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Supplementary Information

An unexpected highly selective ratiometric fluorescent probe for ATP and its application in cell imaging

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1. General experimental procedures

Reagents: Adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-diphosphate disodium salt (ADP), adenosine 5'-monophosphate monohydrate (AMP), cytidine 5'-triphosphate disodium salt (CTP), guanosine 5'-triphosphate disodium salt (GTP), uridine 5'-triphosphate trisodium salt (UTP), thymidine 5'-triphosphate sodium salt (TTP) were purchased from Sigma-Aldrich and their chemical structures were shown in Fig. S1. And all other chemicals were of analytical reagent grade, purchased from Shanghai Chemical Reagent Corporation (Shanghai, China), and used without further purification.

Apparatus: UV-vis absorption spectra were collected on a Perkin Elmer Lambda 25 spectrophotometer (USA). All fluorescence measurements were recorded with a Perkin Elmer LS-55 fluorescence spectrometer (USA). The pH measurements were obtained using a Mettler-Toledo Delta 320 pH meter (Switzerland). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance II spectrometer (Germany). ^{1}H NMR and ^{13}C NMR were operated at 400 and 100 MHz (TMS as internal standard), respectively. MS spectrometry was performed on a Bruker Autoflex MALDI-TOF MS spectrometer (Germany). Element analysis was conducted on Perkin Elmer 2400 elemental analyzer (USA).

Fluorescence measurements: The fluorescence emission spectra were recorded at excitation wavelength of 420.0 nm with emission wavelength range from 460.0 to 670.0 nm with excitation slit set at 10.0 nm and emission at 10.0 nm. A 1.0×10^{-4} M stock solution of NR was prepared by dissolving NR in H_2O/EtOH (10:1, v/v) solution. A stock standard solution of ATP (1.0×10^{-2} M) was prepared by dissolving an appropriate amount

of ATP in water and adjusting the volume to 500 mL in a volumetric flask. These solutions were further diluted to 1.0×10^{-3}-1.0×10^{-7} M stepwise. The solution of NR-ATP was prepared by adding 1.0 mL stock solution of NR and 1.0 mL stock solution of ATP in a 10 mL volumetric flask. Then the mixtures were diluted to 10 mL with HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) buffer solution (pH= 7.2). In the solutions thus obtained, the concentrations of NR were 1.0×10^{-5} M and ATP were 1.0×10^{-3}- 1.0×10^{-8} M (H_2O/EtOH: 99:1, v/v).

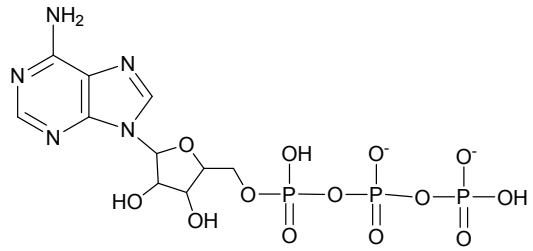
Calculation of association constants: The association constant (K_s) of the ATP-NR complex was determined by the Eq. (1) [S1]:

$$\frac{R-R_0}{R_{\text{lim}}-R}\frac{a_2}{b_2}=K_S[M] \qquad (1)$$

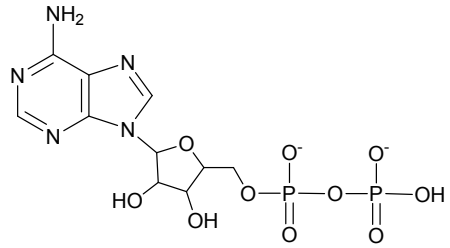
Here R_0 is the fluorescence intensity ratio (I_{580} / I_{530}) of NR in the blank solution and R_{lim} represents the fluorescence intensity ratio of NR in the solution when NR is completely coordinated with ATP. R is the fluorescence intensity ratio of NR measured when in contact with ATP solutions of a given concentration. And a_2/b_2 represents the ratio of the fluorescent intensities of the free ligand (NR) and the complex (ATP-NR) at λ_2 (530 nm). Eq. (1) can be used for the determination of K_s only if the concentration in free ATP ([M]) can be approximated to the total concentration c_M. $\frac{R-R_0}{R_{\text{lim}}-R}\frac{a_2}{b_2}$ is plotted as a function of c_M and the plot should be linear and the slope yields K_s. The association constant (K_s) between NR and ATP was determined to be 1.2×10^6 M^{-1}.

Cell incubation and imaging: The living HeLa cells were provided by XiangYa School of Medicine, Central South University (China). HeLa cells were cultured in Dulbecco's modified eagle's medium (DMEM) and supplemented with 10% fetal bovine

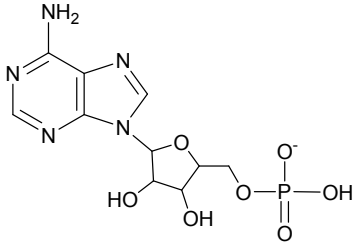
serum (FBS) at 37 ◦C and 5% CO_2. Two groups of cells were plated on glass-bottomed dishes and allowed to adhere for 24 h. One group of cells was treated with apyrase in phosphate buffered saline (PBS) for 60 min, and another group was not treated. Then the two groups of cells were exposed to 10 μM of NR for 30 min at 37 °C and washed with PBS solution three times and imaged. Fluorescence imaging experiments were carried out in living cells on an OLYMPUS FV1000 fluorescence microscope (Japan). The excitation wavelength of laser was 458 nm and the emissions were centered at both red (580 ± 10 nm) and green (520 ± 10 nm) channels.



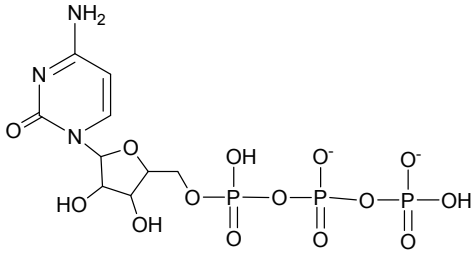
ATP



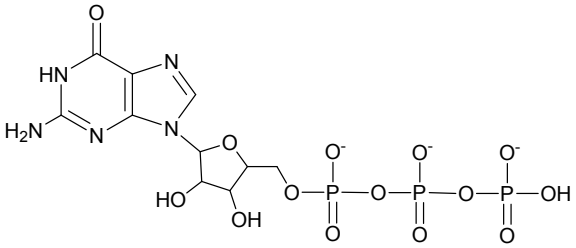
ADP



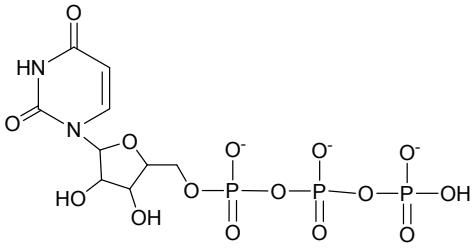
AMP



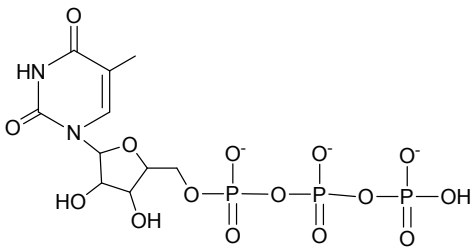
CTP



GTP



UTP



TTP

Fig. S1 The chemical structures of organic phosphate anions.

