Supporting Information

A “turn-on” fluorescent probe for detection of Cu$^{2+}$ in living cells based on signaling mechanism of N=N isomerization

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1. Comparison of representative “turn-on” probes for Cu$^{2+}$

**Table S1** Comparison of “turn-on” fluorescent probes for Cu$^{2+}$ based on various mechanisms

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sensing mechanism</th>
<th>Medium</th>
<th>LOD (nM)</th>
<th>Optimal pH range</th>
<th>Biological application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Cu$^{2+}$-binding blocked PET" /></td>
<td>Cu$^{2+}$-binding blocked PET</td>
<td>H$_2$O containing 50% CH$_3$CN</td>
<td>7.81</td>
<td>5.5 - 8.5</td>
<td>Living cells</td>
<td>1</td>
</tr>
<tr>
<td><img src="image2" alt="Complexation with Cu$^{2+}$ blocked PET and restricted C=N isomerization" /></td>
<td>Complexation with Cu$^{2+}$ blocked PET and restricted C=N isomerization</td>
<td>80% aqueous CH$_3$CN (PB/PBS)</td>
<td>~</td>
<td>5 - 8</td>
<td>Living cells, living organs</td>
<td>2</td>
</tr>
<tr>
<td><img src="image3" alt="Cu$^{2+}$ promoted hydrolysis" /></td>
<td>Cu$^{2+}$ promoted hydrolysis</td>
<td>Tris-HCl containing 1% DMSO</td>
<td>35</td>
<td>7</td>
<td>Fetal equine serum</td>
<td>3</td>
</tr>
<tr>
<td><img src="image4" alt="Cu$^{2+}$ promoted oxidation" /></td>
<td>Cu$^{2+}$ promoted oxidation</td>
<td>HEPES containing 50% CH$_3$CN</td>
<td>58</td>
<td>4 - 11</td>
<td>~</td>
<td>4</td>
</tr>
<tr>
<td><img src="image5" alt="Cu$^{2+}$ induced ring opening of rhodamine spirolactam" /></td>
<td>Cu$^{2+}$ induced ring opening of rhodamine spirolactam</td>
<td>HEPES containing 40% ethanol-</td>
<td>160</td>
<td>5 - 9</td>
<td>Living cells</td>
<td>5</td>
</tr>
<tr>
<td><img src="image6" alt="Coordination with Cu$^{2+}$ inhibited PET" /></td>
<td>Coordination with Cu$^{2+}$ inhibited PET</td>
<td>CH$_3$CN</td>
<td>360</td>
<td>~</td>
<td>~</td>
<td>6</td>
</tr>
<tr>
<td><img src="image7" alt="Cu$^{2+}$-binding blocked PET" /></td>
<td>Cu$^{2+}$-binding blocked PET</td>
<td>PBS containing 40% CH$_3$CN</td>
<td>40</td>
<td>5 - 10</td>
<td>Living cells</td>
<td>7</td>
</tr>
<tr>
<td><img src="image8" alt="Cu$^{2+}$-catalyzed cyclization suppressed N=N isomerization" /></td>
<td>Cu$^{2+}$-catalyzed cyclization suppressed N=N isomerization</td>
<td>PBS containing 10% CH$_3$CN</td>
<td>20</td>
<td>3 - 10</td>
<td>Living cells</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Note:* LOD means limit of detection, and ~ means not mentioned.
2. Synthesis and characterization of compounds

7-(diethylamino)-3-nitro-2H-chromen-2-one (3)

