Real-time detection and imaging for exogenous and endogenous Zn$^{2+}$ in PC12 cells model of depression with NIR fluorescence probe

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Table S1 Comparison of fluorescence probes for sensing Zn$^{2+}$.

Table S2 Photophysical properties of the rhb, ISO-DPA, ISO-DPA$^+$Zn$^{2+}$ in ethanol.
1. Comparison of fluorescence probes for sensing Zn\(^{2+}\)

### Table S1 Comparison of fluorescence probes for sensing Zn\(^{2+}\):

<table>
<thead>
<tr>
<th>Probe</th>
<th>(\lambda_{\text{ex}}/\lambda_{\text{em}}) (nm)</th>
<th>Stokes shift (nm)</th>
<th>Detection limit (nM)</th>
<th>Quantum yield</th>
<th>Application</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="NHSOON" /></td>
<td>360/532</td>
<td>172</td>
<td>42</td>
<td>1.5%-5.5%</td>
<td>HeLa cells</td>
<td>Inorg. Chem. Commun. 2011, 14, 304-307.</td>
</tr>
<tr>
<td><img src="image2.png" alt="NNBFN" /></td>
<td>568/578</td>
<td>10</td>
<td>480</td>
<td>NR-23%</td>
<td>MCF-7 cells, NSCs cells and HeLa cells</td>
<td>Biomater. Sci. 2014, 2, 89-97.</td>
</tr>
<tr>
<td><img src="image4.png" alt="OCOH" /></td>
<td>576/701</td>
<td>125</td>
<td>190</td>
<td>NR</td>
<td>HUVEC-C cells and MDA-MB231 cells</td>
<td>ACS Sens. 2016, 1, 1408-1415.</td>
</tr>
<tr>
<td><img src="image5.png" alt="OHN" /></td>
<td>444/536 (two-photon)</td>
<td>/</td>
<td>180</td>
<td>3%-23%</td>
<td>NIH 3T3 cells, mouse brain tissues</td>
<td>Chem. Commun. 2016, 52, 124-127.</td>
</tr>
<tr>
<td><img src="image6.png" alt="ONHN" /></td>
<td>390/525</td>
<td>135</td>
<td>41</td>
<td>45%-27%</td>
<td>HepG-2 cells</td>
<td>RSC Adv. 2017, 7, 40615-40620.</td>
</tr>
<tr>
<td><img src="image7.png" alt="NRO" /></td>
<td>475/600</td>
<td>125</td>
<td>NR</td>
<td>NR</td>
<td>C6 glioma cells and hippocampal slices</td>
<td>Sci. Rep. 2018, 8, 9069.</td>
</tr>
</tbody>
</table>
### 2. Synthesis of intermediate compounds

$$\text{O} + \text{NC-CN} \xrightarrow{\text{MeCN/piperidine/acetic acid}} \text{NC-CN}$$

**Scheme S1 Synthesis of compound ISO.**

Isophorone (6.91 g, 50 mmol) and malononitrile (3.96 g, 60 mmol) were dissolved in 50 mL
acetonitrile. Piperidine (0.5 mL, 5 mmol) and anhydrous acetic acid (0.30 g, 5 mmol) were added into the solution. The reaction mixture was refluxed at 80 °C for six hours and monitored by TLC. The solvent was removed under reduced pressure. Dichloromethane (50 mL) was added and the solution was washed with water (50 mL×2). The organic phase was collected and the solvent was removed under reduced pressure. The residue was purified by silica column using petroleum and dichloromethane (2:1) as eluent to give ISO as a pale-yellow solid (7.26 g, 78% yield). 1H NMR (400 MHz, CDCl3) δ(ppm): 6.62 (1H, s), 2.51 (2H, s), 2.18 (2H, s), 2.03 (3H, s), 1.01 (6H, s).

Scheme S2 Synthesis of compound ISO-OH.

