Electronic Supporting Information

Two-Photon Fluorescent Probe for Lysosomal Zinc Ions†

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Contents
1. Materials and methods
2. Spectroscopic methods
3. Preparation of cell lines for imaging
4. Preparation of mouse brain tissues for imaging
5. Cell viability assay
6. Synthesis of probe 1
7. Supporting figures and tables
8. References
9. NMR (1H & 13C) spectra
10. HR-FAB mass spectra of probe 1
1. Materials and methods

Unless otherwise noted, the chemicals were purchased from Sigma-Aldrich and used as received. LysoTracker® Deep Red, MitoTracker® Red, ER-Tracker® Red was purchased from Invitrogen. All solvents were purified and dried by standard methods prior to use. DI water was used to prepare all aqueous solutions. 1H NMR and 13C NMR spectra were recorded on a Bruker 400 MHz spectrometer using tetramethylsilane as the internal reference. All chemical shifts are reported in the standard notation of parts per million (ppm) using residual solvent protons as internal standard. Mass spectroscopic data were obtained from the Korea Basic Science Institute (Daegu) with a JEOL JMS 700 high resolution mass spectrometer. The experimental procedures regarding mouse tissues herein were performed in accordance with protocols approved by the Pohang University of Science and Technology (POSTECH) Committee on Animal Research and followed the guidelines for the use of experimental animals established by The Korean Academy of Medical Science. We made every effort to minimize animal suffering and reduce the number of animals used to prepare samples for imaging (for details, see preparation of mouse brain tissues for imaging).

2. Spectroscopic methods

All of the solvents used were of analytical grade. Solutions of metal ions were prepared from NaCl, MgCl₂, FeCl₃·6H₂O, CoCl₂·6H₂O, NiCl₂, CuCl₂, AgNO₃, CdCl₂ and Zn(ClO₄)₂ dissolved in distilled water. All stock solutions of 5 mM for metals were prepared. Probe was dissolved in ethanol (EtOH) at a concentration to 1 mM as stock solution. Aliquots were diluted to 10 μM in 20 mM MES buffer solutions of different pH (pH 4–8) as needed. Fluorescence spectra were recorded on a Photon Technical International Fluorescence system. Absorption spectra were measured using a HP agilent 8453 spectrophotometer. All pH measurements were made with a Thermo scientific, orion 2 star pH benchtop.

3. Preparation of cell lines for imaging

NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (HyClone) supplemented with 10% fetal bovine serum (HyClone) and 1% antibiotics (WelGENE), and maintained in a humidified atmosphere containing 5% CO₂. NIH 3T3 cells were seeded in the gelatin-coated 8-well chamber slide (Nunc™). After 24 h incubation, live cells were treated with probe (30 μM) and Lysotracker (1 μM) in serum free DMEM for 30 min and then washed with PBS buffer. For exogenous Zn(II) supplement, the probe treated cells were incubated with an mixture of 60 μM of Zn(ClO₄)₂ and pyrithione (1:1 mixture) in serum free DMEM for 10 min. After incubation, the cells were washed with PBS buffer (1 times) and fixed on the slide with 4% paraformaldehyde. Then the slides were subjected to microscopic imaging. For cellular zinc suppression experiment, 150 μM TPEN was
Confocal and two-photon fluorescence imaging experiments were performed on Leica TCS SP5 II Adv. System. The microscope was equipped with multiple visible laser lines (405, 458, 476, 488, 496, 514, 561, 594, 633 nm) and fluorescence signals were obtained through Hyd PMT (Hybrid detector PhotoMultiplier Tube; Leica) with green channel (500–630 nm) and red channel (670–750 nm).

