Supporting Information

for

In vivo ratiometric Zn\(^{2+}\) imaging in zebrafish larva
using a new visible light excitable fluorescent sensor

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S1. Materials and methods

All reagents and solvents for synthesis were commercially available and of analytical grade. For spectroscopic study, the water was purified by MilliQ system (<18 MΩ), and other solvents were of chromatographic grade. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), TPEN, and SBD-Cl \( \text{I} \) were purchased from Aldrich. The \(^1\text{H}\)-NMR and \(^{13}\text{C}\)-NMR spectra were recorded on a Bruker DRX-500 spectrometer with TMS as internal standard in MeOD. Mass spectrometric data were determined with a LCQ (ESI-MS, Thermo Finnigan) mass spectrometer.

S2. Synthesis of SBD-TPEA

SBD-Cl (817.7 mg, 3.5 mmol), Et\(_3\)N (571 mg, 4.13 mmol), and TPEA (232 mg, 0.70 mmol) were mixed in 10 mL of THF and refluxed for 48 h. Then the solids were filtered off and washed with CH\(_2\)Cl\(_2\). After combining the filtration and the CH\(_2\)Cl\(_2\) solution, the solvents were removed by evaporation \textit{in vacuo}. The hygroscopic product was obtained by purifying the resulting mixture with silica gel chromatography. Ethyl acetate/methanol (\(\text{v/v}\), 20:1) was used as the eluent. \( R_f = 0.3 \). Yield, 44.5%. M.p. 70–72 °C. \(^1\text{H}\)-NMR (500 MHz, CD\(_3\)OD, \(\delta\), ppm): 8.50 (d, 1H, \( J = 4.8 \text{ Hz, Py-H}\)), 8.37 (d, 2H, \( J = 4.5 \text{ Hz, Py-H}\)), 7.74 (t, 1H, \( J = 7.8 \text{ Hz, Py-H}\)), 7.70 (d, 1H, \( J = 8.4 \text{ Hz, SBD-H}\)), 7.68 (t, 2H, \( J = 7.8 \text{ Hz, Py-H}\)), 7.52 (d, 2H, \( J = 8.0 \text{ Hz, Py-H}\)), 7.29 (t, 1H, \( J = 6.2 \text{ Hz, Py-H}\)), 7.27 (d, 1H, \( J = 7.8 \text{ Hz, Py-H}\)), 7.20 (t, 2H, \( J = 6.2 \text{ Hz, Py-H}\)), 6.04 (d, 1H, \( J = 8.4 \text{ Hz, SBD-H}\)), 5.17 (s, 2H, PyC\(_2\)H\(_2\)), 4.10 (t, 2H, \( J = 6.1 \text{ Hz, -CH}_2\text{CH}_2\)-), 3.88 (s, 4H, PyC\(_2\)H\(_2\)), 2.98 (t, 2H, \( J = 6.1 \text{ Hz, -CH}_2\text{CH}_2\)-). \(^{13}\text{C}\)-NMR (125 MHz, CD\(_3\)OD, \(\delta\), ppm): 158.65, 156.68, 148.95, 148.14, 144.67, 141.39, 137.40, 135.23, 123.65, 122.53, 122.41, 121.26, 114.64, 102.43, 60.43, 57.85, 51.27, 50.90. Element analysis for C\(_{26}\)H\(_{26}\)N\(_8\)O\(_3\)S, Found: C, 58.71; H, 5.06; N, 21.01. Calcd. C,
58.85; H, 4.94; N, 21.12 %. ESI-MS (positive mode, \textit{m/z}): Calcd. 553.17, found: 553.25 for [M+ Na]⁺.

**Figure S1.** \textsuperscript{1}H NMR spectrum of SBD-TPEA in MeOD.

**Figure S2.** \textsuperscript{13}C NMR spectrum of SBD-TPEA in MeOD.

**Figure S3.** 2D H-H COSY of SBD-TPEA in MeOD.
Figure S4. 2D H-C HMQC of SBD-TPEA in MeOD.

Figure S5. ESI-MS spectrum of SBD-TPEA.
Figure S6. ESI-MS spectrum of SBD-TPEA/Zn2+ complex. The sample used for MS determination were obtained from 1H NMR titration experiment after 1.5 equiv Zn2+ has been added (See S6 and Figure S10). The determined isotopic distribution patterns (IDPs) of the peaks with m/z of 595.25 and 613.17 were shown as insets a and c. The related simulated IDPs for [2M+2Zn-2H]+ and [M+Zn+D2O-H]+ were shown as insets b and d.