3 was prepared from 4-diethylamino salicylaldehyde via a one-pot reaction. A mixture of 4-diethylamino salicylaldehyde (483 mg, 2.5 mmol), ethyl nitroacetate (399 mg, 3 mmol), catalytic amount of piperidine (0.1 mL) and acetic acid (0.2 mL) were dissolved in n-butanol (10 mL) in a 50 mL round-bottomed flask. The reaction system was refluxed with stirring for 24 h, then cooled to room temperature, followed by adding ice-water into it. Next, the mixture was filtered and washed with cold n-butanol to collect the solid as the crude product, which was further purified by recrystallization from petroleum ether/ethyl acetate to afford the purified 3 as a bright yellow solid (73% yield). HRMS: Mr calculated for C_{13}H_{14}N_{2}O_{4}, 262.27, found m/z 263.1038 [M + H]^+, 285.0858 [M + Na]^+. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz): \delta 1.17 (t, J = 7.2 Hz, 6 H), 3.43 (q, J = 7.2 Hz, 4 H), 6.39 (s, 1 H), 6.65 (d, J = 6.6 Hz, 1 H), 7.37 (d, J = 9.3 Hz, 1 H), 8.60 (s, 1 H); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75 MHz): \delta 12.56, 45.92, 96.97, 106.54, 111.66, 126.96, 132.99, 143.72, 153.68, 155.12, 159.19.

3-amino-7-(diethylamino)-2H-chromen-2-one (4)

In a 50 ml round-bottomed flask were taken 3 (47 mg, 0.18 mmol), SnCl\textsubscript{2}·2H\textsubscript{2}O (162 mg, 0.72 mmol), ethanol (5 mL) and ethyl acetate (2.5 mL). The reaction system was stirred and refluxed for about 4 h before cooled to room temperature, then ice-water (20 mL) was poured into the system. 5 M NaOH was added dropwise into the system to neutralize the excess acid and adjust pH to 12. The mixture was filtered through diatomaceous earth to collect the aqueous phase, which was extracted with ethyl acetate for three times. The combined organic layer was washed with saturated brine, dried over anhydrous Na\textsubscript{2}SO\textsubscript{4} and evaporated \textit{in vacuo} to obtain the crude product, which was purified twice by column chromatography to give 4 as a yellow solid (61.6%, yield). HRMS: Mr calculated for C_{13}H_{16}N_{2}O_{3}, 232.12, found m/z 233.12 [M + H]^+. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz): \delta 1.16 (t, J = 6.9 Hz, 6 H), 3.35 (q, J = 6.9 Hz, 4 H), 4.16 (s, 2 H), 6.50 (s, J = 1.8 Hz, 1 H), 6.54 (d, J = 6.6 Hz, 1 H), 6.70 (s, 1 H), 7.08 (d, J = 8.4 Hz, 1 H); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75 MHz): \delta 11.41, 28.92, 43.92, 96.94, 107.79, 108.92, 115.29, 126.50, 135.40, 147.62, 151.08, 160.29.

\textit{(E)}-3-((2, 4-diaminophenyl)diazenyl)-7-(diethylamino)-2H-chromen-2-one (A)
To a stirred MeCN solution (2 mL) of 4 (130 mg, 0.56 mmol) was added dropwise HCl (4 equiv.) and water (3.6 mL). After 0.5 h, the resulting salt solution was kept in ice salt bath for further use. A pre-cold aqueous solution (100 μL) of NaNO₂ (43 mg, 0.63 mmol) was added dropwise into the above solution, during which some crushed ice was added into the system to ensure the temperature below -2 °C. The reaction was stirred for 1 h in an ice salt bath. Then the resulted diazonium salt solution was added slowly into the solution of m-phenylenediamine (67 mg, 0.62 mmol), and pH was adjusted to 6 with Na₂CO₃. The system was stirred for 3 h while the temperature was maintained at 3 - 5 °C. Water (50 mL) was added to quench the reaction, inducing precipitation of a deep purple solid, which was filtered, washed with water and purified by column chromatography to furnish A (63.4% yield). HRMS: Mr calculated for C₁₉H₂₁N₅O₂, 351.17, found m/z 352.17 [M + H]⁺. 

