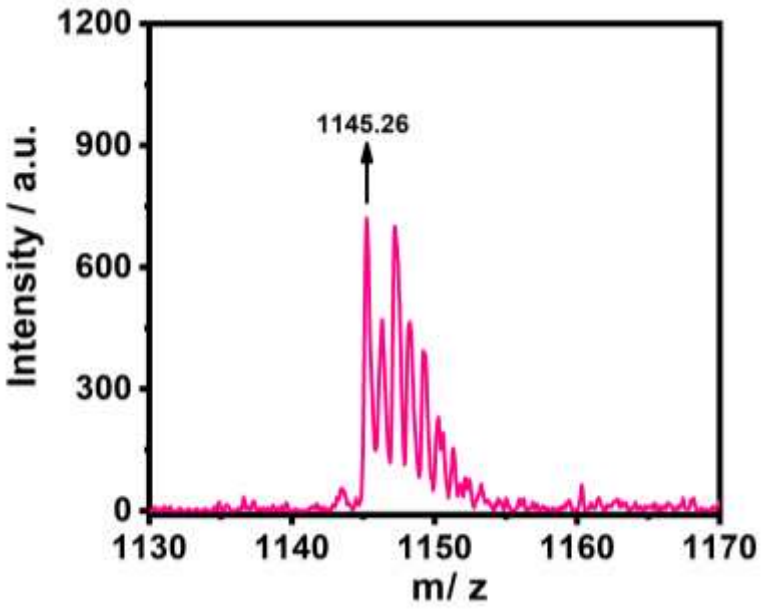


Fig. S11 HR-MS spectrum of the reaction product between **Mito-Zn** and Zn^{2+}.

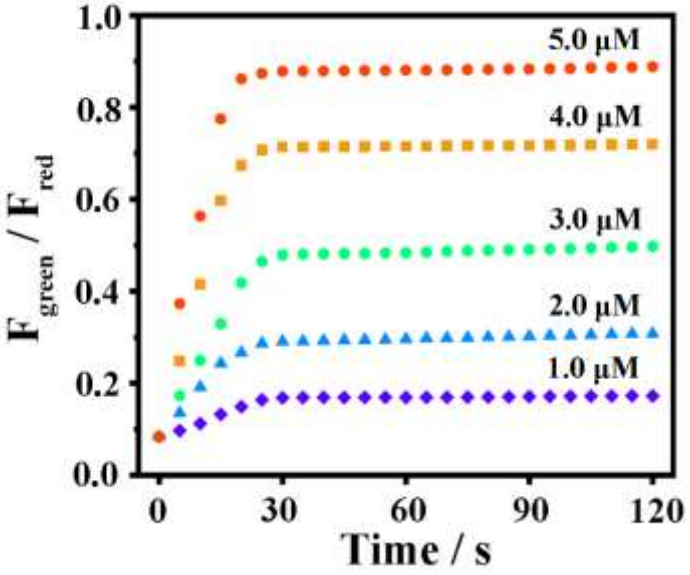


Fig. S12 Time-dependent changes of F_{green}/F_{red} in the presence of different amounts of Zn^{2+}.