S3. Determination of the water solubility of SBD-TPEA

The water solubility of SBD-TPEA was determined according to the method used in reference. Therefore, the DMSO stock solution of SBD-TPEA (1.93 ×10^{-3} M) was added to a cuvette containing 3.0 mL of H2O by using a micro syringe. The volume of DMSO in H2O was maintained to be 0.2 % during the titration experiment. The plot of fluorescence intensity at 585 nm against the concentration of SBD-TPEA was shown in Figure S6. The solubility of SBD-TPEA in water was estimated to be about 3.0 μM.

S4. Fluorescence spectroscopic study

The emission and excitation spectra of SBD-TPEA (3 μM) was determined in HEPES buffer (0.15% DMSO, v/v; 50 mM HEPES, 100 mM KNO3; pH = 7.20) using AMINCO Bowman series 2
The fluorescent pH dependence of **SBD-TPEA** was determined in aqueous solution (10 μM, DMSO/water, 1:9 v/v) at different pH values, and the solution pH values were adjusted by 5 M HNO₃ and 5 M NaOH. The pH values of sample solutions were monitored by a PHS-3 system.

**Figure S8.** Fluorescence pH titration profile of **SBD-TPEA** (3 μM) in HEPES buffer (0.15% DMSO, v/v; 50 mM HEPES, 0.1 M KNO₃; pH 7.2) based on $F_{545}/F_{585}$.

### S5. Absorption titration of SBD-TPEA

All UV-vis spectra were recorded by a Shimadzu UV-3100 spectrophotometer and the UV titration experiment of **SBD-TPEA** was carried out respectively by adding aliquots of 2.5 μL of Zn(NO₃)₂ aqueous solution (1.2 mM) to 3 mL of sample solution (10 μM, 1:9, DMSO/water v/v; 50 mM HEPES, 100 mM KNO₃; pH = 7.20) in a cuvette. The spectra were recorded after the solution was completely mixed in several seconds.

**Figure S9.** Absorption spectra of 10 μM **SBD-TPEA** in HEPES buffer (50 mM, 0.1 M KNO₃, DMSO/water = 1 : 9, v/v, pH 7.2) when titrated with Zn²⁺ (1.2 mM) solution. The final [Zn²⁺]total in the mixed solutions are 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0 and 14.0 μM, respectively. Insets, titration profiles according to the absorbance at 386 nm and 456 nm.

### S6. ¹H NMR Zn²⁺ titration of SBD-TPEA and NMR spectra of Zn²⁺/SBD-TPEA complex

The ¹H NMR titration experiments of **SBD-TPEA** were carried out in CD₃OD on Bruker DRX-500 at 25 °C. Chemical shift was referenced to an external sample of TMS ($\delta$, 0.00 ppm). The initial sensor concentration was 27.0 mM. The total concentration of Zn(NO₃)₂ in the mixtures varied...
from 0 to 1.5 equivalent of the sensor.

Figure S10. $^1$H NMR spectra of SBD-TPEA (initial $c = 2.7 \times 10^{-2}$ M) in CD$_3$OD obtained upon Zn$^{2+}$ titration ($c = 9.0 \times 10^{-2}$ M in CD$_3$OD): (a) at [Zn$^{2+}$]$_{total}$/[sensor] ratio of 1:1; (b) at [Zn$^{2+}$]$_{total}$/[sensor] ratio of 0.5:1, (c) free sensor. The signals marked with ☆ and ✂ are for the protons from free sensor and zinc-bound sensor, respectively.

Figure S11. 2D H-H COSY of Zn$^{2+}$/SBD-TPEA complex in MeOD.
**Figure S12.** 2D H-C HMQC of Zn\(^{2+}\)/SBD-TPEA complex in MeOD.

