# Electronic supplementary information

## Analysis of Protein Phosphorylation in Solution and Cells Combining ATP Analogue with Fluorescent Techniques

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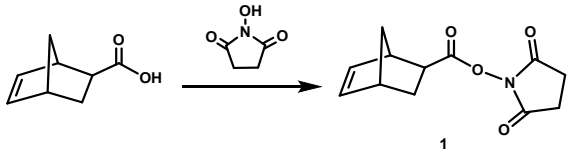
## Synthesis of Compounds

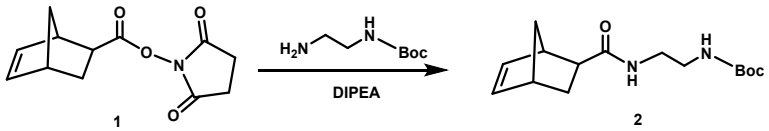
### 2,5-dioxopyrrolidin-1-yl (1R,4R)-bicyclo[2.2.1]hept-5-ene-2-carboxylate (1)



1

**1** was synthesized according to literature.[1] Dissolved 5-norbornene-2-carboxylic acid (9.97 g, 0.07 mol) and N-hydroxysuccinimide (9.12 g, 0.08 mol) in 150 mL of $CH_2Cl_2$ at 0 $^oC$, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCl) was added dropwise, and the reaction was stirred overnight. The reaction mixture was washed with 1 M HCl followed by NaCl (sat.) three times, and the organic layer was dried with $Na_2SO_4$. The product was purified by column chromatography, and a white solid compound **1** (8.5 g, yield: 84.4%) was obtained. $^1H$ NMR (400 MHz, $CDCl_3$): δ 6.24 (dd, *J* = 8.0, 4.0 Hz, 1H), 6.12 (dd, *J* = 8.0, 4.0 Hz, 1H), 3.26-3.22 (m, 1H), 2.99 (bs, 1H), 2.83-2.80 (m, 4H), 2.52-2.49 (m, 1H), 2.04-1.98 (m, 1H), 1.52-1.47 (m, 3H). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 169.96, 169.31, 138.13, 132.17, 49.66, 46.41, 42.53, 40.62, 29.58, 25.62. Calculated for $C_{12}H_{13}NO_4Na$ $[M+Na]^+$ 258.0742 found 258.0747.

### tert-butyl-(2-((1R,4R)-bicyclo[2.2.1]hept-5-ene-2-carboxamido)ethyl)carbamate (2)

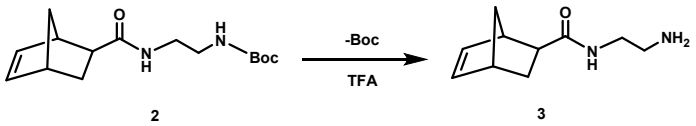



1 2

**2** was synthesized according to literature.[2] Dissolve **1** (500.00 mg, 2.13 mmol) with 3 mL of $CH_2Cl_2$, and slowly add mono-Boc protected ethylenediamine (410.14 mg, 2.56 mmol) and DIPEA (413.57 mg, 3.20 mmol), the reaction was stirred overnight. The reaction mixture was wash 3 times with water, and organic layer dried with $Na_2SO_4$. The product was purified by column chromatography (450 mg, yield: 75.5%). $^1H$ NMR (400 MHz, $CDCl_3$): δ 6.22 (dd, *J* = 8.0, 4.0 Hz, 1H), 6.13 (bs, 1H), 5.96 (dd, *J* = 8.0, 4.0 Hz, 1H), 4.93 (bs, 1H), 3.31-3.24 (m, 4H), 3.31 (s, 1H), 2.90-2.85 (m, 2H), 1.94-1.88 (m, 1H), 1.44 (s, 9H), 1.35-1.29 (m, 2H). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 175.27, 157.17, 137.95, 132.47, 79.89, 50.17, 46.33, 44.97, 42.89, 40.94, 40.50, 29.89, 28.58. Calculated for $C_{15}H_{25}N_2O_3$ $[M+H]^+$ 281.1820 found

281.1873.

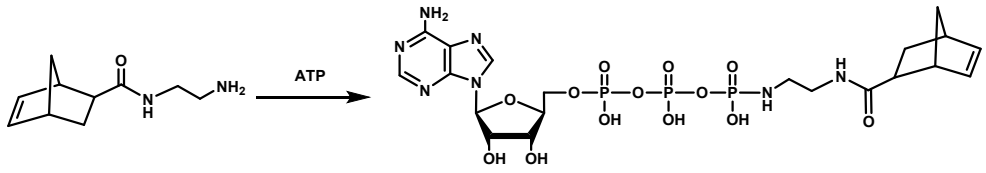
**(1R,4R)-N-(2-aminoethyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (3)**




2 3

In the $CH_2Cl_2$ solution of compound **2** (200.00 mg, 0.71 mmol), 4 mL of a mixed solution of TFA and $CH_2Cl_2$ ($V_{TFA}:V_{DCM}=1:1$) was added dropwise, the reaction was Stirring for 2 h. The product obtained is a white oily liquid after rotary evaporation (120 mg, yield>95%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 6.09 (dd, $J$ = 8.0, 4.0 Hz, 1H), 5.83 (dd, $J$ = 8.0, 4.0 Hz, 1H), 3.29-3.19 (m, 2H), 3.13 (s, 1H), 2.87-2.81 (m, 5H), 1.77-1.71 (m, 1H), 1.44 (s, 9H), 1.33-1.22 (m, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 174.31, 137.39, 137.39, 49.76, 45.88, 43.86, 42.51, 39.17, 37.02, 28.85. Calculated for $C_{10}H_{17}N_2O$ $[M+H]^+$ 181.1296 found 181.1339.