4. Preparation of mouse brain tissues for imaging
C57BL6 type mouse (6 weeks, male) was used for this experiment. The experiment was done on light protected conditions in a dark-room and covered with aluminum foil. The mouse brain was dissected within 2 min and sliced to the thicknesses of 300 μm under PBS buffer solution by using Vibratome (Leica vt1000s model). After preparation of the staining solution of probe (10 μM in PBS buffer), the prepared tissue sample slides were dipped into the staining solution at 37 °C for 20 min and washed three times with PBS buffer. Prepared tissues were mounted on the microscope slides by using buffer solution and subjected to two-photon microscopic imaging.

Two-photon microscopy (TPM) with a Ti-Sapphire laser (Chameleon Vision II, Coherent) at 140 fs pulse width and 80 MHz pulse repetition rate was used (TCS SP5 II, Leica, Germany). TPM imaging was performed by using a 20× objective lens (obj. HCX APO 20x/ 1.00 W, 11507701, Leica, Germany). The excitation laser was tuned to 900 nm. Emission light was captured using an emission filter; λ_{em} = 500–650 nm. Power of the excitation laser was approximately 22.5 mW. The imaging field-of-view (FOV) was 300 × 300 μm consisting of 1024 × 1024 pixels. Acquired images were processed by using LAS AF Lite (Leica, Germany).

5. Cell viability assay
Cell viability was assessed by measuring their ability to metabolize 3-(4,5-dimethyl-diazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Cells were seeded onto 96-well plates at a density of about 5*10^3 cells per well in the growth medium and incubated until about 70–80% confluence. Following the probe treatments for 30 min as indicated, 25 μL of MTT solution (5 mg/ml) was added to each well, and cells were maintained for 2 h at 37 °C. 100 μL of solubilizing solution (4 mM HCl, 0.1% Nondet P-40 in isopropanol) was then added. After incubation for 12 h at 37 °C, absorbance at 570 nm was measured.
6. Synthesis of probe 1

Scheme 1.

\[ \text{NO}_2 \text{Cl} \xrightarrow{135^\circ \text{C, 1.5 h, 97\%}} \text{H}_2\text{N} \xrightarrow{\text{NH}_2} \text{H} \xrightarrow{\text{NaBH(OAc)}_3, \text{CH}_2\text{Cl}_2, 2\, \text{h, 95\%}} \text{H} \xrightarrow{\text{SnCl}_2\text{H}_2\text{O}, \text{CH}_2\text{CN/ EtOH, 90\%, 2 h, 99\%}} \text{H} \]

\[ \text{N}^1\text{-(2-nitrophenyl)ethane-1,2-diamine} (\text{1a}) \]

Compound 1a was synthesized following the known procedure.\(^2\) 2-Chloronitrobenzene (788 mg, 5 mmol) was added to ethylenediamine (3.3 mL, 50 mmol), and refluxed for 1.5 h at 135 °C. After cooling to room temperature, the reaction mixture was extracted with ethyl acetate and water and organic layer were dried over Na\(_2\)SO\(_4\) and filtered. The residue was evaporated under vacuum and purified by silica gel column chromatography with 20% MeOH/CH\(_2\)Cl\(_2\) to give compound 1a (869 mg, 97% yield).

\[ \text{N}^1\text{-(2-nitrophenyl)-N}^2\text{,N}^2\text{-bis(pyridin-2-ylmethyl)ethane-1,2-diamine} (\text{1b}) \]

To a solution of compound 1a (694 mg, 3.8 mmol) in CH\(_2\)Cl\(_2\) (38 mL, 0.1 M), sodium triacetoxyborohydride (3.2 g, 15.2 mmol) and 2-pyridinecarboxaldehyde (1.1 mL, 11.4 mmol) were added and stirred for 2 h at room temperature. After reaction finished, saturated NaHCO\(_3\) solution was added to the reaction mixture, then extracted with ethyl acetate. The organic layer was dried over MgSO\(_4\) and solvent was evaporated under reduced pressure. The residue was further purified by silica gel column chromatography with 5% MeOH/ethyl acetate to obtained compound 1b (1.317 g, 95% yield).