**Table 1.** Assignment of \(^1\)H NMR signals for SBD-TPEA and its Zn\(^{2+}\) complex in CD\(_3\)OD.

<table>
<thead>
<tr>
<th>Protons</th>
<th>Signals for free SBD-TPEA</th>
<th>Signals for Zn(^{2+})/SBD-TPEA (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_a)</td>
<td>7.70 (d, 1H, (J = 8.39) Hz)</td>
<td>7.66-7.73 (m, 4H, H(_o), H(_f), H(_m) and H(_n))</td>
</tr>
<tr>
<td>H(_f)</td>
<td>7.52 (d, 2H, (J = 7.96) Hz)</td>
<td></td>
</tr>
<tr>
<td>H(_m)</td>
<td>7.27 (d, 1H, (J = 7.78) Hz)</td>
<td></td>
</tr>
<tr>
<td>H(_g)</td>
<td>7.68 (t, 2H, (J = 7.78) Hz)</td>
<td>8.06-8.22 (m, 6H, H(_g), H(_i), H(_j) and H(_l))</td>
</tr>
<tr>
<td>H(_i)</td>
<td>8.37 (d, 2H, (J = 4.49) Hz)</td>
<td></td>
</tr>
<tr>
<td>H(_j)</td>
<td>8.50 (d, 1H, (J = 4.75) Hz)</td>
<td></td>
</tr>
<tr>
<td>H(_l)</td>
<td>7.74 (t, 1H, (J = 7.78) Hz)</td>
<td></td>
</tr>
<tr>
<td>H(_b)</td>
<td>6.04 (d, 1H, (J = 8.39) Hz), 6.44 (d, 1H, (J = 7.58) Hz)</td>
<td></td>
</tr>
<tr>
<td>H(_c)</td>
<td>4.10 (t, 2H, (J = 6.10) Hz)</td>
<td>3.76 (brs, 2H)</td>
</tr>
<tr>
<td>H(_d)</td>
<td>2.98 (t, 2H, (J = 6.06) Hz)</td>
<td>3.33 (overlaid by CD(_3)OD peak)</td>
</tr>
<tr>
<td>H(_e)</td>
<td>3.88 (s, 4H)</td>
<td>4.25 (s, 4H)</td>
</tr>
<tr>
<td>H(_h)</td>
<td>7.20 (t, 2H, (J = 6.22) Hz)</td>
<td>7.44 (t, 2H, (J = 6.22) Hz)</td>
</tr>
<tr>
<td>H(_k)</td>
<td>7.29 (t, 1H, (J = 6.22) Hz)</td>
<td>7.57 (t, 1H, (J = 6.22) Hz)</td>
</tr>
<tr>
<td>H(_n)</td>
<td>5.17 (s, 2H)</td>
<td>5.21 (s, 2H)</td>
</tr>
</tbody>
</table>

**Chart S1.** Proposed Zn\(^{2+}\) binding mode of SBD-TPEA

**S7. Dissociation constant determination for Zn\(^{2+}\) Complexes of SBD-TPEA in HEPES Buffer**

A series of buffered Zn\(^{2+}\) solutions were prepared for the determination of the dissociation constant of Zn\(^{2+}\)/SBD-TPEA complex.\(^2\) Thus, various amounts of ZnNO\(_3\) (0 ~ 20 mM) were added to the solutions of \(2\cdot[4\,(2\text{-hydroxyethyl})\cdot1\text{-piperazinyl}]\text{ethanesulfonic acid}\) (HEPES 50 mM, pH 7.20, 0.1 M KNO\(_3\)) containing 10 mM of EGTA (ethylenbis(oxyethylenenitrilo) tetraacetic acid). The concentration of free Zn\(^{2+}\) was calculated with \([\text{EGTA}]_{\text{total}}, [\text{Zn}\(^{2+}\)]_{\text{total}},\) and \(K'_{\text{Zn-EGTA}} = 3.80 \times 10^8\) M\(^{-1}\).

For the determination of dissociation constant, the above mentioned buffered Zn\(^{2+}\) solutions were added with free sensor solution. The final concentration of total sensor in the mixture is around 3 μM, and the emission spectra of the mixed solutions were determined respectively after complete mixing.
**Figure S13.** Fluorescence ratio \((F_{545}/F_{585})\) of SBD-TPEA (3 μM) as a function of free \(\text{Zn}^{2+}\) concentration in 50 mM HEPES buffer (pH 7.2, 0.1 M KNO₃) containing 10 mM EGTA and 0 - 20 mM \([\text{Zn}^{2+}]_{\text{total}}\).