2. Synthesis and characteristic data

Compound 1: 1-[2-(2-Hydroxyethoxy)ethyl]piperazine (0.87 g, 5.0 mmol) and 4-bromo-1, 8-naphthalic anhydride (1.4 g, 5.0 mmol) were dissolved in ethylene glycol monomethylether (50 mL). The reaction mixture was stirred and refluxed for 6 h. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using CH_2Cl_2/C_2H_5OH (8:1, v/v) as eluent to afford a yellow solid product. Yield: 1.1 g (59 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.50 (d, J = 8.0 Hz, 1H), 8.43 (d, J = 8.0 Hz, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.68 (t, J = 8.0 Hz, 1H), 7.21 (d, J = 8.0 Hz, 1H), 3.81 (t, J = 4.0 Hz, 2H), 3.72 (d, J = 4.0 Hz, 2H), 3.65 (d, J = 4.0 Hz, 2H), 3.44 (s, 4H), 3.07 (s, 4H), 2.91(t, J = 4.8 Hz, 2H). MS (TOF) m/z 371.2.

Compound 2: Compound **1** (0.53 g, 1.0 mmol) and diethylenetriamine (0.54 mL, 5 mmol) were dissolved in ethanol (30 mL). The reaction mixture was stirred and refluxed for 8 h. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using CH_2Cl_2/C_2H_5OH (4:1, v/v) as eluent to afford a yellow solid product. Yield: 0.13 g (28 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.49 (d, J = 8.0 Hz, 1H), 8.41 (d, J = 8.0 Hz, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.67 (t, J = 8.0 Hz, 1H), 7.20 (d, J = 8.0 Hz, 1H), 3.79 (t, J = 4.0 Hz, 2H), 3.70 (d, J = 4.0 Hz, 2H), 3.64 (d, J = 4.0 Hz, 2H), 3.41 (s, 4H), 3.20 (t, J = 8.0 Hz, 2H), 3.05 (s, 4H), 2.90 (t, J = 4.8 Hz, 2H), 2.68 (t, J = 8.0 Hz, 2H), 2.50 (t, J = 8.0 Hz, 2H), 2.17 (t, J = 8.0 Hz, 2H). MS (TOF) m/z 456.3.

Compound 3: Rhodamine B (2.0 g, 4.2 mmol) and diethylenetriamine (10 mL, 92 mmol) were dissolved in ethanol (50 mL). The reaction mixture was stirred and refluxed for 24 h. After the solvent was evaporated under reduced pressure, the crude product was

purified by silica gel column chromatography using CH_2Cl_2/C_2H_5OH (10:1, v/v) as eluent to afford a pink solid product. Yield: 0.15 g (6.4 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, J = 8.0 Hz, 1H), 7.42 (m, 2H), 7.08 (m, 1H), 6.42 (d, J = 8.0 Hz, 2H), 6.36 (s, 2H), 6.26 (d, J = 8.0 Hz, 2H), 3.35-3.18 (m, 10H), 2.71 (d, J = 8.0 Hz, 2H), 2.55 (t, J = 8.0 Hz, 2H), 2.20 (t, J = 8.0 Hz, 2H), 1.16 (t, J = 8.0 Hz, 12H). MS (TOF) m/z 528.3.

Compound NR: Compound **1** (0.37 g, 1.0 mmol) and compound **3** (0.53 g, 1.0 mmol) were dissolved in ethanol (30 mL). The reaction mixture was stirred and refluxed for 16 h. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using CH_2Cl_2/C_2H_5OH (6:1, v/v) as eluent to afford a yellow solid product. Yield: 0.41 g (47 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.52 (d, J = 8.0 Hz, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.37 (d, J = 8.0 Hz, 1H), 7.76 (d, J = 4.0 Hz, 1H), 7.65 (t, J = 8.0 Hz, 1H), 7.40 (t, J = 4.0 Hz, 2H), 7.19 (d, J = 8.0 Hz, 1H), 7.05 (d, J = 4.0 Hz, 1H), 6.43-6.35 (m, 4H), 6.25 (d, J = 8.0 Hz, 2H), 4.16 (t, J = 6.0 Hz, 2H), 3.73 (d, J = 4.0 Hz, 4H), 3.66 (d, J = 4.0 Hz, 2H), 3.48 (s, 1H), 2.87 (s, 4H), 2.76 (d, J = 8.0 Hz, 4H), 2.43 (t, J = 6.0 Hz, 2H), 1.14 (t, J = 6.0 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.5, 164.5, 164.0, 155.6, 153.7, 153.3, 148.9, 132.5, 132.2, 131.0, 130.0, 128.7, 127.8, 125.6, 123.7, 123.4, 122.7, 115.0, 108.2, 105.7, 97.9, 72.4, 67.8, 62.0, 57.9, 53.4, 52.7, 47.6, 47.2, 44.3, 39.9, 39.7, 12.6. MS (TOF) m/z 880.5. Anal. calcd. for $C_{52}H_{61}N_7O_6$ (NR): C, 70.97; H, 6.99; N, 11.14; O, 10.91. Found: C, 70.90; H, 7.03; N, 11.13; O, 10.94.