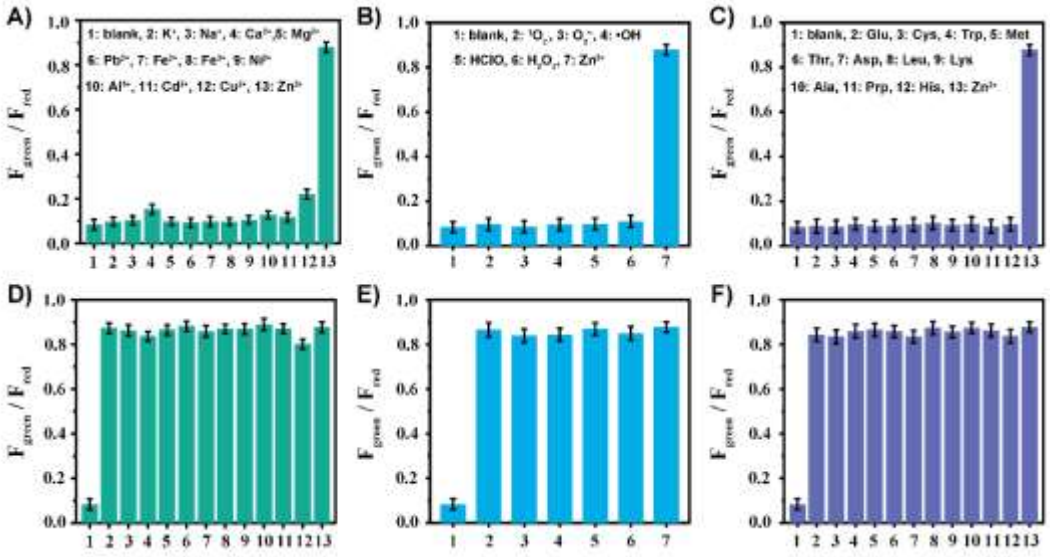


Fig. S13 Selectivity and competition tests of **Mito-Zn** (5.0 μM) in HEPES buffer (pH = 7.4, 10 mM, 1% CH_3CN) towards various (A, D) metal ions, (B, E) ROS and (C, F) amino acids. The competition tests were conducted with the coexistence of other metal ions, ROS or amino acids with Zn^{2+}. The concentration of metal ions, except K^+, Na^+, Ca^{2+} and Mg^{2+}, ROS and amino acids is 25 μM. The concentration of K^+, Na^+, Ca^{2+}, and Mg^{2+} is 1 mM. Data were acquired after incubation **Mito-Zn** with the analytes for 1 min.

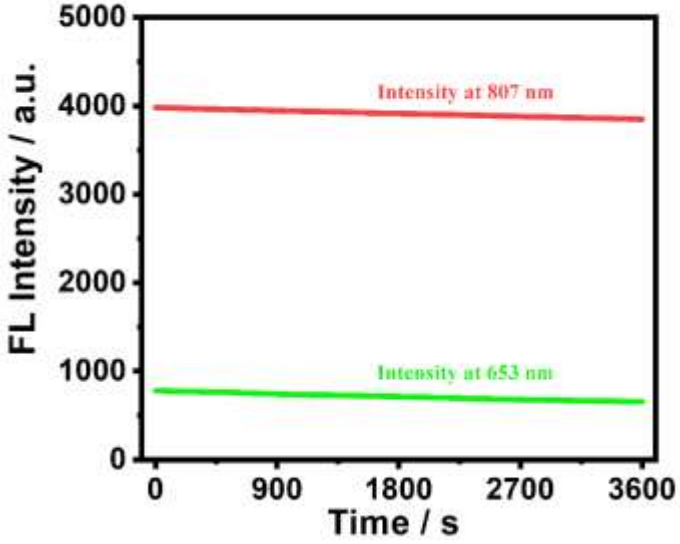


Fig. S14 Time courses of the fluorescence intensities at 807 and 653 nm, as measured by fluorescence spectrophotometer equipped with a 90 W Xenon lamp under 552 nm irradiation for 1 h.