**S8 Determination the detection limit of SBD-TPEA**

The ratio of emission intensity \((F_{545}/F_{585})\) of SBD-TPEA (3 μM) was collected for 20 times to determine the background noise \(\sigma\). Then the solution was treated with various concentration of \([\text{Zn}^{2+}]_\text{free}\) from 0.6 – 2.2 nM (see S7), and all fluorescence spectra were collected after thoroughly mixing. A linear regression curve was then fitted according to the ratio of emission intensity \((F_{545}/F_{585})\) in the range of 0.6–2.2 μM, and the slope of the curve was obtained (Figure S14). The detection limit was then determined to be 0.5 nM.

**S9. Cell culture methods and confocal fluorescence imaging.**

HepG2 and HeLa cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 mg/ml) and 5% CO₂ at 37 °C. After removing the incubation media and rinsing with 1× PBS for three times, the cells were stained by SBD-TPEA via incubation with 10 μM SBD-TPEA solution (in 1× PBS containing 0.5% DMSO) for 20 min at 25 °C. Then the cells were washed three times with PBS and imaged with Olympus FV10-ASW microscope equipped with a 60× oil-immersion objective. For the imaging of HepG2 cells with exogenous \(\text{Zn}^{2+}\), the exogenous \(\text{Zn}^{2+}\) was introduced by incubating the cells with 5 μM \(\text{ZnSO}_4/2\)-mercaptopyridine-N-oxide solution (prepared by diluting 5 mM \(\text{ZnSO}_4/2\)-mercaptopyridine-N-oxide stock solution with 1× PBS). Then the cells were treated with SBD-TPEA solution in a similar procedure described above and imaged. After the imaging,
the cells of exogenous Zn\textsuperscript{2+} were further treated with 25 µM TPEN solution (prepared by diluting the TPEN stock solution with 1× PBS) for 10 min to scavenge the intracellular Zn\textsuperscript{2+}. Then the cells were rinsed with 1× PBS and imaged. For all imaging, the samples were excited at 488 nm, and emissions were collected at 510 ~ 560 nm and 580 ~ 630 nm, respectively.

Colocalization experiments were carried out by co-staining the cells with Golgi dye BODIPY TR ceramide (Invitrogen) and SBD-TPEA. The cells were incubated in 5 µM BODIPY TR ceramide solution at 4 °C for 30 min, then the cells were washed with 1× PBS for 2 times followed by incubation with 1× PBS for 30 min at 37 °C. After rinse with 1× PBS for 2 times, the cells were further incubated with SBD-TPEA solution (10 µM) for 20 min at 25 °C. Finally the cells were washed with 1× PBS for 2 times before imaging. The ceramide marked images were obtained upon irradiation at 543 nm with a band path from 555 to 650 nm, while the SBD-TPEA stained images were obtained upon irradiation at 488 nm with a band path from 500-600 nm.

**Figure S15.** Confocal fluorescence images of intracellular Zn\textsuperscript{2+} in HepG2 cells with SBD-TPEA-staining. HepG2 cells incubated with SBD-TPEA (10 µM) at 25 °C for 20 min (top); the stained cells were exposed to 5 µM ZnSO\textsubscript{4}/2-mercaptopyridine-N-oxide solution at 25 °C for 5 min, followed by washing with SBD-TPEA solution (middle), the cells in middle further treated by TPEN solution (25 µM, 10 min, bottom). (a) Bright-field transmission images. (b, e, h) Fluorescence images obtained according to the emission collected at 510–560 nm. (c, f, i) Fluorescence images obtained according to the emission collected at 580–630 nm. (d, g, j) Ratiometric images generated from (b, e, h) and (c, f, i). \( \lambda_{\text{ex}} \), 488 nm.
Figure S16. Confocal fluorescence images of intracellular Zn$^{2+}$ in HeLa cells via SBD-TPEA-staining. HeLa cells incubated with SBD-TPEA (10 μM) at 25 °C for 20 min (top); the stained cells were exposed to 5 μM ZnSO$_4$/2-mercaptopyridine-N-oxide solution at 25 °C for 5 min followed by washing with SBD-TPEA solution (middle); the cells in middle further treated by TPEN solution (25 μM, 10 min, bottom). (a) Bright-field transmission images. (b, e, h) Fluorescence images obtained according to the emission collected at 510–560 nm. (c, f, i) Fluorescence images obtained according to the emission collected at 580–630 nm. (d, g, j) Ratiometric images generated from (b, e, h) and (c, f, i). $\lambda_{\text{ex}}$, 488 nm.