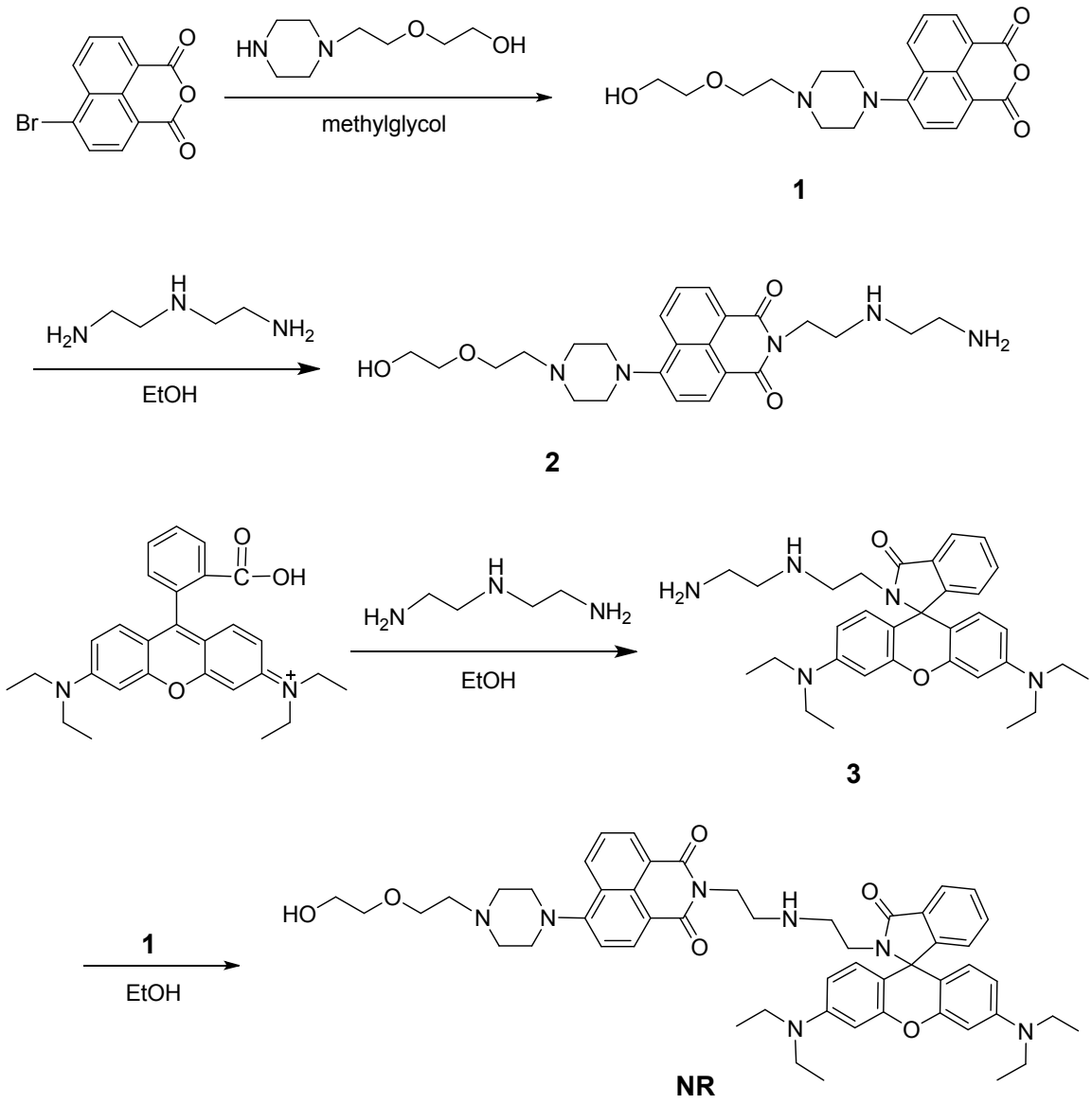


Fig. S2 Synthetic route of compound NR.

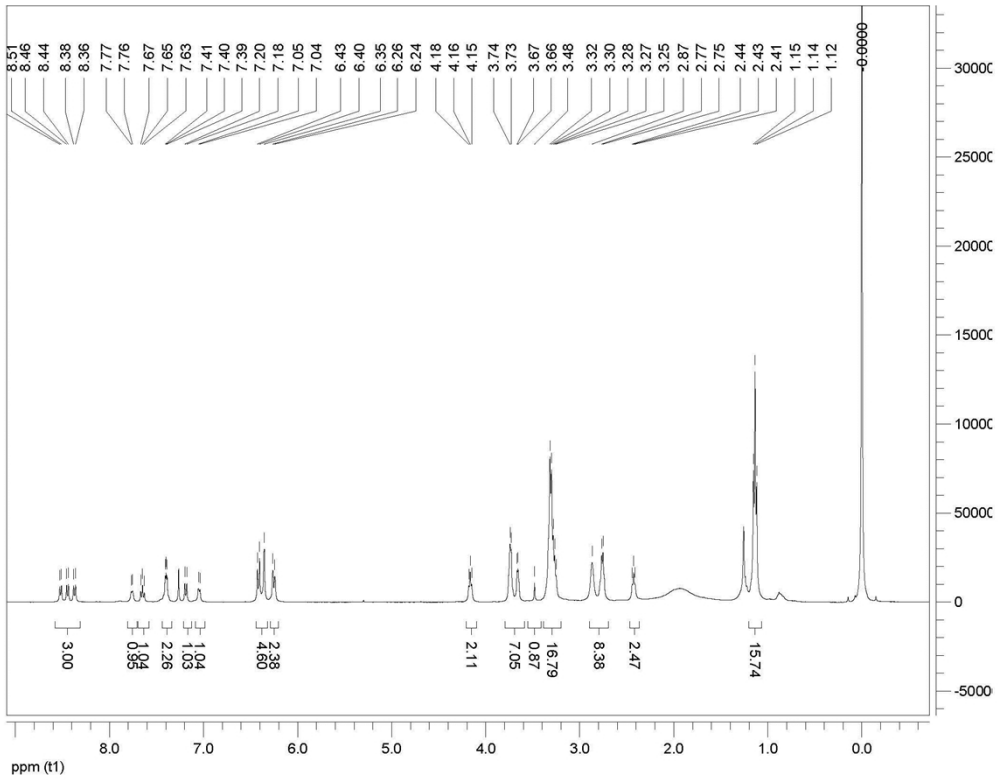


Fig. S3 ^{1}H NMR spectra of compound NR in $CDCl_3$.

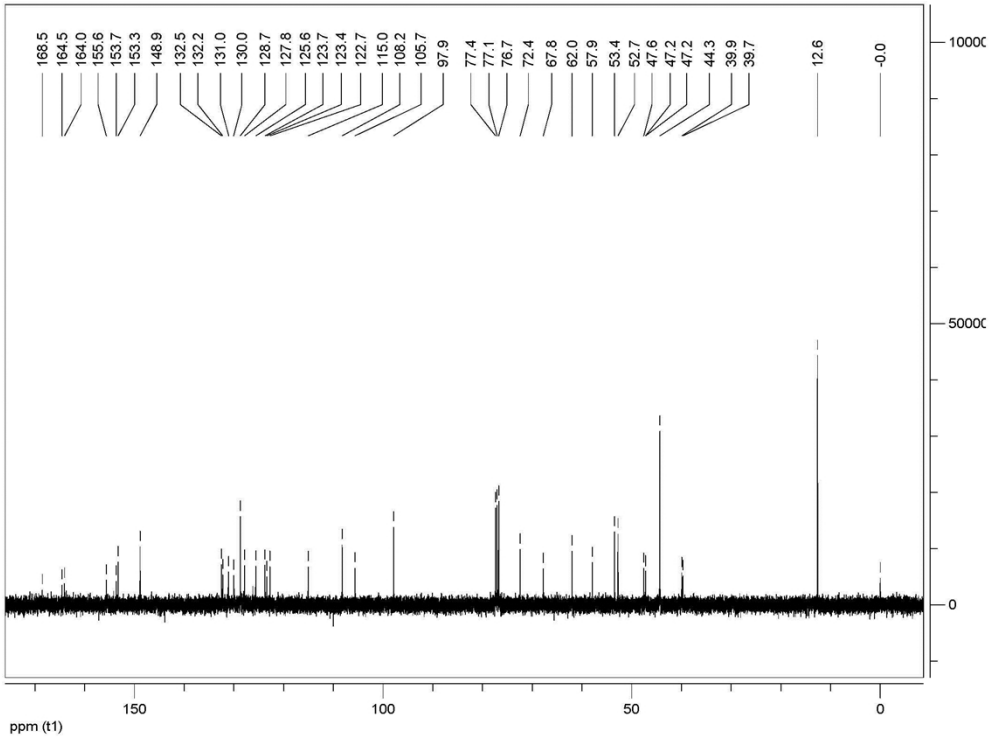


Fig. S4 ^{13}C NMR spectra of compound NR in $CDCl_3$.