\[ ^1\text{H NMR (400 MHz, CDCl}_3\text{)} \delta 8.50 (\text{ddd}, J = 4.8, 1.2, 1.2 \text{ Hz}, 2 \text{ H}), 8.47 (\text{br s}, 1 \text{ H}), 8.17 (\text{dd}, J = 8.8, 1.6 \text{ Hz}, 1 \text{ H}), 7.69–7.67 (\text{m}, 4 \text{ H}), 7.37 (\text{ddd}, J = 8.4, 6.8, 1.2 \text{ Hz}, 1 \text{ H}), 7.16–7.12 (\text{m}, 2 \text{ H}), 6.73–6.71 (\text{m}, 1 \text{ H}), 6.61 (\text{ddd}, J = 8.4, 6.8, 1.2 \text{ Hz}, 1 \text{ H}), 3.88 (\text{s}, 4 \text{ H}), 3.39–3.35 (\text{m}, 2 \text{ H}), 2.93 (\text{t}, J = 6.4 \text{ Hz}, 2 \text{ H}); ^{13}\text{C NMR (100 MHz, CDCl}_3\text{)} \delta 158.6, 148.6, 144.9, 136.5, 135.9, 131.5, 126.5, \text{S4} \]
SnCl$_2$·H$_2$O (4 g, 18 mmol) was added to a solution of compound 1b (1.317 g, 3.6 mmol) in mixture of acetonitrile (24 mL, 0.15 M) and EtOH (24 mL, 0.15 M), and the reaction mixture was stirred for 2 h at 90 °C. After cooling to room temperature, the reaction mixture was extracted with CH$_2$Cl$_2$ and brine. The organic layer was dried over MgSO$_4$, and filtered. The residual solvent was evaporated under reduced pressure, and crude mixture was further purified by silica gel column chromatography with 5% MeOH/CH$_2$Cl$_2$ to obtained compound 2 (1.188 g, 99% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.54–8.53 (m, 2 H), 7.62 (ddd, $J$ = 7.6, 7.6, 2.0 Hz, 2 H), 7.48–7.46 (m, 2 H), 7.14 (ddd, $J$ = 7.6, 4.8, 1.2 Hz, 2 H), 6.77–6.61 (m, 3 H), 6.54 (dd, $J$ = 7.6, 1.2 Hz, 1 H), 4.41 (br s, 1 H), 3.88 (s, 4 H), 3.60 (br s, 2 H), 3.18 (t, $J$ = 6.0 Hz, 2 H), 2.93 (t, $J$ = 6.0 Hz, 2 H);

$^1$C NMR (100 MHz, CDCl$_3$) $\delta$ 159.1, 148.9, 137.4, 136.3, 134.1, 123.0, 121.9, 120.0, 117.8, 115.6, 111.2, 60.0, 52.5, 41.3.

A mixture of 4-bromo-1,8-naphthalic anhydride (415 mg, 1.5 mmol) and compound 2 (601 mg, 1.8 mmol) in DMF-EtOH (1 mL, 1.5 M : 5 mL, 0.3 M) was stirred at 160 °C. After overnight, the reaction mixture was cooled down to room temperature, and the solvent was evaporated. The crude mixture was purified by recrystallization with CH$_2$Cl$_2$/hexanes. Compound 3 was obtained as yellow powder (490 mg, 55% yield). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.71 (dd, $J$ = 7.2, 1.2 Hz, 1 H), 8.65 (dd, $J$ = 8.4, 1.2 Hz, 1 H), 8.45 (d, $J$ = 8.0 Hz, 1 H), 8.36–8.17 (m, 2 H), 8.09 (d, $J$ = 8.0 Hz, 1 H), 7.91 (dd, $J$ = 8.4, 7.2 Hz, 1 H), 7.36–7.32 (m, 1 H), 7.12–7.03 (m, 5 H), 6.89–6.79 (m, 4 H), 4.69 (br s, 1 H), 3.68 (s, 4 H), 3.27 (t, $J$ = 5.2 Hz, 2 H), 2.76 (t, $J$ = 6.0 Hz, 2 H); $^1$C NMR (100 MHz, CDCl$_3$) $\delta$ 163.2, 163.1, 158.7, 148.5, 144.4, 135.8, 133.4, 132.2, 131.4, 131.1, 130.6, 130.4, 130.2, 129.3, 128.9, 128.1, 123.3, 122.7, 122.4, 121.5, 120.7, 117.2, 112.1, 59.7, 51.6, 41.1.