Fig. S17. Confocal fluorescence images of HeLa cells co-stained by 5 μM BODIPY TR ceramide and 10 μM SBD-TPEA. (a) Fluorescence image of cells visualized by green emission of SBD-TPEA ($\lambda_{\text{ex}}, 488$ nm); (b) fluorescence image visualized by BODIPY TR ceramide ($\lambda_{\text{ex}}, 543$ nm); (c) overlay of (a) and (b).

S10. Ratiometric in vivo Zn$^{2+}$ imaging in Zebrafish Larva stained by SBD-TPEA.

Zebrafish larvae were incubated in pure water produced by MilliQ system at 28.5 °C. PTU (Sigma) was added into the incubation media to depress the development of pigment after 8 h, and the final concentration was 0.003%. The fluorescence images of the larvae at certain development stage were obtained after incubation with 5 μM SBD-TPEA solution at 28.5 °C for 1.5 h followed by being embedded in methyl cellulose. Then, the head of the larva was imaged by a Zeiss LS-710 confocal fluorescence microscope. The imaging of Zn$^{2+}$-fed zebrafish larvae was also performed. The 3-day-old zebrafish larvae were fed with 100 μM Zn$^{2+}$ solution at 28.5 °C for 1 h. Then the larvae were washed with 1 × PBS three times followed by SBD-TPEA staining in a procedure similar to that described above. Then the larvae were imaged by the fluorescence microscope. After removing the SBD-TPEA solution, we rinsed the Zn$^{2+}$-fed zebrafish larvae three times with 1 × PBS. Then, these larvae were incubated further with 50 μM TPEN solution. After removing the TPEN solution and rinsing with PBS three times, we dyed the larvae by SBD-TPEA.
solution and imaged. All the confocal imaging of zebrafish larva were carried out on the larvae head after embedding the larvae in methyl cellulose with a Zeiss LS-710 confocal microscope equipped with a 20× objective. The samples were excited at 488 nm, and images were collected from the band paths 500 ~ 560 nm and 570 ~ 650 nm, respectively.

**Figure S18.** Confocal fluorescence ratiometric images (left) and the average ratio in regions of interest (ROIs) (right) of three-day-old zebrafish larva head at 28.5°C. (a) zebrafish larva incubated with SBD-TPEA (50 μM) at 28.5°C for 1.5 h; (b) zebrafish larva was fed in Zn²⁺ (100 μM) solution at 28.5°C for 1 h and then incubated with SBD-TPEA (50 μM) at 28.5°C for 1.5 h; (c) zebrafish larva was incubated with SBD-TPEA (50 μM) at 28.5°C for 1.5 h and then treated with TPEN (50 μM) at 28.5°C for 20 min.

**S11. Determination of chelatable [Zn²⁺] in Golgi apparatus of HepG 2 cells and zebrafish larva.**

The chelatable [Zn²⁺] on Golgi of HepG 2 cells and zebrafish larva were determined according to the following equation:

\[
R = \frac{R_{\text{min}} K_d + R_{\text{max}} [\text{Zn}^{2+}]}{(K_d + [\text{Zn}^{2+}])} \quad (\text{eq. 1})
\]

R is the ratio of Golgi apparatus obtained in the ratiometric images. \(R_{\text{max}}\) and \(R_{\text{min}}\) are the ratios of Golgi apparatus after treated with \(\text{ZnSO}_4/2\text{-mercaptopyridine-N-oxide}\) and TPEN solution respectively. \(K_d\) is the dissociation constant of SBD-TPEA/Zn²⁺ complex obtained in S7. All the data are the average values according to 10 independent cells.

**S12. References**