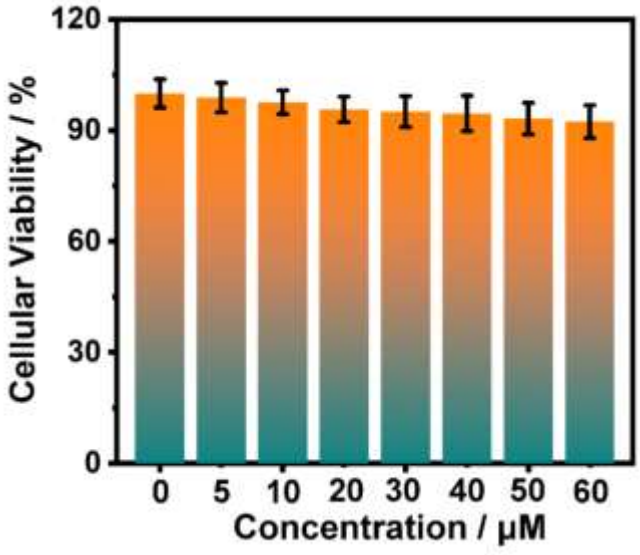


Fig. S15 Cytotoxicity of **Mito-Zn** against HeLa cells as determined by MTT assay. HeLa cells were cultured with **Mito-Zn** of various concentrations (0-60 μM) for 48 h.

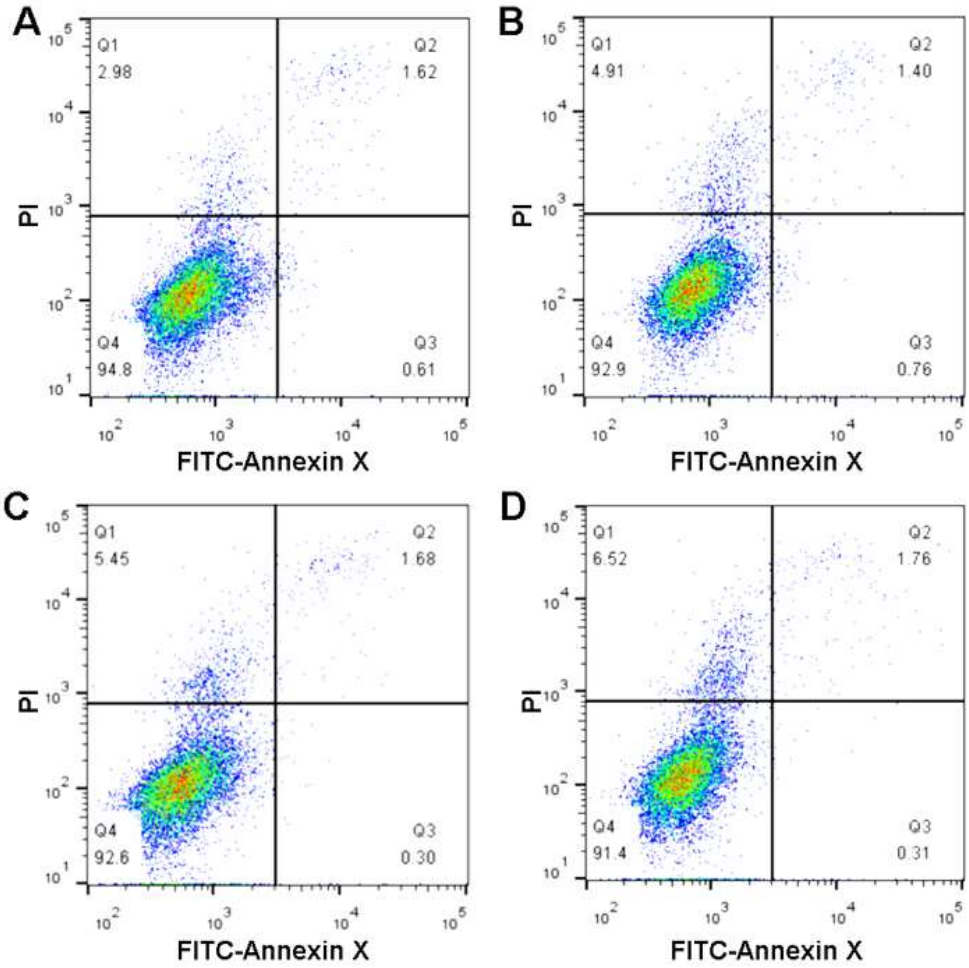


Fig. S16 Apoptosis assay of HeLa cells incubated with **Mito-Zn** at concentrations of (A) 0 μM, (B) 20 μM, (C) 40 μM, and (D) 60 μM. Q1, Q2, Q3, and Q4 represent the region of dead cells, late apoptotic cells, early apoptotic cells, and normal cells, respectively.