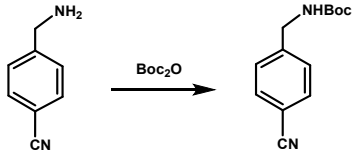
**ATP analogue ATP-NB (4)**




3 4

Dissolved disodium salt of ATP (27.60 mg 0.05 mmol) in 5 mL water, and adjust the pH to 7.0 with 1 M NaOH. Then EDCl (383.40 mg, 2.00 mmol) was add, the Ph was adjust to 5.6-5.8 with 1M HCl. At this time, compound **3** (125.70 mg 0.70 mmol) dissolved in water was added to the previous mixed solution, and the mixed solution was stirred for 4 h. The pH value of the solution should be controlled between 5.6-5.8 during the reaction. After the reaction is over, adjust the pH to 8.5 with 25% ammonium hydroxide. The product was separated and purified by Hilic-Amind semi-preparative column, and the obtained compound **4**, ATP-NB (11.04 mg, yield: 33%) was lyophilized at low temperature, and Store at -80 $^o$C. $^1$H NMR (400 MHz, $D_2O$): δ 8.44 (s, 1H), 8.14 (s, 1H), 7.14 (s, 2H), 6.08 (dd, $J$ = 8.0, 4.0 Hz, 1H), 6.04 (d, $J$ = 8.0 Hz, 1H), 5.77 (dd, $J$ = 8.0, 4.0 Hz, 1H), 4.45 (s, 2H), 4.29 (s, 2H), 4.14 (s, 2H), 3.11-2.97 (m, 11H), 1.79-1.77 (m, 4H). $^{31}$P NMR (162 MHz, $D_2O$): 1.78 (d), -11.44 (s) -22.94 (s). Calculated for $C_{20}H_{29}N_7O_{13}P_3$ $[M-H]^-$ 668.1114 found 668.1037. UV/Vis spectroscopy ($H_2O$): λ 260 nm.

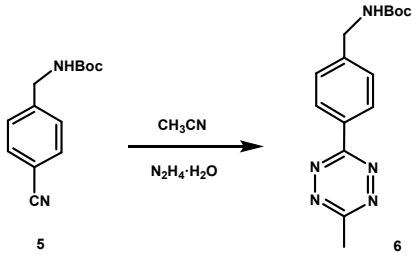
**tert-butyl (4-cyanobenzyl)carbamate (5)**




5

**5** was synthesized according to literature.[3] Mix 4-(aminomethyl)benzonitrile·HCl (5.00 g, 30.00 mmol) and toluene in $CH_2Cl_2$, add di-tert-butyl dicarbonate (8.30 g , 45.60 mmol) and triethylamine (76.00 mmol), the resulting mixture was stirred at room temperature for 16 h. The product was purified by column chromatography to obtain compound **5** (6.9 g, yield: 88%) as a white powder. $^1$H NMR (400 MHz, $CDCl_3$): δ 7.62 (d, $J$ = 8.0 Hz, 2H), 7.39 (d, $J$ = 8.0 Hz, 2H), 5.00 (s, 1H), 4.37 (s, 2H), 1.46 (s, 9H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 156.05, 144.83, 132.60, 127.96, 118.99, 111.26, 80.27, 44.38, 28.54. Calculated for $C_{13}H_{17}N_2O_2$ $[M+H]^+$ 233.1290 found 233.1299.

**tert-butyl (4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamate (6)**

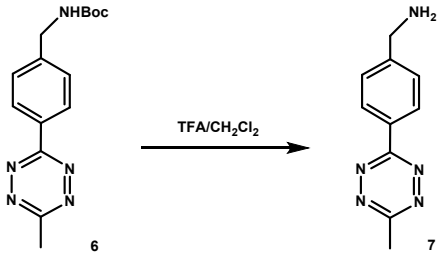



5 6

**6** was synthesized according to literature.[3] In a high pressure reaction tube, add compound **5** (232 mg, 1 mmol), acetonitrile (525 μL, 10 mmol), $Ni(OTf)_2$ (178 mg), and hydrazine hydrate (3.1 mL, 50 mmol). Sealed and heated the tube to 60 °C. After the mixture was stirred for 24 h, it turned dark purple in color. To the mixture, 5 mL of an solution of $NaNO_2$ (1.4 g, 20 mmol) was added, and then 1 M HCl was added dropwise until the pH value reached 3 and gas evolution ceased, at which time the mixture had turned bright red. Extracted with ethyl acetate, combined organic layer to washed with water and brine, dried over anhydrous $Na_2SO_4$ to obtain a bright pink oily liquid. Purification by column chromatography gave compound **6** (77 mg, yield: 61%) as a bright red powder. $^1$H NMR (400 MHz, $CDCl_3$): δ 8.56 (d, $J$ = 8.0 Hz, 2H), 7.50 (d, $J$ = 8.0 Hz, 2H), 4.97 (s, 1H), 4.43 (s, 2H), 3.09 (s, 3H), 1.48 (s, 9H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 167.45, 164.13, 156.56, 144.15, 131.00, 128.42, (d, $J$ =

