Supporting Information (SI)

Near-infrared mito-specific fluorescent probe for ratiometric detection and imaging of alkaline phosphatase activity with high sensitivity

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1. Synthesis and characteristic of probe Cy-OP.

\[ \text{Scheme S1 Synthesis route of Cy-OP.} \]

Synthesis of Cy-7.

Cy-7 was synthesized according to the literature.[1]

Synthesis of Cy-O.

Compound Cy-O was also synthesized according to the literature.[2]

To 0.638 g (1.0 mmol) of Cy-7 and 0.245 g (1.5 mmol) of sodium acetate was added 20 mL of anhydrous DMF. The contents were heated at 80 °C for 4 h in nitrogen atmosphere after which the heating was discontinued and allowed to cool to r.t. Solvents were distilled off in vacuo and the resulting residue was purified by the silica gel chromatography (CH\_2Cl\_2 and MeOH 100:1), compound Cy-O was obtained as a red solid (0.374 g, 76%).

Synthesis of Cy-OP.

Cy-O (0.246 g, 0.5 mmol) was stirred into dry CH\_2Cl\_2 (50 mL) at 0 °C. And POCl\_3 (0.2 mL) was added slowly though syringe. After that, the reaction solution was stirred at room temperature for 1 h in nitrogen atmosphere. Then, ice water (50 mL) was added, and the reaction solution was extracted with CH\_2Cl\_2 (3 \times 30ml). The combined organic phase was dried with Na\_2SO\_4, and concentrated under reduced pressure. After purified by the silica gel chromatography (CH\_2Cl\_2/MeOH, 3:1, v/v), the pure compound Cy-OP was obtained as a green solid (0.170 g, 56%). FT-IR: (KBr, cm\(^{-1}\)) \(v\): 3438 (OH), 3050 (ArH), 2964, 2930, 2866 (Alkyl CH), 1626 (C=N), 1577, 1509, 1455 (ArC=C), 1432 (O=P-OH), 1318 (C–O), 1256 (P=O), 1210 (C–N), 1074
(C–C). $^1$H NMR (500 MHz, DMSO-$d_6$) δ (ppm) δ 8.36 (d, $J = 14.0$ Hz, 1H), 8.25 (d, $J = 14.1$ Hz, 1H), 7.56 (t, $J = 6.6$ Hz, 2H), 7.43 – 7.29 (m, 4H), 7.20 (dt, $J = 14.2$, 7.2 Hz, 2H), 6.09 (dd, $J = 24.4$, 14.2 Hz, 2H), 4.21 – 4.11 (m, 4H), 2.58 (s, 4H), 1.79 (d, $J = 5.7$ Hz, 2H), 1.66 (d, $J = 6.2$ Hz, 12H), 1.27 (t, $J = 7.0$ Hz, 6H). $^{13}$C NMR (126 MHz, DMSO-$d_6$): δ (ppm) 171.62 (s), 161.26 (s), 145.62 – 143.91 (m), 143.32 (d, $J = 112.2$ Hz), 142.17 (s), 141.44 (d, $J = 93.7$ Hz), 128.84 (s), 124.94 (s), 122.85 (s), 122.11 (s), 111.13 (s), 100.01 (s), 49.20 (s), 27.24 (s), 24.62 (s), 12.39 (s). $^{31}$P NMR (202 MHz, DMSO-$d_6$): δ (ppm) -5.49 (s). ESI-MS m/z: [M+CH$_3$OH-H$_2$O]$^+$ Calcd for C$_{35}$H$_{44}$N$_2$O$_4$P$^+$ 587.3033; Found 587.3013.
2. $^1$H, $^{13}$C, and $^{31}$P NMR spectra of probe Cy-OP.

Figure S1 $^1$H NMR spectrum of Cy-OP in DMSO-$d_6$.

Figure S2 $^{13}$C NMR spectrum of Cy-OP in DMSO-$d_6$. 
Figure S3 $^{31}$P NMR spectrum of Cy-OP in DMSO-$d_6$.

3. Spectral titration profiles

Figure S4 Normalized absorbance (red line) and fluorescence (blue line) spectra of Cy-O (solid line) and Cy-OP (dotted line) in TBS buffer. $\lambda_{\text{ex}}$: 516 nm for Cy-O and 700 nm for Cy-OP.
Figure S5 Absorption spectra of Cy-OP in different concentrations from 1 to 18 μM in 10 mM TBS buffer of pH 8.0. Inset: Plots of absorbance at 736 nm versus [Cy-OP].

Figure S6 Fluorescence spectra of Cy-OP in different concentrations from 1 to 20 μM in 10 mM TBS buffer of pH 8.0. Inset: Plots of intensity at 766 nm versus [Cy-OP], λex = 700 nm.[3]
Figure S7 Plots of $I_{616\text{ nm}}/I_{766\text{ nm}}$ of Cy-OP without (black squares) or with (red dots) 200 mU/mL ALP versus solution pH between 5.0 and 9.5. Solution pH was tuned by TBS buffer. [Cy-OP] = 5 μM, $\lambda_{\text{ex}} = 516$ nm.
Figure S8 Plots of $A_{516\text{ nm}}/A_{736\text{ nm}}$ (a) and $I_{616\text{ nm}}/I_{766\text{ nm}}$ (b) of Cy-OP in TBS buffer solution pH 8.0 upon addition of ALP from 0 to 120 min at different temperatures (0 °C: black line; 10 °C: red line; 20 °C: blue line; 37 °C: pink line; 45 °C: green line). $[\text{Cy-OP}] = 5 \mu M$, $[\text{ALP}] = 400 \text{ mU/mL}$, $\lambda_{\text{ex}} = 516 \text{ nm}$.