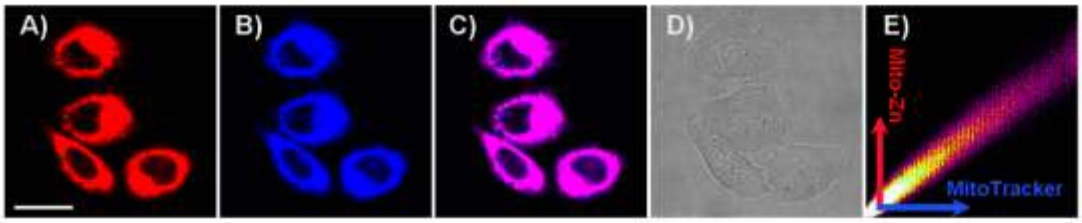


Fig. S17 Subcellular localization investigations in HeLa cells that were counterstained with **Mito-Zn** and MitoTracker green: (A) red channel: fluorescence from **Mito-Zn** (λ_{ex} = 552 nm; λ_{em} = 760-840 nm), (B) blue channel: fluorescence from MitoTracker green (λ_{ex} = 488 nm; λ_{em} = 500-570 nm), (C) the merged image of A with B, (D) bright-field image, and (E) the emission correlation plot of the red channel with blue channel. Scale bar: 20 μm.

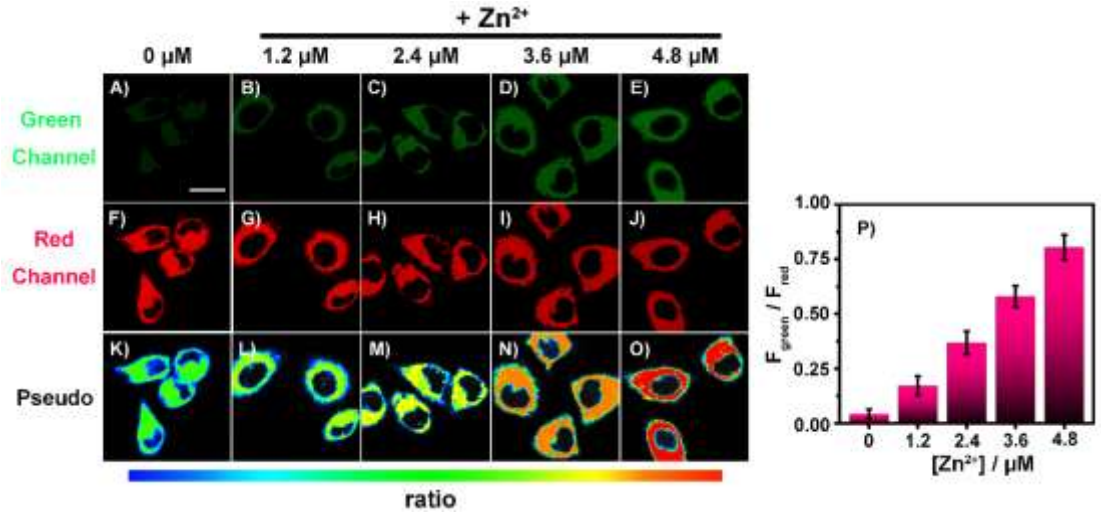


Fig. S18 Fluorescence images of **Mito-Zn** labelled HeLa cells stimulated with varied concentrations of Zn^{2+}: (A, F, K) 0 μM, (B, G, L) 1.2 μM, (C, H, M) 2.4 μM, (D, I, N) 3.6 μM and (E, J, O) 4.8 μM. (A-E) Fluorescence images from the green channel (λ_{em} = 610-690 nm). (F-J) Fluorescence images from the red channel (λ_{em} = 760-840 nm). (K-O) Pseudo images obtained from the green and red channels. (P) The corresponding F_{green}/F_{red} vs. Zn^{2+} concentrations. Scale bar: 20 μm.