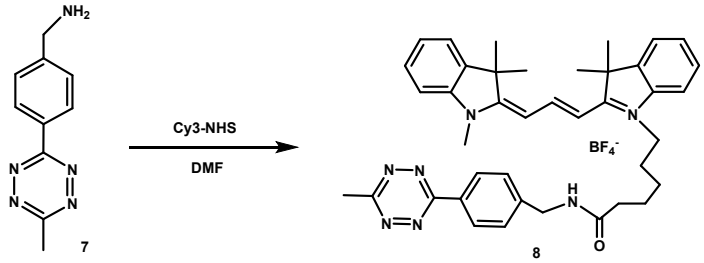
14.0 Hz), 80.01, 44.58, 28.61, 21.39. Calculated for $C_{15}H_{20}N_5O_2$ $[M+H]^+$ 302.1539 found 302.1626.

**[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]methanamine (7)**




**7** was synthesized according to literature.[3] Dissolved compound **6** (200.00 mg, 0.67 mmol) in $CH_2Cl_2$, drop 4 mL of a mixed solution of TFA and $CH_2Cl_2$ ($V_{TFA}$:$V_{DCM}$=*1:1*), the reaction solution was stirred for 2 h. The product obtained was a bright red solid compound **7** (129 mg) after rotary evaporation, Yield>95%). $^1$H NMR (400 MHz, $D_2O$): δ 8.43 (d, *J* = 8.0 Hz, 2H), 7.67 (d, *J* = 8.0 Hz, 2H), 4.28 (s, 2H), 3.03 (s, 3H). $^{13}$C NMR (100 MHz, $D_2O$) δ 167.37, 163.75, 137.03, 131.96, 129.61, 128.48, 42.60, 20.02. Calculated for $C_{10}H_{12}N_5$ $[M+H]^+$ 202.1093 found 202.1092.

**Probe TZ-Cy3 (8)**




Compound **7** (0.63 mg, 3.12 μmol) was dissolved in anhydrous DMF in a brown reaction bottle, then added N,N-diisopropylethylamine (DIPEA, 1.2 μL, 6.6 μmol), Cyanine3 NHS Ester (1.3 mg, 2.2 μmol). Stirred overnight at room temperature in the dark. The crude product is The reaction mixture was purified by reversed phase C18 column to obtained bright red compound **8** (0.89 mg, yield: 70%). Calculated for $C_{40}H_{46}N_7O$ $[M-BF_4]^+$ 640.3764 found 640.3783.

**Phosphorylation level evaluated by capillary electrophoresis with laser induced fluorescence detection (CE-LIF)**

CE-LIF detection system was used to evaluate the phosphorylation efficiency of ATP-BN. Place 50 μL of Abl reaction solution (containing 1 μM peptide labeled with FITC, 100 μM ATP-NB, 300 nM kinase Abl) and 15 mM Tris-HCl (containing 5 mM $MgCl_2$) buffer) in 37 $^oC$ for 60 minutes. After that, the CE-LIF detection system (home made) is used to detect the fluorescent signal of the FITC-labelled peptide substrate in the buffer (100 mM HEPES, 40 mM NaCl, pH 8.0) to determine the degree of phosphorylation. The dimension of the uncoated fused-silica capillary was 75 mm ID and 45 cm length (30 cm to the detection window). The samples were introduced by electrokinetic injection at 10 kV for 10 s. The separating potential was 15 kV.