The similar synthesis route of ISO-OH can refer to ref 1. ISO (0.75 g, 4 mmol) and p-hydroxybenzaldehyde (0.49 g, 4 mmol) were dissolved in 50 mL acetonitrile. Eight drops of piperidine was added into the solution. The reaction mixture was refluxed at 80 °C for six hours and monitored by TLC. The solvent was removed under reduced pressure. Dichloromethane (60 mL) was added and then washed with water (60 mL×2). The organic phase was collected and the solvent was removed under reduced pressure, and the residue was purified by silica column using petroleum and dichloromethane (1:1) as eluent to give ISO-OH as an orange solid (0.98 g, 85% yield). 1H NMR (400 MHz, DMSO-d6) δ(ppm): 10.00 (1H, s), 7.55 (2H, d, J = 8.4 Hz), 7.21 (2H, dd, J = 16.1 Hz, J = 16.2 Hz), 6.79 (2H, d, J = 8.5 Hz), 6.79 (1H, s), 2.60 (2H, s), 2.53 (2H, s), 1.01(6H, s).

Scheme S3 Synthesis of compound DPA.

The similar synthesis route of DPA can refer to ref 2. To a solution of 12.00 g (110.8 mmol) of 2-(aminomethyl) pyridine in 30 mL methanol, 12.00 g (112.0 mmol) of pyridine-2-carboxaldehyde in 30 mL methanol were slowly added at 0 °C. A dark yellow solution was obtained and stirred for 1
h at room temperature. Then cooled to 0 °C, 1.56 g (110.8 mmol) of NaBH₄ was added slowly to the solution and the reaction mixture was stirred for 12 h. After the reaction completed, the solution color changed into light yellow, and methanol was removed under reduced pressure. The solid product was dissolved in dilute hydrochloric and adjusted the pH to 3. The clear yellow solution was washed with dichloromethane (3 × 50 mL) to remove the unreacted substrates. A saturated solution of Na₂CO₃ was added to the aqueous layer to adjust the pH to 10. The solution was then extracted with dichloromethane (3 × 100 mL) and dried over magnesium sulfate. The solvent was removed under vacuum to obtain DPA as a yellow oil (14 g, 70% yield).

\[
\text{\(\delta(ppm):8.55(2H, d, J = 4.0 \text{ Hz}), 7.66 (2H, t, J = 8.0 \text{ Hz}), 7.36 (2H, d, J = 8.0 \text{ Hz}), 7.17 (2H, t, J = 4.0 \text{ Hz}), 4.0 (4H, s).\)}
\]

Scheme S4 Synthesis of compound ISO-DPA.

To a solution of DPA (0.19 g, 1 mmol) in 10 mL of acetonitrile, aqueous formaldehyde (37%) (0.11 mL, 1.2 mmol) was added. The mixture was heated at 60 °C for two hours. Then a solution of ISO-OH (0.29 g, 1 mmol) in 10 mL of acetonitrile was slowly added. The reaction mixture was refluxed at 60 °C for twelve hours monitored by TLC. The solvent was removed under reduced pressure. The residue was purified by silica column using petroleum and ether-ethyl acetate (1:2) as eluent to give ISO-DPA as an orange solid (0.27 g, 54% yield). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta(ppm):11.36 (1H, s), 8.54 (2H, d, J = 4.0 \text{ Hz}), 7.78 (2H, td, J = 8.0 \text{ Hz}, J = 1.7 \text{ Hz}), 7.66 (1H, d, J = 1.7 \text{ Hz}), 7.48 (1H, dd, J = 8.0 \text{ Hz}, J = 2.0 \text{ Hz}), 7.44 (2H, d, J = 7.8 \text{ Hz}), 7.30-7.15(4H, m), 6.82(1H, d, J = 8.4 \text{ Hz}), 6.78 (1H, s), 3.83 (4H, s), 3.74 (2H, s), 2.60 (2H, s), 2.53 (2H, s), 1.01 (6H, s). \(^{13}\)C NMR (100 MHz, CDCl₃) \(\delta(ppm):169.36, 159.96, 158.03, 154.73, 148.70, 137.47, 137.11, 130.12, 129.20, 126.70, 126.13, 123.79, 123.19, 122.42, 117.66, 113.90,113.11, 77.12, 58.77, 56.79, 43.05, 39.24, 32.04, 28.06. HRMS (ESI), m/z calculated for C\(_{32}\)H\(_{30}\)N\(_5\)O \([\text{M - H}^-]:500.2450, \text{ found: 500.2451}].\)
3. Preparation of ROS / RNS