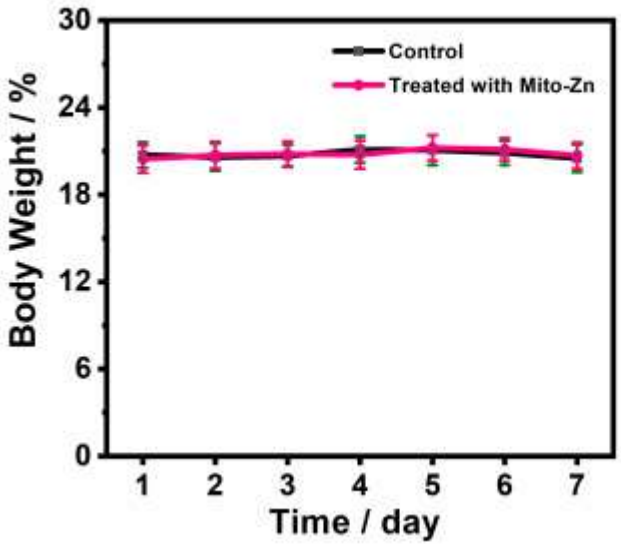


Fig. S19 Body weights change of mice treated with PBS (50 μL, 10 mM) or **Mito-Zn** (50 μL, 500 μM) for 7 days.

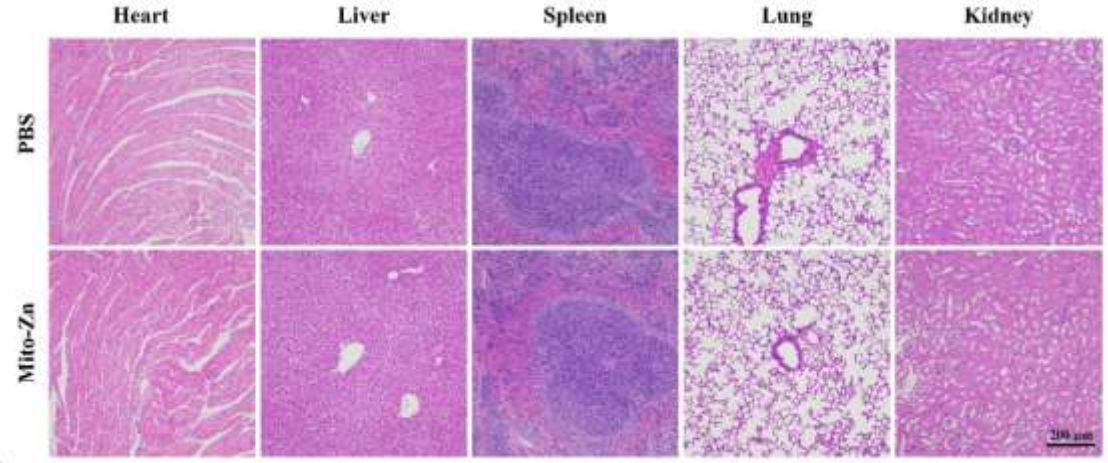


Fig. S20 HE stained histological images of main organs (heart, liver, spleen, lung, and kidney) from mice intravenously treated with PBS (50 μL, 10 mM) versus **Mito-Zn** (50 μL, 500 μM) after 7 days.