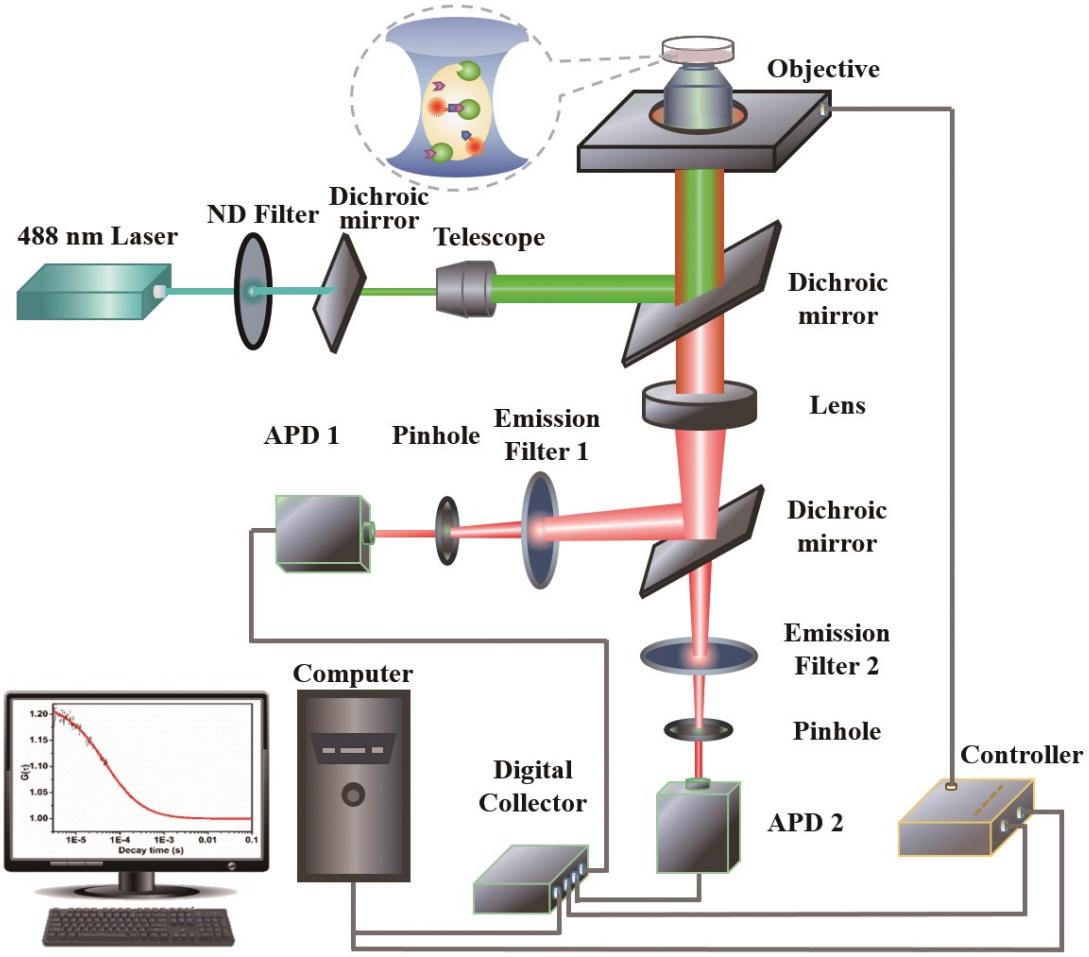



**Fig. S1** The setup of a FRET-FCS system

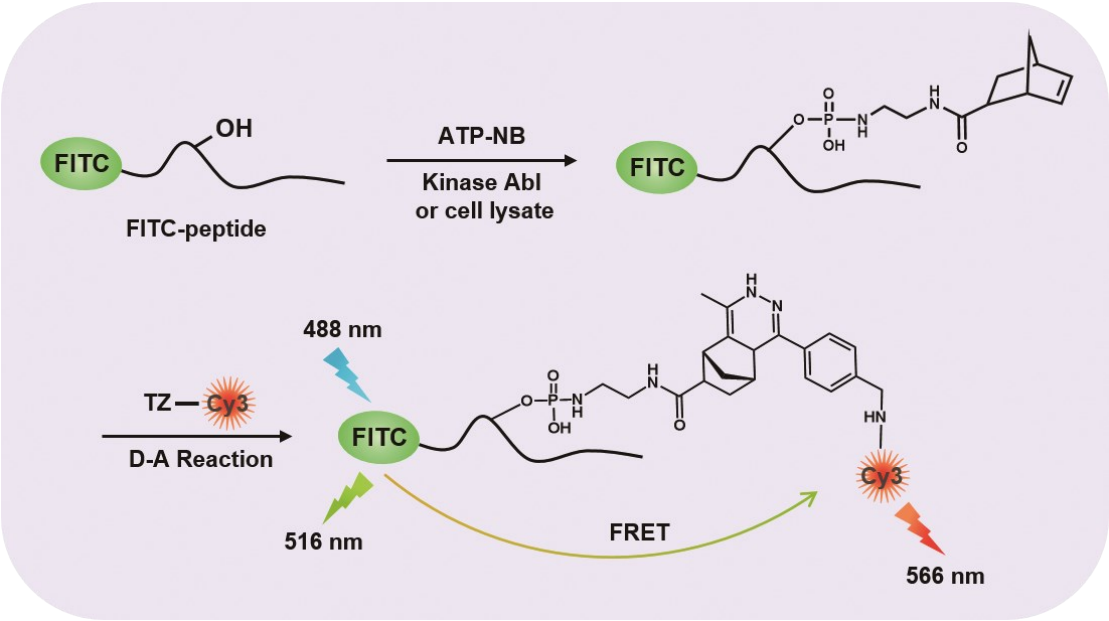



**Fig. S2** The principle of peptide phosphorylation detection by FRET-FCS with the ATP-NB in solution.

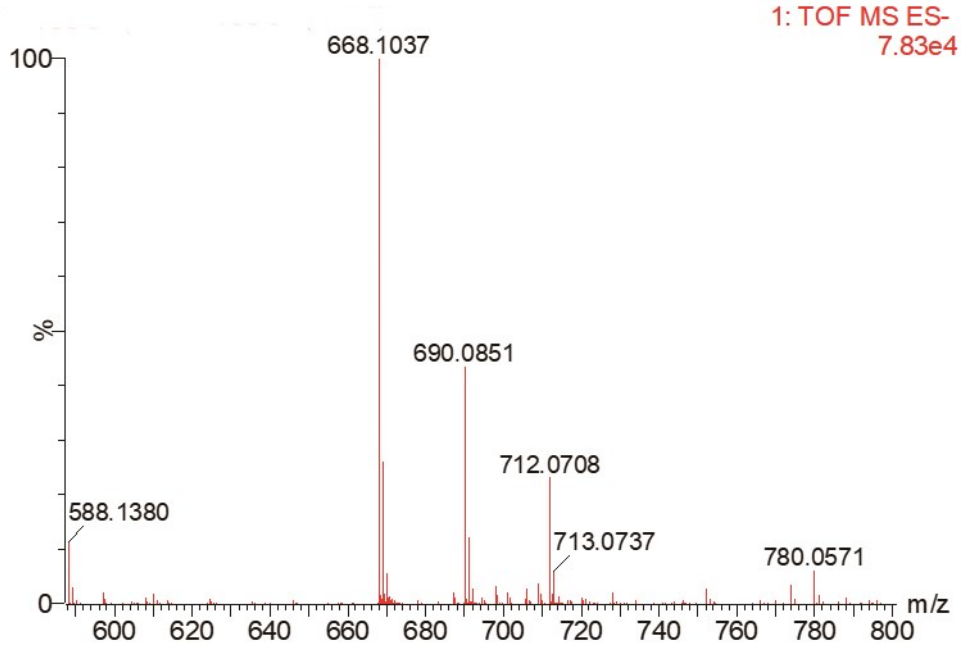