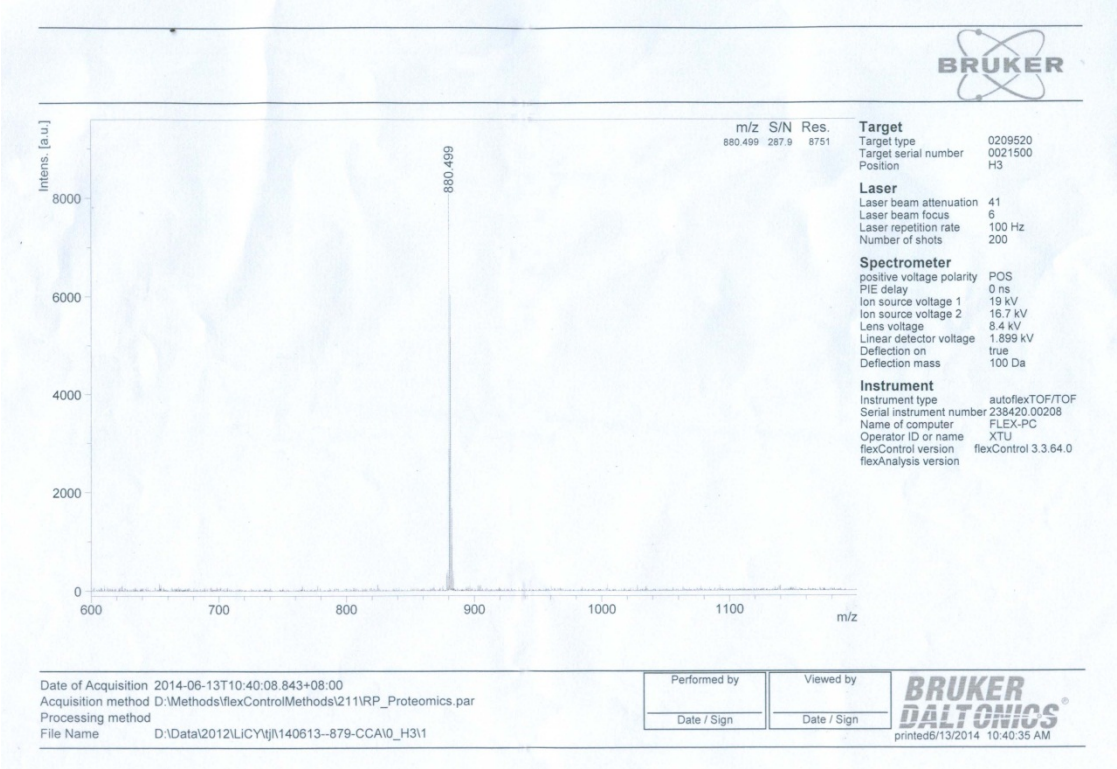


Fig. S5 Mass spectra of compound NR.

3. Selectivity

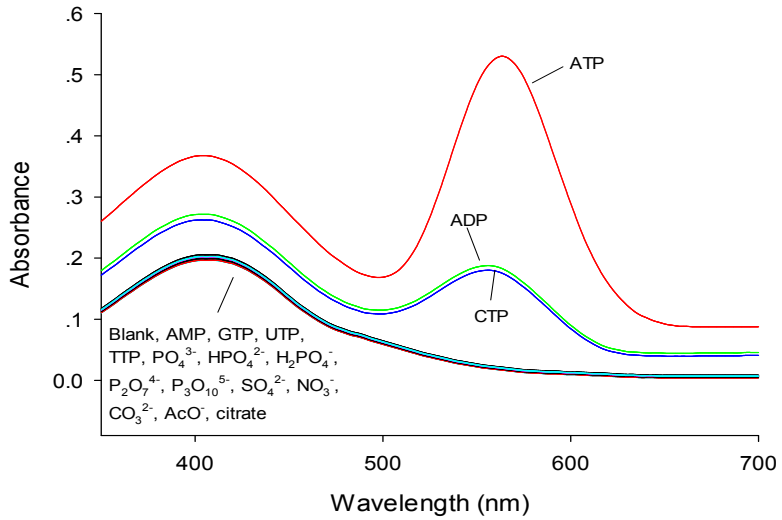


Fig. S6 Absorption spectra of NR (10 μM) upon the addition of ATP (10 μM) and other anions (1 mM) in HEPES buffer solution (pH= 7.2).

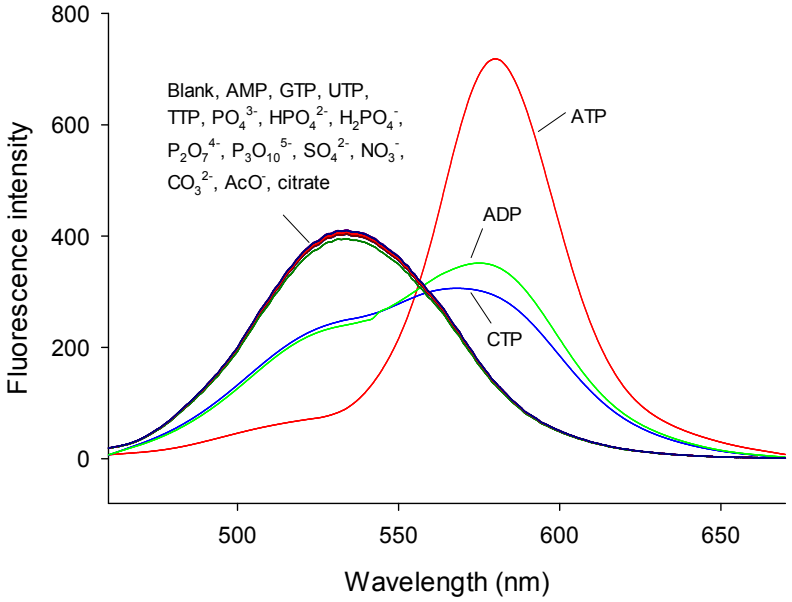


Fig. S7 Fluorescence spectra of NR (10 μM) upon the addition of ATP (10 μM) and other anions (1 mM) in HEPES buffer solution (pH= 7.2).

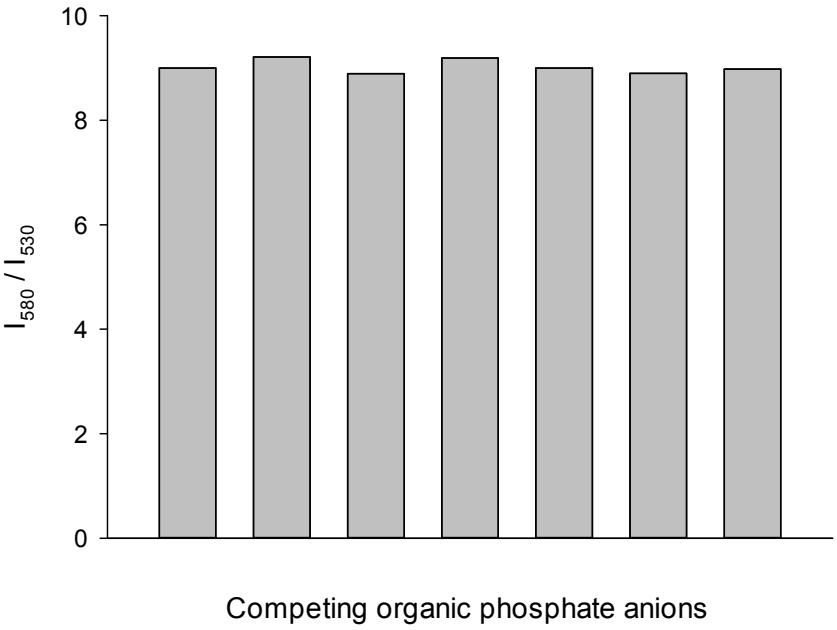


Fig. S8 The fluorescence intensity ratio of NR (10 μM) with ATP (10 μM) upon the addition of other organic phosphate anions such as ADP, AMP, CTP, GTP, UTP, TTP (1 mM) in HEPES buffer solution (pH= 7.2).