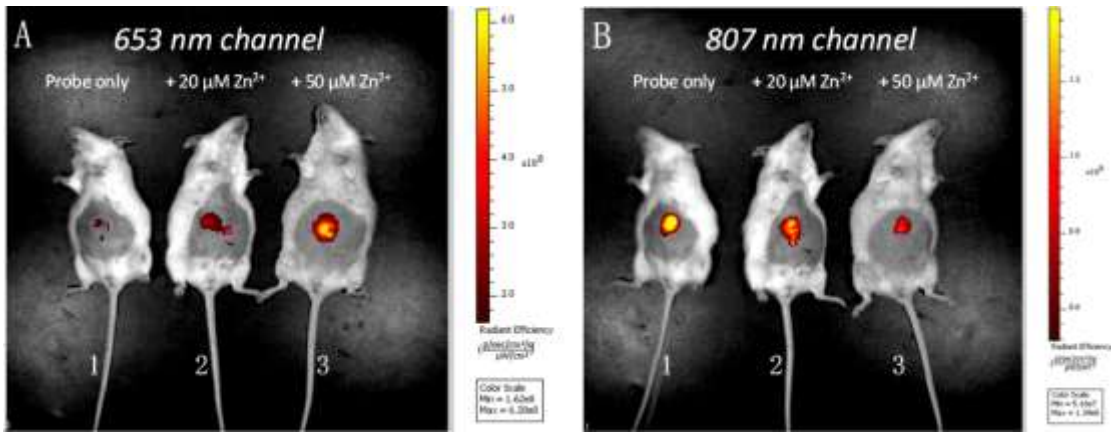
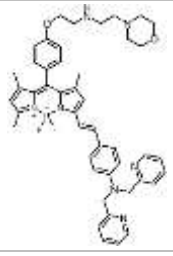
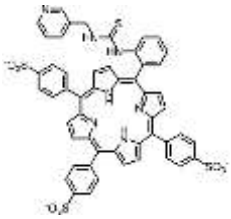
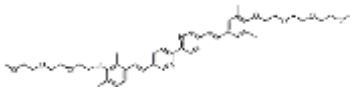
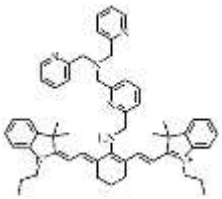
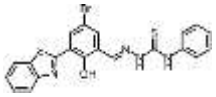
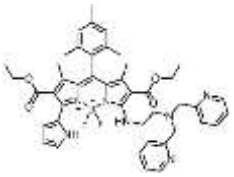
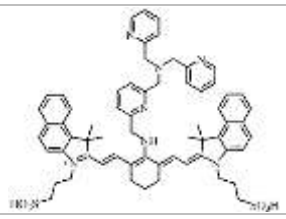


Fig. S21 In vivo imaging on female Kunming mice. The living animals were first subcutaneously injected with 20 μL probe of 500 μM, and different concentrations of Zn^{2+} (0, 20, and 50 μM) solutions were also injected in situ. The fluorescence signals were collected from two separate channels (A) 653 nm and (B) 807 nm. Excitation wavelength: 552 nm.

Table S1 Sensing performance comparison between **Mito-Zn** and other Zn^{2+} ratiometric probes.

Structure of probes	λ_{ex} (nm)/λ_{em} (nm)	Linear Range (μM)	LOD (nM)	Testing media	Reference
	545/578, 647	0 ~ 10	480	EtOH/Tris-HCl (9/1, v/v)	S2
	416/610, 648	0.08 ~ 5	31.1	Phosphate buffer solution	S3
	404/465, 550	0 ~ 6	50	MeOH/HEPES (1/1, v/v)	S4
	560/590, 730	0 ~ 5.0	No data	CH_3CN/HEPES (1/9, v/v)	S5
	362/488, 570	0.04 ~ 4.0	387	MeOH/HEPES (3/2, v/v)	S6
	550/605, 670	0 ~ 3	29	CH_3CN/HEPES (1/1, v/v)	S7
	552/653, 807	0.5 ~ 5.0	26.7	CH_3CN/HEPES (1/99, v/v)	This work

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