**Fig. S3** MS analysis result of ATP-NB (**4**), calculated for $C_{20}H_{30}N_7O_{13}P_3$ $[M]^-$ 668.1114 found 668.1037.

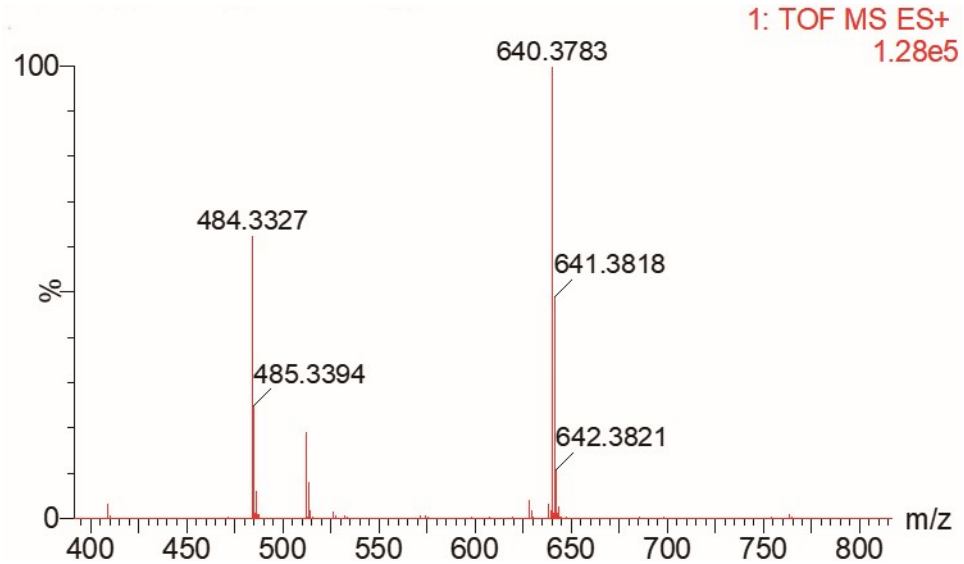



**Fig. S4** MS analysis result of TZ-Cy3 (**8**), calculated for $C_{40}H_{46}N_7O$ $[M\text{-}BF_4]^+$ 640.3764 found 640.3783.