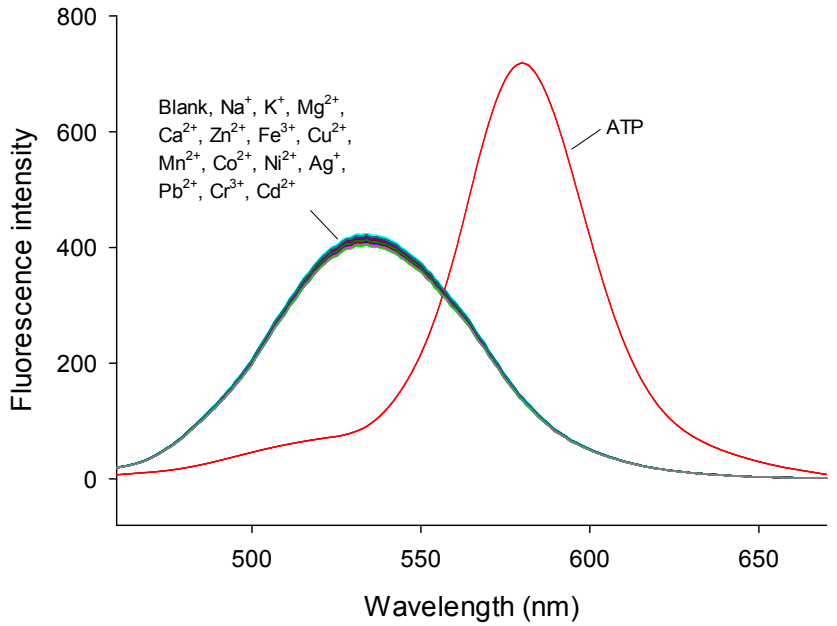


Fig. S9 Fluorescence spectra of NR (10 μM) upon the addition of ATP (10 μM) and other cations (1 mM) in HEPES buffer solution (pH= 7.2).

4. Effect of pH

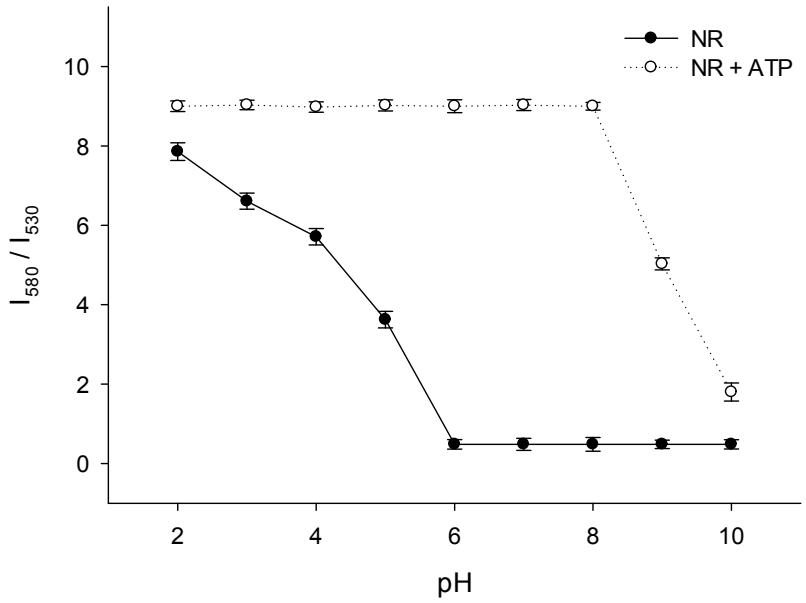


Fig. S10 Effect of pH on the fluorescence intensity ratio (I_{580}/I_{530}) of NR (solid line) and NR + ATP (dashed line).

5. Job's plot

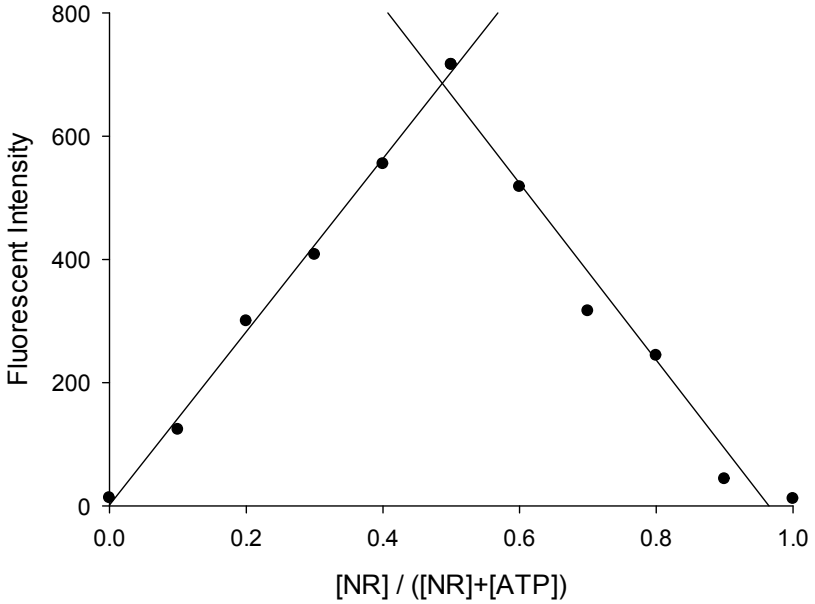


Fig. S11 The Job Plot of NR and ATP using fluorescent intensity at 580 nm in HEPES buffer solution (pH= 7.2) with an excitation at 520 nm. The total concentration of [NR] and [ATP] is 10 μM.

6. Mass spectrum of NR-ATP

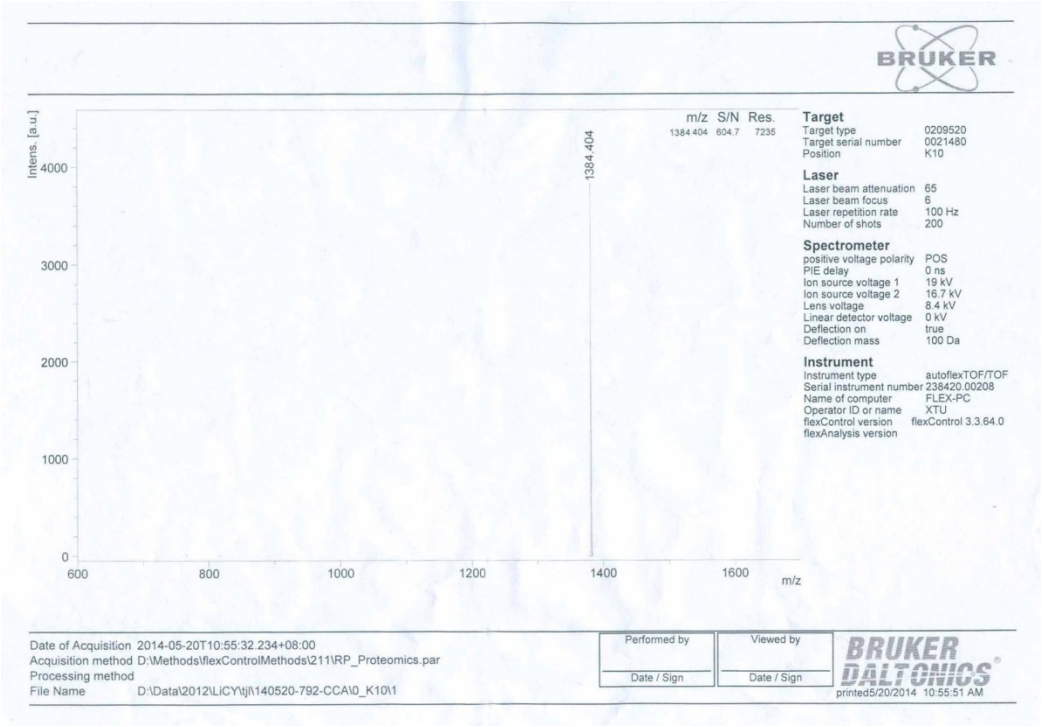


Fig. S12 Mass spectra of NR-ATP.