The solutions of H$_2$O$_2$, ·OCI, ·OH, ONOO$^-$ were prepared according to ref 3.

**H$_2$O$_2$:** The concentration of H$_2$O$_2$ was determined from the absorption at 240 nm ($\varepsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$).

**·OCI:** The concentration of ·OCI was determined from the absorption at 292 nm ($\varepsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$).

**·OH:** Hydrogen peroxide (H$_2$O$_2$, 10 eq) was added to FeSO$_4$·7H$_2$O in ultrapure water, hydroxyl radical (·OH) was achieved by the Fenton reaction. The concentration of ·OH was determined by the concentration of FeSO$_4$·7H$_2$O.

**ONOO$:** KNO$_2$ (0.6 M, 10 mL), H$_2$O$_2$ (0.7 M, 10 mL) and HCl (0.6 M, 10 mL) were successively added to a NaOH solution (3 M, 10 mL) at 0 °C. The concentration of ONOO$^-$ was determined by the absorption at 302 nm ($\varepsilon =1670 \text{ cm}^{-1} \text{ M}^{-1}$) in 0.1 M NaOH solution.

4. Cytotoxicity of ISO-DPA

![Cell viability of PC12 cells incubated with probe ISO-DPA (100, 10, 1, 0 µM) at 37 °C for 24 h. Error bars represent the standard deviation (±S.D.) with n ≥3.](image)

**Fig. S1**

5. Determination of excitation wavelength of confocal laser scanning microscope via fluorescence spectra

The maximum absorption wavelength and maximum emission wavelength of ISO-DPA (10 µM) after adding Zn$^{2+}$ (10 µM) is 475 nm and 660 nm. And confocal laser scanning microscope has three
suitable excitation wavelengths including 488 nm, 541 nm and 561 nm. So we carried out fluorescence spectra measurement of ISO-DPA with three different excitation wavelengths. The following spectra show that ISO-DPA exhibits weakest fluorescence intensity at 561 nm excitation. We chose 561 nm as excitation wavelength of confocal laser scanning microscope to avoid the fluorescence of the probe itself in PC12 cells.

![Fluorescence spectra](image)

**Fig. S2** Fluorescence intensities of ISO-DPA (10 µM) before (black) and after (red) adding Zn$^{2+}$ (10 µM) in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1) (a): $\lambda_{ex} = 488$ nm, (b): $\lambda_{ex} = 514$ nm, (c): $\lambda_{ex} = 561$ nm.

6. NMR and MS data for compounds

![NMR spectrum](image)

**Fig. S3** The $^1$H NMR spectrum of compound ISO in CDCl$_3$. 
**Fig. S4** The $^1$H NMR spectrum of compound ISO-OH in DMSO.

**Fig. S5** The $^1$H NMR spectrum of compound DPA in CDCl$_3$. 
Fig. S6 The $^1$H NMR spectrum of compound ISO-DPA in DMSO.