$^4$-(2-(2-(bis(pyridin-2-ylmethyl)amino)ethyl)amino)phenyl)-6-bromo-1H-benzo[de]isoquinoline-1,3(2H)-dione (Probe 1)

4-(2-Aminoethyl)morpholine (0.15mL, 1.16 mmol) was added to a solution of compound 3 (170 mg, 0.29 mmol) in dimethyl sulfoxide (1 mL, 0.3 M), and the reaction mixture was stirred overnight at 90 °C. After reaction complete, reaction mixture was cooled to room temperature and poured into ice water. The yellow precipitation was filtered and purified by silica gel column chromatography with 10% MeOH/CH$_2$Cl$_2$. After column chromatography, the compound was recrystallized using CH$_2$Cl$_2$/hexanes to get yellow precipitate of probe 1 (98 mg, 53% yield). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.67 (d, $J$ = 7.2 Hz, 1 H), 8.56 (d, $J$ = 8.4 Hz, 1 H), 8.29–8.28 (m, 2 H), 8.19 (d, $J$ = 8.4 Hz, 1 H), 8.13–8.12 (m, 2 H), 7.67–7.64 (m, 2 H), 7.52–7.51 (m, 2 H), 7.37–7.34 (m, 2 H), 7.16–7.14 (m, 2 H), 7.05–7.01 (m, 2 H), 6.56–6.54 (m, 3 H), 6.37–6.34 (m, 3 H), 4.41 (br s, 1 H), 3.70–3.08 (m, 13 H), 3.18 (t, $J$ = 6.0 Hz, 2 H), 2.93 (t, $J$ = 6.0 Hz, 2 H); $^1$C NMR (100 MHz, CDCl$_3$) $\delta$ 163.2, 163.1, 158.7, 148.5, 144.4, 135.8, 133.4, 132.2, 131.4, 131.1, 130.6, 130.4, 130.2, 129.3, 128.9, 128.1, 123.3, 122.7, 122.4, 121.5, 120.7, 117.2, 112.1, 59.7, 51.6, 41.1.
7.74 (t, J = 7.6 Hz, 1 H), 7.30 (ddd, J = 8.8, 7.2, 1.6 Hz, 1 H), 7.09–7.07 (m, 3 H), 7.03 (ddd, J = 7.6, 7.2, 1.6 Hz, 2 H), 6.92–6.89 (m, 2 H), 6.84 (ddd, J = 7.6, 7.6, 1.2 Hz, 1 H), 6.79–6.74 (m, 2 H), 6.40 (br s, 1 H), 4.45 (br s, 1 H), 3.80 (t, J = 4.4 Hz, 4 H), 3.71–3.62 (m, 4 H), 3.49-3.45 (m, 2 H), 3.26–3.21 (m, 2 H), 2.88–2.85 (m, 2 H), 2.71 (t, J = 5.6 Hz, 2 H), 2.63–2.57 (m, 4 H); 13C NMR (100 MHz, CDCl₃) δ 164.2, 163.6, 158.9, 149.6, 148.5, 144.5, 136.0, 134.8, 131.5, 130.3, 129.7, 129.0, 126.3, 124.8, 123.3, 122.7, 121.8, 121.6, 120.4, 117.3, 111.9, 110.2, 104.5, 66.9, 60.0, 55.7, 53.0, 51.9, 40.6, 38.9.