7. Photostability

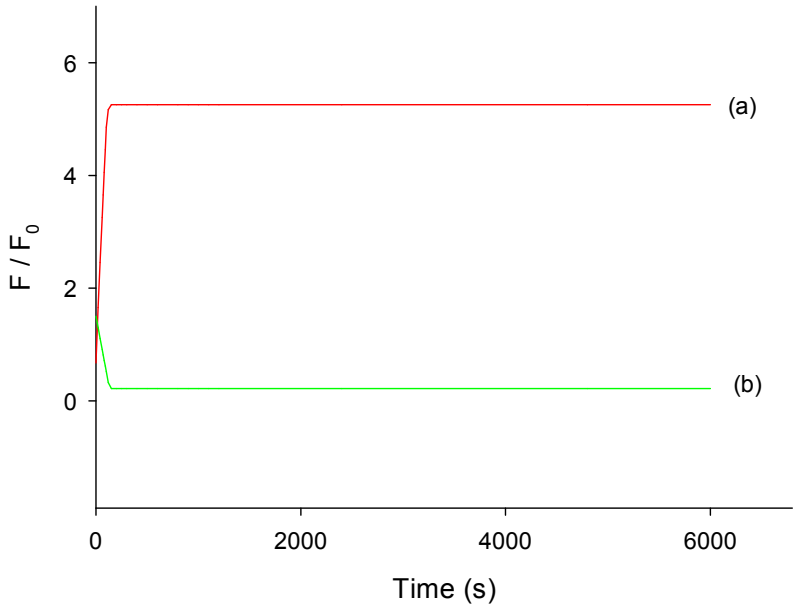


Fig. S13 The Kinetics of the fluorescence of NR (10 μM) upon the addition of ATP (10 μM). (a) Fluorescence intensity is recorded at 580 nm. (b) Fluorescence intensity is recorded at 530 nm. Excitation wavelength is fixed at 420 nm.

8. Comparison of ATP probes

Table S1 A comparison of the performance of this probe with previously reported ATP probes.

Probe	Receptor	Change of signal	Absorption wavelength (nm)	Emission wavelength (nm)	Operating range (μM)	Interferents
Ref. 3a	Zinc complex	Fluorescence	450	535	0 − 10	ADP
Ref. 3b	Zinc complex	Fluorescence	509	523	—	ADP, GTP, CTP, $P_2O_7^{4-}$
Ref. 3c	Zinc complex	Fluorescence	500	559	0 − 10	CTP
Ref. 3d	Zinc complex	Fluorescence	670	723	0 − 350	ADP
Ref. 3e	Zinc complex	Color	463	—	—	CTP
Ref. 3f	Zinc complex	Fluorescence	—	383	—	UTP, ADP
Ref. 3g	Zinc complex	Color	463	—	2 − 260	ADP
Ref. 3h	Zinc complex	Fluorescence	410	560	0 − 20	ADP, $P_2O_7^{4-}$
Ref. 3i	Zinc complex	Fluorescence	—	434	0 − 5	ADP
Ref. 3j	Lanthanide complex	Fluorescence	—	615	—	No
Ref. 3k	Polymer	Color	600	—	0 − 1500	CTP
Ref. 3l	Quaternary ammonium	Fluorescence	—	415	0 − 300	GTP
Ref. 3m	Zinc complex	Fluorescence	341, 513	454, 525	0 − 5	ADP, GTP, CTP, $P_2O_7^{4-}$
Ref. 3n	Imidazolium	Fluorescence	—	375, 478	0 − 200	ADP
This probe	Rhodamine	Fluorescence	400, 560	530, 580	0.1 − 10	ADP, CTP

9. Binding mechanism

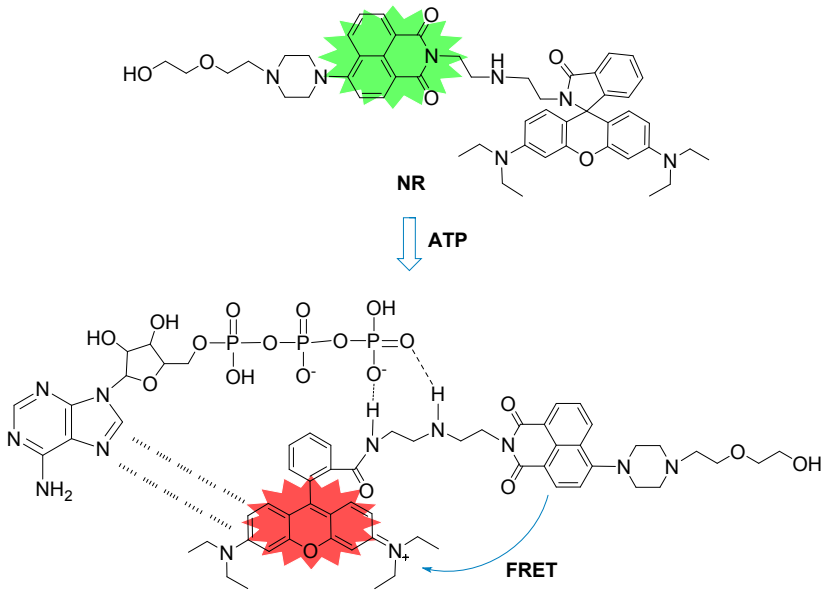


Fig. S14 Proposed binding mechanism of the probe.

10. NMR studies for NR

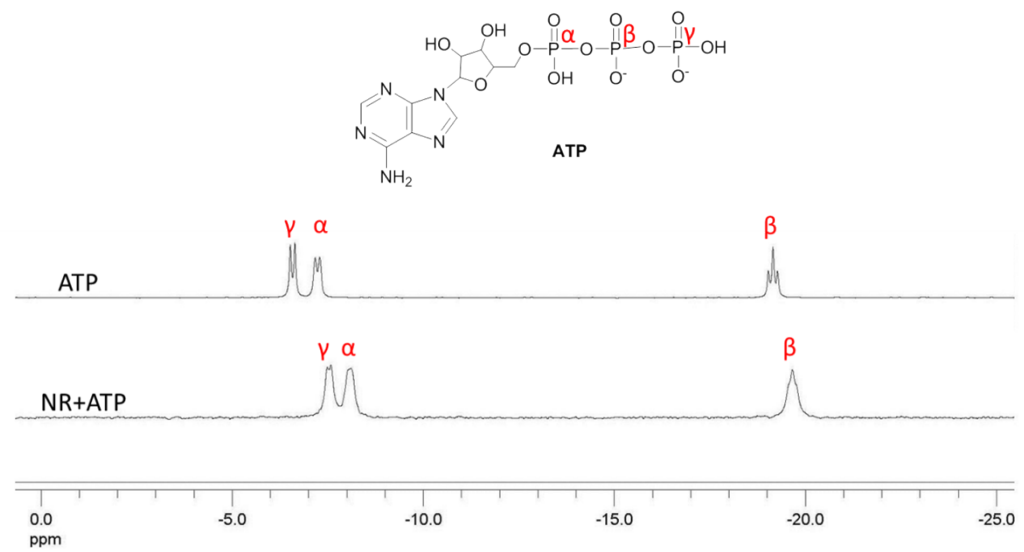


Fig. S15 ^{31}P NMR spectra of ATP and NR+ATP. NMR solvent: 10% D_2O in d_6-DMSO.