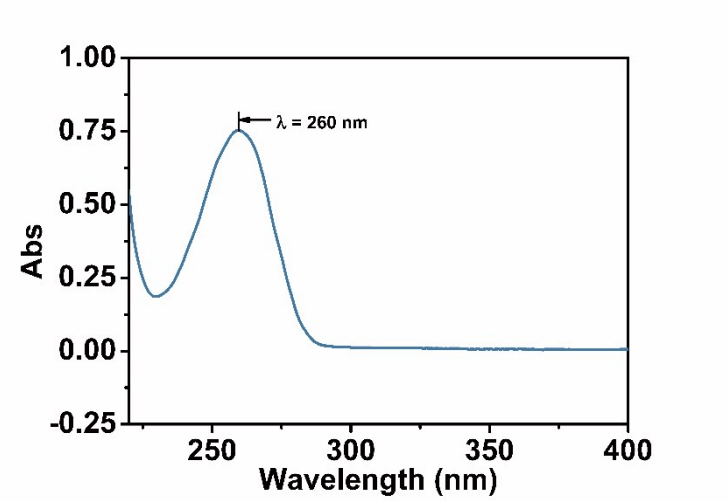



**Fig. S5** UV-VIS spectrum of ATP-NB (**4**) in water and the maximum absorption peak appears at 260 nm.

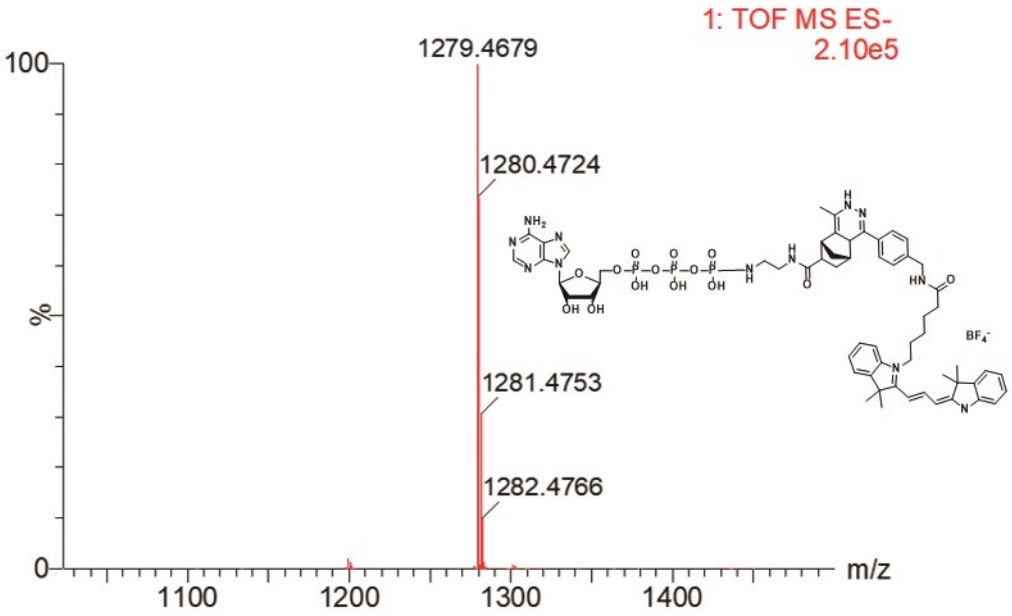



**Fig. S6** MS analysis results of D-A reaction between ATP-NB and TZ-Cy3, calculated for $C_{60}H_{75}N_{12}O_{14}P_3$ $[M\text{-}2H\text{-}BF_4]^-$1297.4817 found 1279.4679.

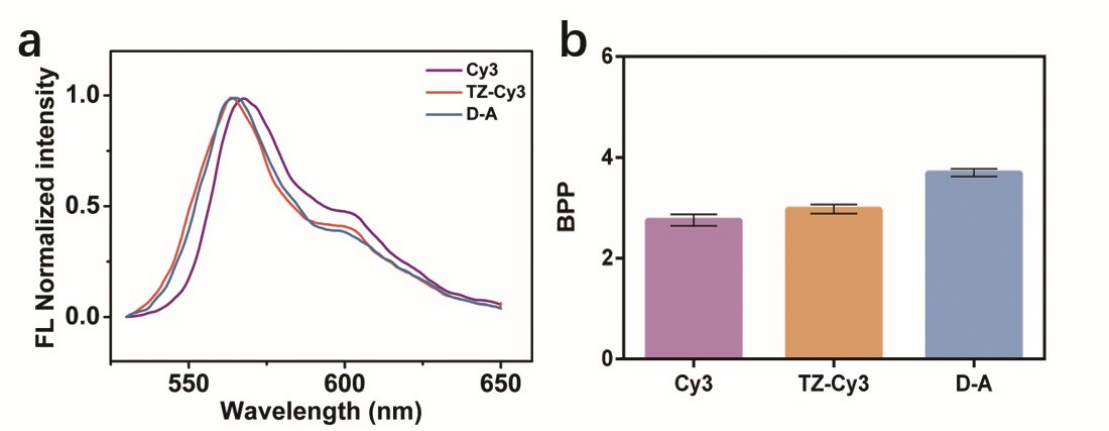



**Fig. S7** Fluorescence detection of Cy3, TZ-Cy3 and TZ-Cy3 after D-A reaction. (a) Fluorescence spectra; (b) The BPP values detected by FCS.