7. Supporting figures and tables

Fig. S1 Absorption spectra of probe 1 (10 μM) before (black line) and after (red line) addition of Zn(II)-salt (10 μM). The spectra were taken in 1% EtOH containing 20 mM MES buffer (pH = 5.0).

Fig. S2 Fluorescence intensity changes of (a) probe 1 (10 μM) and (b) it’s Zn(II) complex (10 μM) under pH = 4.0–8.0 MES buffer (20 mM). All the fluorescence data were obtained by excitation at 450 nm.
**Fig. S3** (a) Fluorescence changes of probe 1 (10 μM) with various concentration of Zn(ClO₄)₂ (0–50 μM) at pH = 5.0 MES buffer containing 1% EtOH. (b) Plot of fluorescence intensity of probe 1 (10 μM) with equiv. of Zn(II) addition at pH = 5.0. All the fluorescence data were obtained by excitation at 450 nm.

**Fig. S4** (a) Fluorescence changes of probe 1 (10 μM) with various concentration of Zn(ClO₄)₂ (0–50 μM) at pH = 7.4 MES buffer containing 1% EtOH. (b) Plot of fluorescence intensity of probe 1 (10 μM) with equiv. of Zn(II) addition at pH = 7.4. All the fluorescence data were obtained by excitation at 450 nm.

**Fig. S5** Plot of fluorescence enhancement of probe 1 (10 μM) with Zn(II) (0–0.9 μM) in pH = 5.0 MES buffer containing 1% EtOH. The fluorescence data were obtained by excitation at 450 nm.
Fig. S6 Fluorescence changes of probe 1 (10 μM in pH = 5.0 MES buffer containing 1% EtOH) upon treatment with 0.18 μM of Zn(ClO₄)₂. The fluorescence data were obtained by excitation at 450 nm. This data shows a signal-to-background ratio of more than three.

Fig. S7 Fluorescence intensity changes of Probe 1 (10 μM) with various metal ions (10 μM) in 1% EtOH containing pH = 5.0 MES buffer. All the fluorescence data were obtained by excitation at 450 nm.

Fig. S8 Two-photon action spectra of probe 1 (10 μM) before (black line) and after (red line) addition of Zn(II)-salt (10 μM). The spectra were taken in 1% EtOH containing 20 mM MES buffer (pH = 5.0). The two-photon action cross-section (Φ₂σTP) values were determined by following the literature (S. K. S8)

**Fig. S9** Cell viability of probe 1 towards NIH 3T3 cell lines by MTT assay.

![Cell viability graph](image_url)

**Fig. S10** One-photon confocal microscopic imaging of Zn(II) in live NIH 3T3 cells with probe 1. (a) The cells were incubated with probe 1 only; (b) The cells were incubated with probe 1, followed by further incubation with an exogenous source of Zn(II), 60 μM of Zn(ClO₄)₂ and pyrithione (1:1 mixture), for 10 min; (c) The cells were incubated with probe 1, followed by further incubation with TPEN (150 μM) for 10 min. In all cases, the incubation of cells with probe was carried out with 30 μM of probe for 30 min. One-photon images were obtained under excitation at 458 nm and emission were collected at 500–630 nm.
Fig. S11 Pseudo-colored fluorescence images of HeLa cells: (a) TPM images of cells co-incubated with probe 1 (30 μM) followed by Zn(ClO₄)₂ and pyrithione (1:1) (60 μM); collected in the green channel window (500–550 nm) under excitation at 880 nm. (b) OPM images of cells incubated with MitoTracker Red (1 μM) for 30 min at 37 °C, collected in the red channel window (650–750 nm) under excitation at 633 nm. (c) Merged images. (d) Intensity profiles measured across the region of interest (ROI) in the HeLa cells.