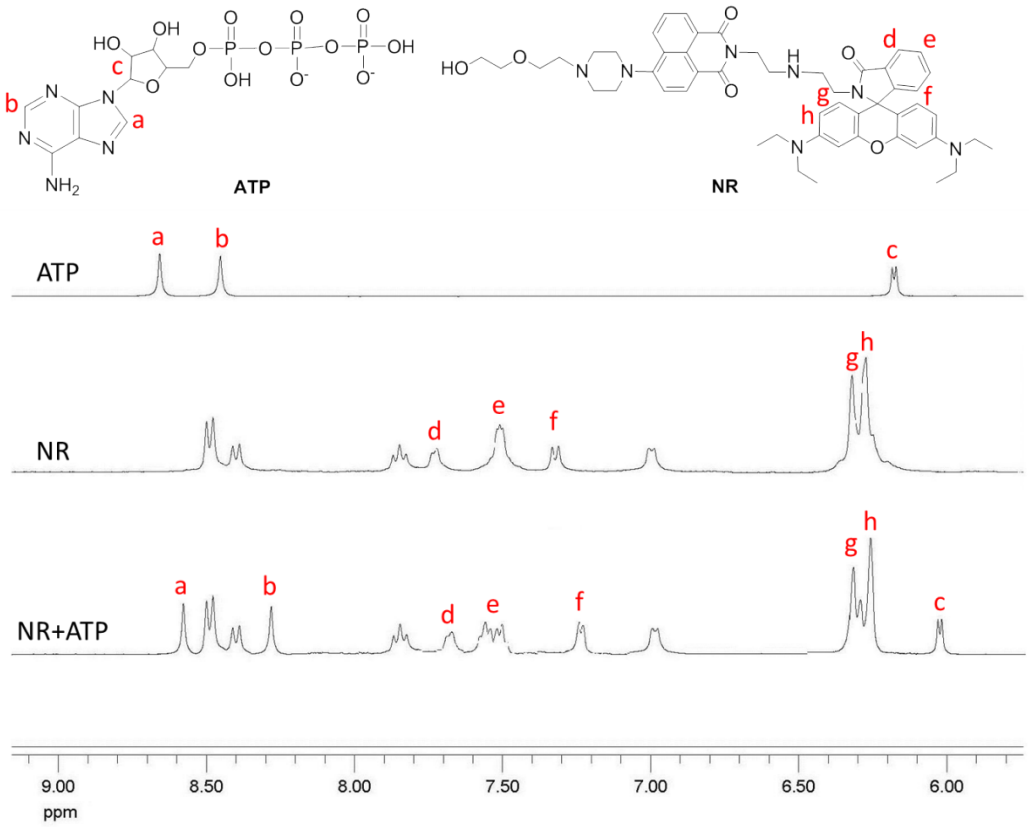


Fig. S16 Partial ^{1}H NMR spectra of ATP, NR and NR+ATP. NMR solvent: 10% D_2O in d_6-DMSO.

11. Fluorescence spectra of control compounds 2 and 3

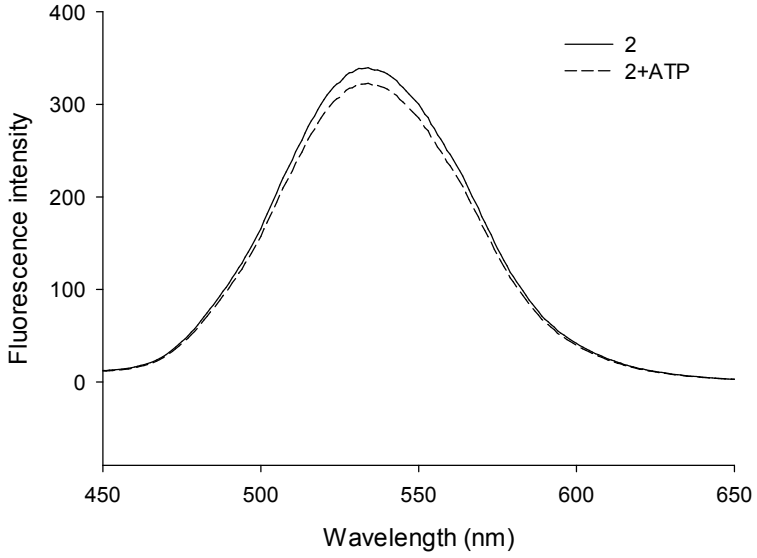


Fig. S17 Fluorescence spectra of compound **2** (10 μM) upon the addition of ATP (10 μM) in HEPES buffer solution (pH= 7.2).

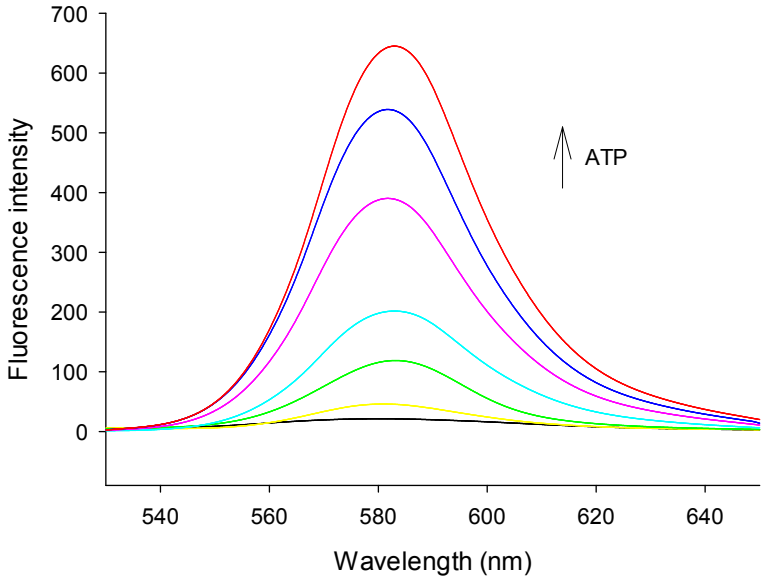


Fig. S18 The fluorescence emission spectra of compound **3** (10 μM) in the presence of different concentrations of ATP (0, 0.1, 0.2, 0.5, 2.0, 5.0, 10 μM) in HEPES buffer solution (pH = 7.2).

12. NMR studies for compound 3

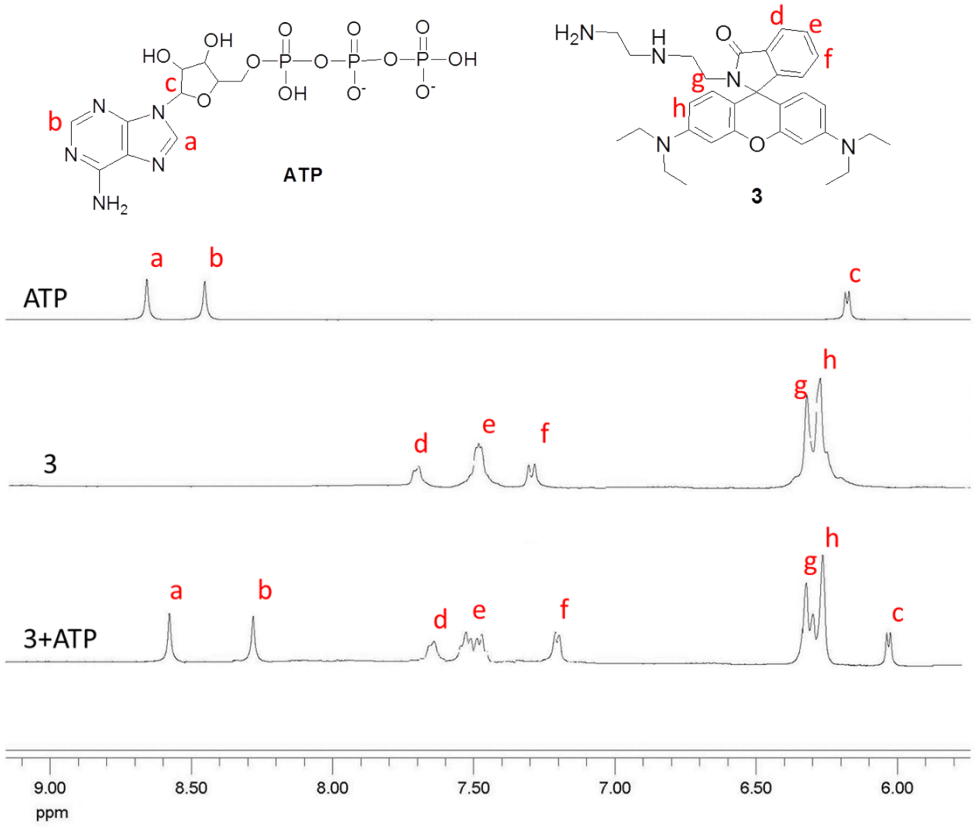


Fig. S19 Partial ^{1}H NMR spectra of ATP, **3** and **3**+ATP. NMR solvent: 10% D_2O in d_6-DMSO.