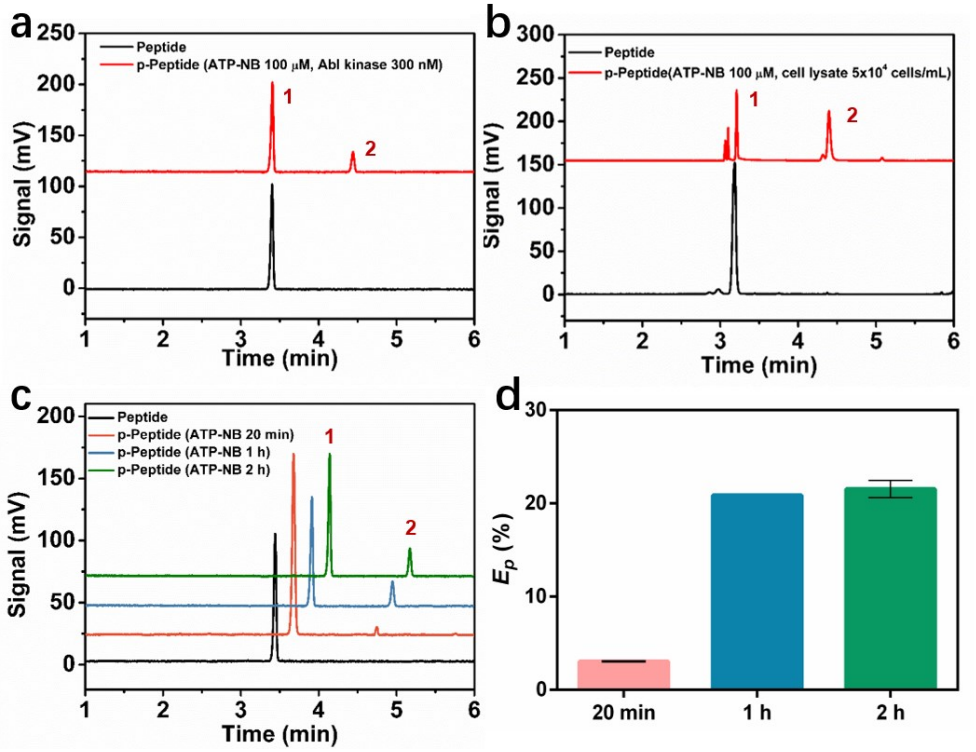



**Fig. S8** CE-LIF analysis of peptide substrate before and after phosphorylated by ATP-NB (the peak 1 was the unphosphorylated substrate peptide and the peak 2 was the phosphorylated substrate peptide). (a) Capillary electropherograms in solution (the phosphorylation efficiency $E_p$ was 21%); (b) Capillary electropherograms in cell lysate (the phosphorylation efficiency $E_p$ was 63%). (c) Capillary electropherograms in solution at different phosphorylation times; (d) The effect of phosphorylation time on phosphorylation efficiency $E_p$. Experimental conditions: $E$ = 333 V/cm, effective length of capillary: 30 cm, samples injection: 10 kV for 10 s, migration buffer: 15 mM Tris-HCl (containing 5 mM $MgCl_2$). Concentrations of peptide, ATP-NB, and Abl kinase are 1 μM, 100 μM, and 300 nM, respectively.

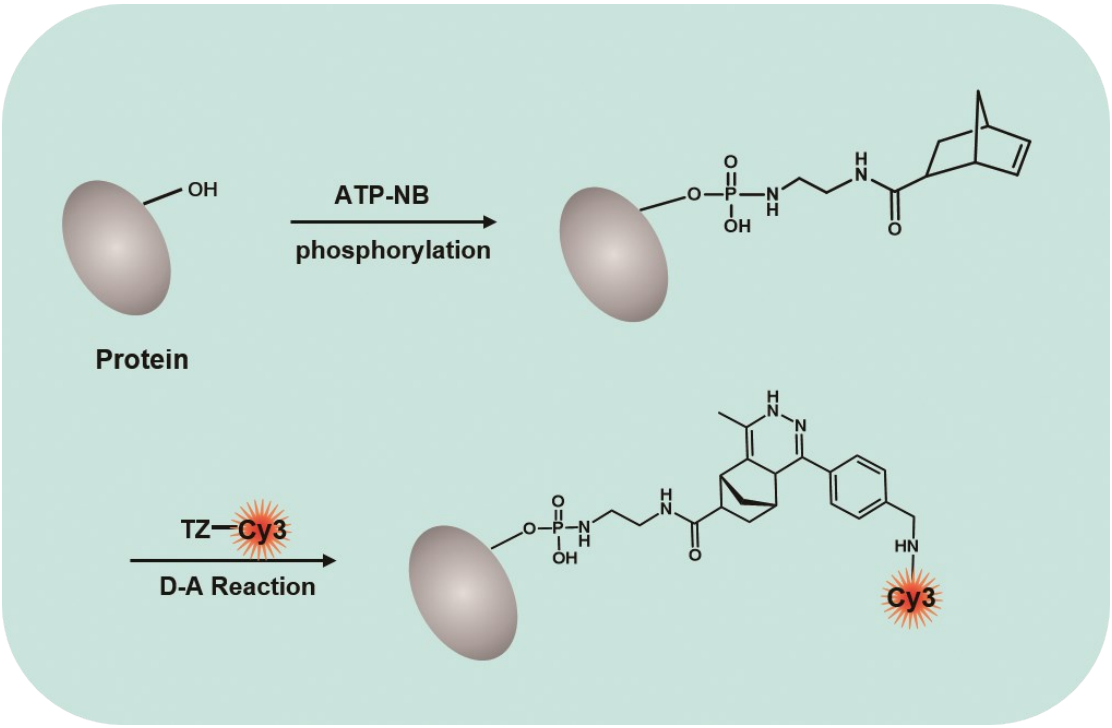



**Fig. S9** The principle of total proteins phosphorylation in cells.

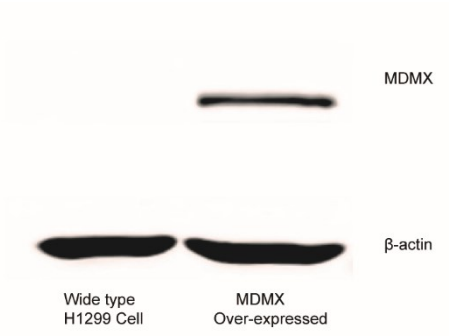



**Fig. S10** Western blotting analysis. H1299 cells stably expressing MDMX-EGFP was lysed and tested by Western blotting. MDMX-EGFP is detected by anti-MDMX. β-actin was used as an internal control. Wide type H1299 cell was used as a negative control.