Fig. S7 The $^{13}$C NMR spectrum of compound ISO-DPA in CDCl$_3$. 
7. Materials and instruments

All reactants and solvents (analysis level) used were get from commercial suppliers. The products were purified by column chromatography. The Ultraviolet-visible light (UV-vis) absorption spectra were performed on Varian Cary 500 spectrophotometer and fluorescence spectra were performed on Agilent Cary Eclipse spectrophotometer. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker AM-400 spectrometer. High resolution mass spectra were recorded on a Waters LCT Permiex XE spectrometer. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry was performed on AB SCIEX 4800 Plus MALDI TOF/TOF™. The fluorescence
quantum yields were determined with rhodamine B ($\Phi_f = 0.89$ in ethanol) as reference. The measurements of pH were done using a pH-10C digital pH meter.

8. Measurement of relative fluorescence quantum yield

The fluorescence quantum yield ($\Phi_f$) of the related compounds in ethanol was determined by using rhodamine B as a reference with a known $\Phi_f$ value of 0.89 in ethanol. The calculation formula is listed as follow:

$$\Phi_{f,\text{sample}} = \Phi_{f,\text{rhb}} \times \frac{F_{\text{sample}}}{F_{\text{rhb}}} \times \frac{\text{Abs}_{\text{rhb}}}{\text{Abs}_{\text{sample}}}$$

Where $\Phi_{f,\text{sample}}$ is fluorescence quantum yield of the samples to be tested; $\Phi_{f,\text{rhb}}$ is fluorescence quantum yield of rhodamine B; $\text{Abs}_{\text{rhb}}$ is absorbance of rhodamine B; $F_{\text{rhb}}$ is the area under the fluorescence spectra of rhodamine B; $\text{Abs}_{\text{sample}}$ is absorbance of the test samples; $F_{\text{sample}}$ is the area under the fluorescence spectra of the test sample.

### Table S2 Photophysical properties of the rhl, ISO-DPA, ISO-DPA+Zn²⁺ in ethanol:

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>Abs</th>
<th>$\Phi_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhl</td>
<td>29281.3600</td>
<td>0.0346</td>
<td>0.89</td>
</tr>
<tr>
<td>ISO-DPA</td>
<td>3773.2577</td>
<td>0.0474</td>
<td>0.083</td>
</tr>
<tr>
<td>ISO-DPA+Zn²⁺</td>
<td>14025.1053</td>
<td>0.0330</td>
<td>0.44</td>
</tr>
</tbody>
</table>

9. Job’s plot

![Job's plot](image)

Fig. S10 Job’s plot of the complexation of fluorescence probe ISO-DPA with Zn²⁺ was plotted as function of the different molar ratio $[\text{Zn}²⁺]/([\text{Zn}²⁺] + [\text{ISO-DPA}])$ by fluorescence method, indicating the 1:1 stoichiometry. The total concentration of ISO-DPA and Zn²⁺ was constantly maintained in 20 µM in a mixture solution of HEPES...
buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). \( \lambda_{\text{ex}} = 475 \, \text{nm}, \lambda_{\text{em}} = 660 \, \text{nm} \).

10. Determination of binding constant

Binding constant (\( K_a \)) was calculated from the fluorescence titration experiment based on the modified Benesi-Hildbrand plot. Fluorescence intensities were obtained from the titration curves of 10 µM ISO-DPA in the presence of different concentration of metal ions at 660 nm in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). \( K_a \) was calculated according to the following equation:

$$\frac{1}{K_a[M]}(I_t - I_0) + \frac{1}{I_t - I_0} = \frac{1}{I - I_0}$$

“\( I \)” stands for the fluorescence intensity of ISO-DPA. \( I_t \) refers to the fluorescence intensity at saturated metal ion concentration. \( I_0 \) means the fluorescence intensity in the absence of metal ion. \( K_a \) is the binding constant and [M] is the concentration of metal ion.

According to the plot of fluorescence intensity versus Zn\(^{2+}\), Fe\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\) concentration, \( K_a \) is calculated as 1.3×10\(^5\) M\(^{-1}\), 3.9×10\(^5\) M\(^{-1}\), 6.3×10\(^4\) M\(^{-1}\), 4.9×10\(^5\) M\(^{-1}\), 1.3×10\(^5\) M\(^{-1}\) respectively.