Probe 1
(Green Channel)
MitoTracker
(Red Channel)
Merged images
Pearson’s Correlation Coefficient
(PCC) = 0.374
Mander’s Overlap Coefficient
(MOC) = 0.419

Fig. S12 Pseudo-colored fluorescence images of NIH 3T3 cells: (a) TPM images of cells co-incubated with probe 1 (30 μM) followed by Zn(ClO₄)₂ and pyrithione (1:1) (60 μM); collected in the green channel window (500–550 nm) under excitation at 880 nm. (b) OPM images of cells incubated with ER-Tracker Red (1 μM) for 30 min at 37 °C, collected in the red channel window (650–750 nm) under excitation at 633 nm. (c) Merged images. (d) Intensity profiles measured across the region of interest (ROI) in the HeLa cells.

Probe 1
(Green Channel)
ER-Tracker
(Red Channel)
Merged images
Pearson’s Correlation Coefficient
(PCC) = 0.131
Mander’s Overlap Coefficient
(MOC) = 0.200
Table S1 Molar extinction coefficient \([\varepsilon \text{ (L mol}^{-1} \text{ cm}^{-1})]\), the maximum absorbance \([\lambda_{\text{abs}} \text{ (nm)}]\) and emission wavelength \([\lambda_{\text{em}} \text{ (nm)}]\), and fluorescence quantum yield \((\Phi_{\text{F}})\) of probe 1 and its Zn(II) complex at various pHs.

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<tr>
<td></td>
<td>(\varepsilon)</td>
<td>(\lambda_{\text{abs}})</td>
<td>(\lambda_{\text{em}})</td>
<td>(\Phi_{\text{F}}^a)</td>
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<td>(\lambda_{\text{abs}})</td>
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<td>545</td>
<td>0.05</td>
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\(^a\)Fluorescence quantum yield determined using rhodamine 6G \((\Phi_{\text{F}} = 0.95)\) as standard.

Table S2 Colocalization coefficient and information of probe 1 and LysoTracker Deep Red during imaging of NIH 3T3 cells in Fig. 3.

<table>
<thead>
<tr>
<th>Colocalization</th>
<th>Pearson’s correlation</th>
<th>Overlap coefficient</th>
<th>Colocalization rate (%)</th>
<th>Colocalization area ((\mu\text{m}^2))</th>
<th>Area Image ((\mu\text{m}^2))</th>
<th>Area Foreground ((\mu\text{m}^2))</th>
<th>Area Background ((\mu\text{m}^2))</th>
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</tbody>
</table>

8. References

9. NMR (\(^1\)H & \(^13\)C) spectra
10. HR-FAB mass spectra of probe 1

[ Elemental Composition ]
Data : Morpholine-C38H39N7O3  Date : 10-Jan-2014 09:57
Sample: -
Note: -
Inlet : Direct  Ion Mode : FAB+
RT : 0.18 min  Scan#: (4,5)
Elements : C 38/0, H 40/1, O 3/1, N 7/1
Mass Tolerance : 1000ppm, 1mmu if m/z < 1, 3mmu if m/z > 3
Unsaturation (U.S.) : -0.5 - 200.0

Observed m/z  Int%  Err[ppm / mmu]  U.S. Composition
642.3195  100.0  +0.3 / +0.2  22.5 C 38 H 40 O 3 N 7
643.3183  43.7
644.3171  10.2

[ Theoretical Ion Distribution ]
Molecular Formula : C38 H40 O3 N7
(m/z 642.3193, MW 642.7807, U.S. 22.5)
Base Peak : 642.3193, Averaged MN : 642.7834(a), 642.7842(w)

m/z  INT.
642.3193  100.0000  *****************************************************
643.3223  45.5507  *****************************************************
644.3252  10.7359  *****
645.3281  1.7414 *
646.3308  0.2176
647.3336  0.0222
648.3363  0.0019
649.3390  0.0001