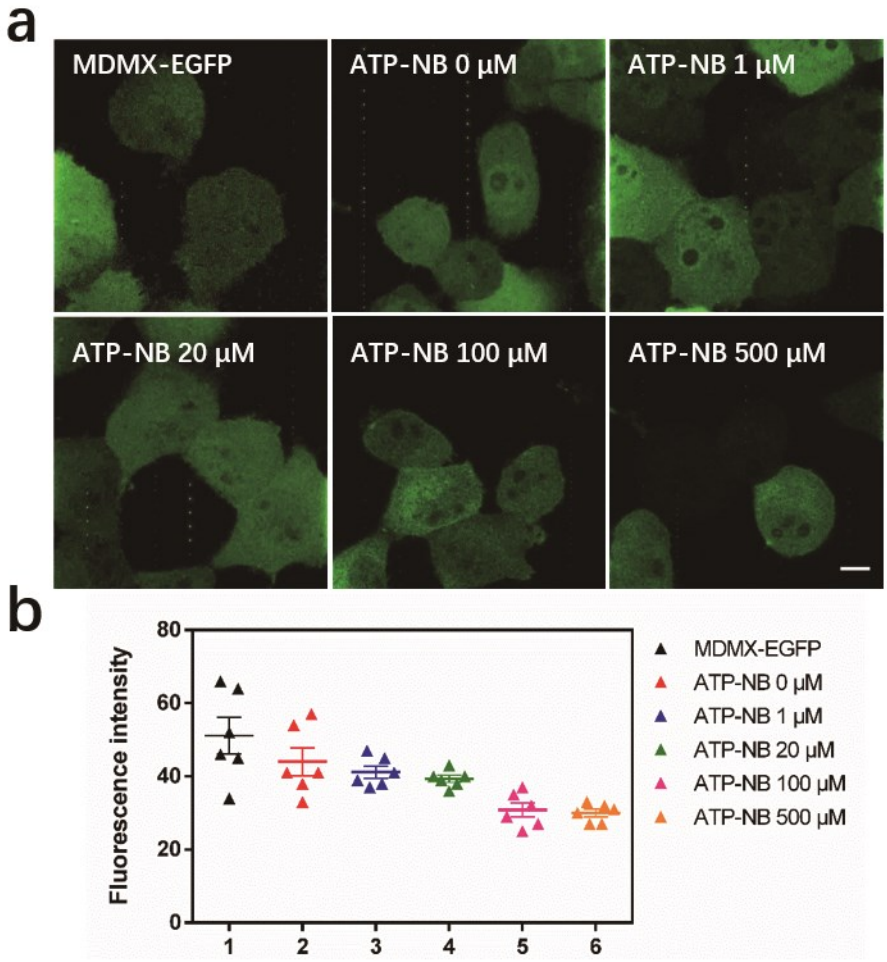



**Fig. S11** The effect of FRET on MDMX-EGFP fluorescence intensity by ATP-NB. (a) The confocal imaging of MDMX-EGFP in H1299 cells. (b) The fluorescence intensity of EGFP under different concentrations of ATP-NB (the number of selected cells in each group is n = 6). Scale bar = 10 μm

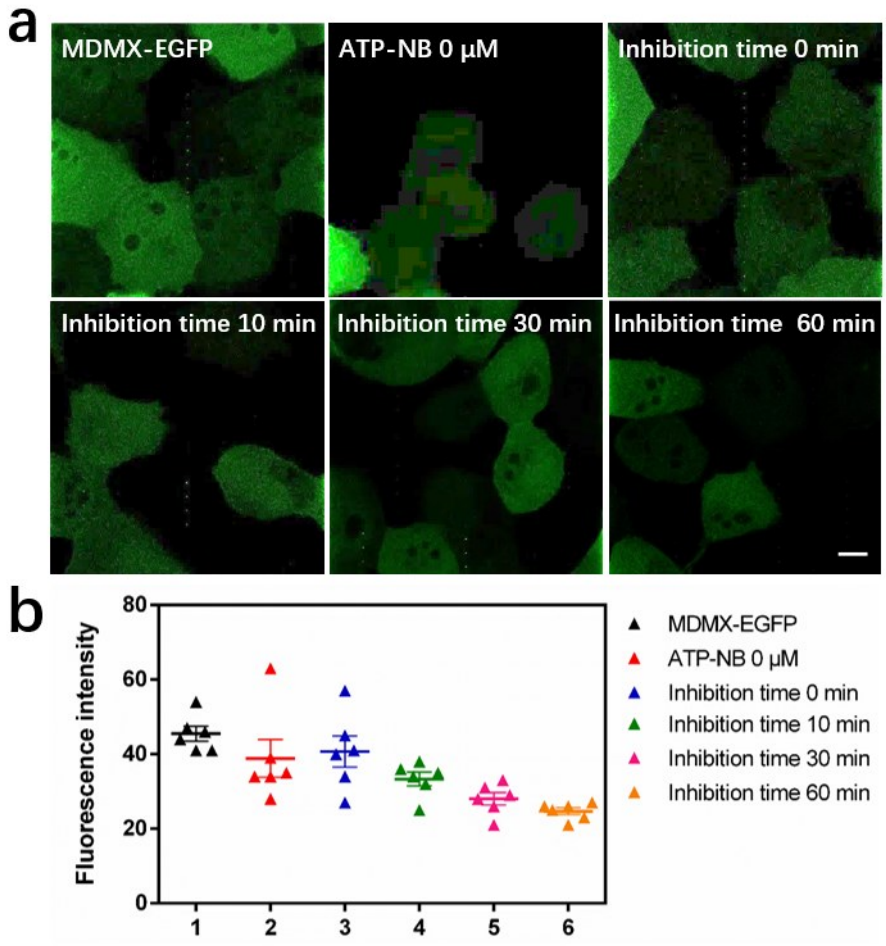



**Fig. S12** The effect of FRET on MDMX-EGFP fluorescence intensity by phosphatase inhibitors. (a) The confocal imaging of MDMX-EGFP in H1299 cells. (b) The fluorescence intensity of EGFP under different inhibition time (the number of selected cells in each group is n = 6). Scale bar = 10 μm

## References

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