1H NMR (CDCl₃, 300 MHz): δ 1.23 (t, J = 6.9 Hz, 6 H), 3.43 (q, J = 7.2 Hz, 4 H), 5.89 (d, J = 2.4 Hz, 1 H), 6.16 (dd, J = 6.3 Hz, 1 H), 6.55 (d, J = 2.4 Hz, 1 H), 6.61 (dd, J = 6.3 Hz, 1 H), 7.38 (d, J = 8.7 Hz, 1 H), 7.60 (d, J = 8.7 Hz, 1 H), 7.92 (s, 1 H), without four active hydrogens showed on it. 

13C NMR (CDCl₃, 75 MHz): δ 12.27, 12.36, 14.03, 54.86, 59.71, 96.21, 96.50, 106.11, 108.19, 109.57, 122.62, 130.45, 130.57, 132.97, 146.37, 46.56, 149.87, 153.03, 155.11, 159.87.

3-(5-aminoo-2H-benzo[d][1,2,3]triazol-2-yl)-7-(diethylamino)-2H-chromen-2-one (B)

A stirred MeCN solution of A and Cu(OAc)₂·H₂O (5 equiv.) was heated at 40 °C for 20 min. The system was quenched with water and extracted with ethyl acetate for three times. The combined extracts were washed with saturated brine, dried over anhydrous Na₂SO₄ and concentrated. Purified by column chromatography, B was obtained as a bright yellow solid. HRMS: Mr calculated for C₁₉H₁₉N₅O₂, 349.15, found m/z 350.16 [M + H]⁺.
3. $^1$H NMR, $^{13}$C NMR and HRMS of compounds

Fig. S1 $^1$H NMR of 7-(diethylamino)-3-nitro-2H-chromen-2-one (3)
Fig. S2 $^{13}$C NMR of 7-(diethylamino)-3-nitro-2H-chromen-2-one (3)

Fig. S3 HRMS of 7-(diethylamino)-3-nitro-2H-chromen-2-one (3)
Fig. S4 $^1$H NMR of 3-amino-7-(diethylamino)-2H-chromen-2-one (4)

Fig. S5 $^{13}$C NMR of 3-amino-7-(diethylamino)-2H-chromen-2-one (4)
Fig. S6 HRMS of 3-amino-7-(diethylamino)-2H-chromen-2-one (4)

Fig. S7 $^1$H NMR of (E)-3-((2, 4-diaminophenyl)diazenyl)-7-(diethylamino)-2H-chromen-2-one (A)
Fig. S8 $^{13}$C NMR of (E)-3-((2, 4-diaminophenyl)diazenyl)-7-(diethylamino)-2H-chromen-2-one (A)

Fig. S9 HRMS of (E)-3-((2, 4-diaminophenyl)diazenyl)-7-(diethylamino)-2H-chromen-2-one (A)
Fig. S10 HRMS of 3-(5-amino-2H-benzo[d][1,2,3]triazol-2-yl)-7-(diethylamino)-2H-chromen-2-one (B)

4. Cell imaging

Fig. S11 Fluorescence imaging of Cu²⁺ in HepG2 cells incubated with probe A. (a - c) Bright-field images of HepG2 cells preloaded with probe A (5 μM) in a mixture of PBS (0.01 M, pH 7.4) and acetonitrile (9: 1, v/ v) for 30 min at 37 °C, then treated with increasing concentrations of Cu²⁺ in PBS (0.01 M, pH 7.4) for additional 30 min: (a) 5 μM, (b) 20 μM, (c) 50 μM. (d - f) Fluorescence images in blue channel of HepG2 cells preloaded with probe A (5 μM) in a mixture of PBS (0.01 M, pH 7.4) and acetonitrile (9: 1, v/ v) for 30 min at 37 °C, then treated with increasing concentrations of Cu²⁺ in PBS (0.01 M, pH 7.4) for additional 30 min: (d) 5 μM, (e) 20 μM, (f) 50 μM. Scale bar represents 100 μm.

5. References

5. C. Yu, T. Wang, K. Xu, J. Zhao, M. Li, S. Weng and J. Zhang, *Dyes Pigm.*, 2013, **96**, 38-44.