Figure S9 Time-dependent absorption (a) and fluorescence (c) spectra of Cy-OP in TBS buffer solution of pH 8.0 at 37 °C from 0 to 120 min. Plots of $A_{736\text{ nm}}$ (b) and $I_{616\text{ nm}}/I_{766\text{ nm}}$ (d) versus time. $[\text{Cy-OP}] = 5 \mu M$, $\lambda_{\text{ex}} = 516 \text{ nm}$.

Figure S10 Plots of $A_{736\text{ nm}}/A_{516\text{ nm}}$ versus ALP concentration from 0 to 300 mU/mL in TBS buffer solution pH 8.0. $[\text{Cy-OP}] = 5 \mu M$. 

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Figure S11 Fluorescence spectra of Cy-OP incubated with ALP of increasing concentration from 0 to 300 mU/mL for 60 min in 10 mM TBS buffer of pH 8.0, $\lambda_{\text{ex}}$ = 516 nm (a) and 700 nm (b). [Cy-OP] = 5 μM.

Figure S12 Plots of $I_{766\text{ nm}}/I_{616\text{ nm}}$ versus ALP concentration from 0 to 300 mU/mL in TBS buffer solution pH 8.0. [Cy-OP] = 5 μM, $\lambda_{\text{ex}}$ = 516 nm.
**Figure S13** Lineweaver-Burk plot for the enzyme-catalyzed reaction of Cy-OP. The Michaelis-Menten equation was described as: \( V = \frac{V_{\text{max}} \cdot [\text{probe}]}{K_m + [\text{probe}]} \), where \( V \) is the initial reaction rate, \([\text{probe}]\) is the probe concentration (substrate), and the \( K_m \) is the Michaelis constant. Conditions: 400 mU/mL ALP, 1, 2, 4, 5, 8, 10 \( \mu \text{M} \) of Cy-OP. The measurements were performed at 37°C.

**Figure S14** \(^{31}\)P NMR spectra of Cy-OP (a), Cy-OP incubated with 400 mU/mL ALP for 4h at 37°C (b) and NaH\(_2\)PO\(_4\) (c) in the mixture of DMSO-\(d_6\) and D\(_2\)O (v/v: 4/6).
**Figure S15** HRMS spectrum of Cy-OP with ALP incubated for 1h at 37 °C in TBS buffer. [Cy-OP] = 5 μM, [ALP] = 400 mU/mL.

**Figure S16** Absorbance (a) and fluorescence spectra of of Cy-OP with 400 mU mL⁻¹ ALP pretreated with different concentrations of Na₃VO₄ from 0 to 300 μM in 10 mM TBS buffer pH 8.0. ALP was incubated with Na₃VO₄ for 30 min at 37°C. Inset: Plots of A 516 nm/A 736 nm (a) and I 616 nm/I 766 nm (b) versus the concentration of Na₃VO₄. [Cy-OP] = 5 μM, λₑₓ = 516 nm.
Figure S17 Absorbance (a) and fluorescence spectra of of Cy-OP with 400 mU mL$^{-1}$ ALP pretreated with different concentrations of NaH$_2$PO$_4$ from 0 to 1.0 mM in 10 mM TBS buffer pH 8.0. ALP was incubated with NaH$_2$PO$_4$ for 30 min at 37°C. Inset: Plots of A$_{516 \text{ nm}}$/A$_{736 \text{ nm}}$ (a) and I$_{616 \text{ nm}}$/I$_{766 \text{ nm}}$ (b) versus the concentration of NaH$_2$PO$_4$. [Cy-OP] = 5 μM, λ$_{ex}$ = 516 nm.

Figure S18 I$_{616 \text{ nm}}$/I$_{766 \text{ nm}}$ of Cy-OP in 10 mM TBS buffer pH 8.0 in the presence of 400 mU / mL ALP upon addition of 100 μM of competition species from 1 to 20: none, Na$^+$, K$^+$, Mg$^{2+}$, Fe$^{3+}$, Al$^{3+}$, Ca$^{2+}$, F$^-$, Cl$^-$, Br$^-$, I$^-$, HCO$_3^-$, CO$_3^{2-}$, NO$_3^-$, SO$_4^{2-}$, AcO$^-$, BSA, telomerase, lysozyme, and inorganic pyrophosphatase. [Cy-OP] = 5 μM, λ$_{ex}$ = 516 nm.
**Figure S19** Cytotoxicity of Cy-OP against HeLa cells as determined by CCK-8 (Cell Counting kit-8) assay: HeLa cells were treated with Cy-OP (2-30 μM) for 1 to 6 hours.

**Figure S20** Fluorescence images of mitochondria in HeLa cells. HeLa cells were incubated with Cy-OP (3 μM) or Mito Tracker Green (200 nM) for 30 min, respectively. Emission from the NIR channel (Cy-OP, $\lambda_{ex} = 633$ nm, $\lambda_{em} = 740$−800 nm), emission from the green channel (Mito Tracker Green, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 500$−540 nm), Scale bar = 25 μm.
4. References