Fig. S11 Benesi-Hildbrand equation plot of 10 µM of ISO-DPA with Zn\(^{2+}\) ions obtained from fluorescence titration data. The binding constant was determined to be 1.3×10\(^5\) M\(^{-1}\). \( \lambda_{\text{ex}} = 475 \, \text{nm}, \lambda_{\text{em}} = 660 \, \text{nm} \).
Fig. S12 Benesi-Hildebrand equation plot of 10 µM of ISO-DPA with Fe$^{2+}$ ions obtained from fluorescence titration data. The binding constant was determined to be $3.9 \times 10^5$ M$^{-1}$. $\lambda_{ex} = 475$ nm, $\lambda_{em} = 660$ nm.

Fig. S13 Benesi-Hildebrand equation plot of 10 µM of ISO-DPA with Co$^{2+}$ ions obtained from fluorescence titration data. The binding constant was determined to be $6.3 \times 10^4$ M$^{-1}$. $\lambda_{ex} = 475$ nm, $\lambda_{em} = 660$ nm.
Fig. S14 Benesi-Hildebrand equation plot of 10 µM of ISO-DPA with Ni$^{2+}$ ions obtained from fluorescence titration data. The binding constant was determined to be $4.9 \times 10^5$ M$^{-1}$. $\lambda_{ex} = 475$ nm, $\lambda_{em} = 660$ nm.

Fig. S15 Benesi-Hildebrand equation plot of 10 µM of ISO-DPA with Cu$^{2+}$ ions obtained from fluorescence titration data. The binding constant was determined to be $1.3 \times 10^5$ M$^{-1}$. $\lambda_{ex} = 475$ nm, $\lambda_{em} = 660$ nm.
11. Time courses of the fluorescence responses

![Graph showing time courses of fluorescence responses](image)

**Fig. S16** Photostability of ISO-DPA (10 μM) in the absence and presence of Zn\(^{2+}\) (10 μM) in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). \(\lambda_{\text{ex}} = 475\) nm, \(\lambda_{\text{em}} = 660\) nm.

12. Selectivity of ISO-DPA

![Absorption and fluorescence spectra](image)

**Fig. S17** Absorption spectra and fluorescence spectra of ISO-DPA (10 μM) in the presence of 100 μM of various ions including (Na\(^+\), Mg\(^{2+}\), Al\(^{3+}\), K\(^+\), Ca\(^{2+}\), Fe\(^{2+}\), Fe\(^{3+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Cd\(^{2+}\), Pb\(^{2+}\), F\(^-\), Cl\(^-\), Br\(^-\), I\(^-\), AcO\(^-\), CO\(_3^{2-}\)) and 10 μM of Zn\(^{2+}\) in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and (V/V = 1:1). \(\lambda_{\text{ex}} = 475\) nm.

![Absorption and fluorescence spectra](image)

**Fig. S18** Absorption spectra and fluorescence spectra of ISO-DPA (10 μM) in the presence of 100 μM of ROS,
RNS and RSS species including (H$_2$O$_2$, ‘OCl, ‘OH, ONOO’, HS’, HSO$_3^-$, S$_2$O$_3^{2-}$, S$_2$O$_5^{2-}$, GSH, Cys, Hcy) and 10 μM of Zn$^{2+}$ in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and (V/V = 1:1). $\lambda_{ex} = 475$ nm.

13. Demetalation of ISO-DPA-Zn$^{2+}$ using TPEN

![Fig. S19](image)

Fig. S19 (a) UV-vis spectra of ISO-DPA (10 μM) upon adding different concentration of TPEN (0-10 μM). (b) Fluorescence spectra changes of ISO-DPA after adding TPEN (0-10 μM). All tests were performed in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). $\lambda_{ex} = 475$ nm.

References