13. Mass spectrum for compound 3 and 3-ATP

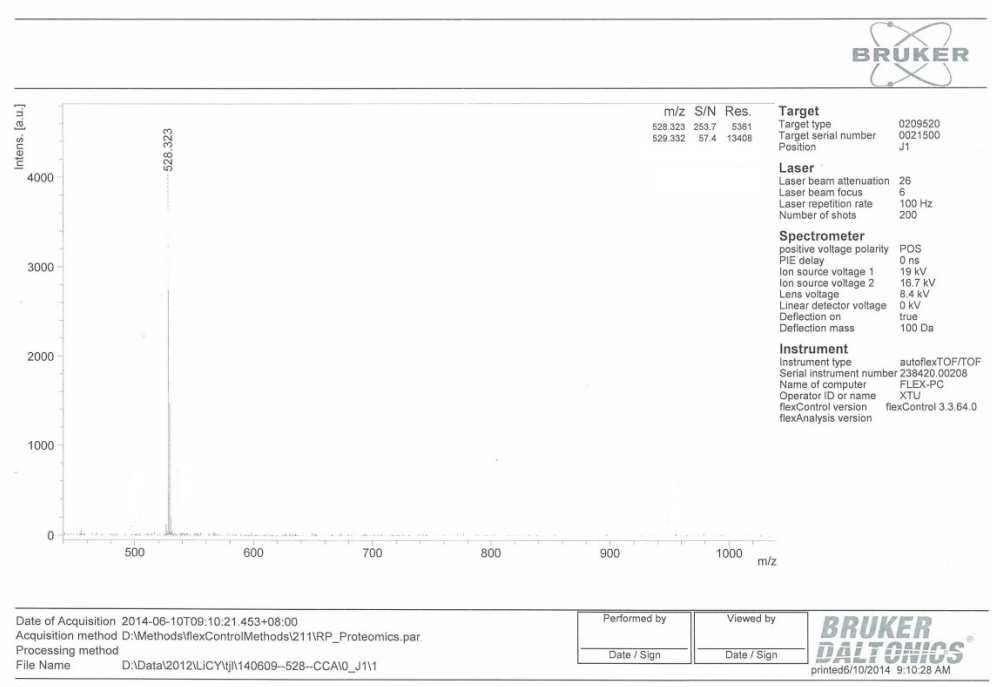


Fig. S20 Mass spectra of compound **3**.

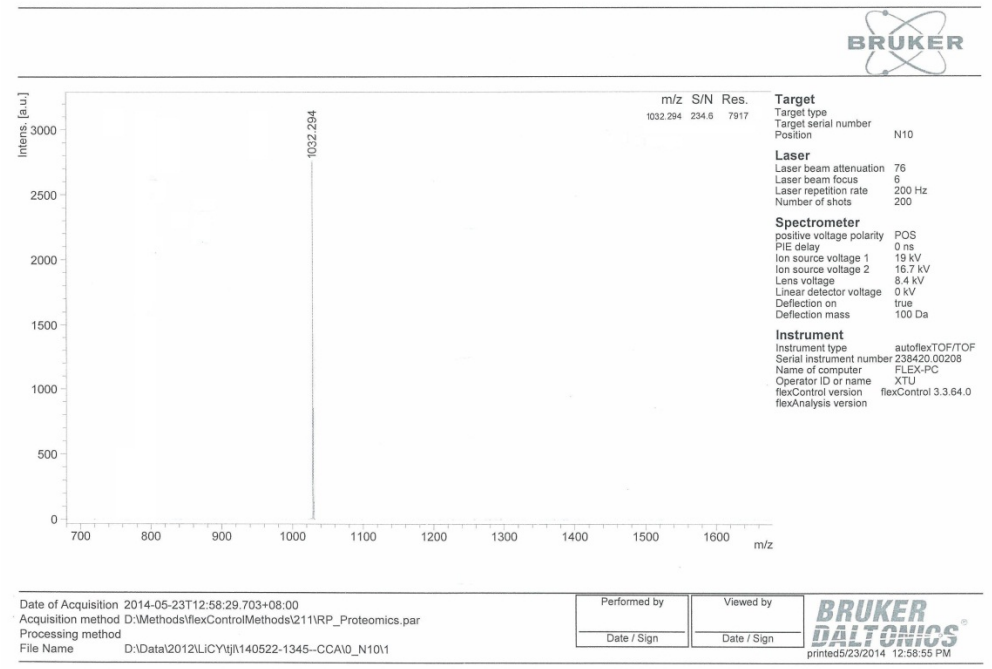


Fig. S21 Mass spectra of **3**-ATP.

14. DFT calculated interactions between NR and GTP

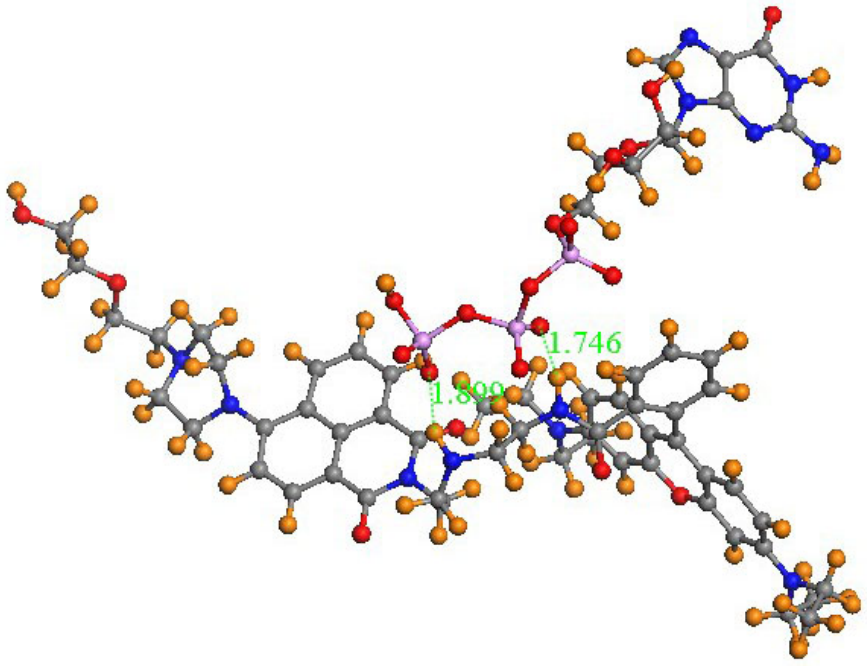


Fig. S22 Plot views of DFT calculated interactions between NR and GTP. Green dashed lines demonstrate the hydrogen bonds interactions.

15. Cell viability

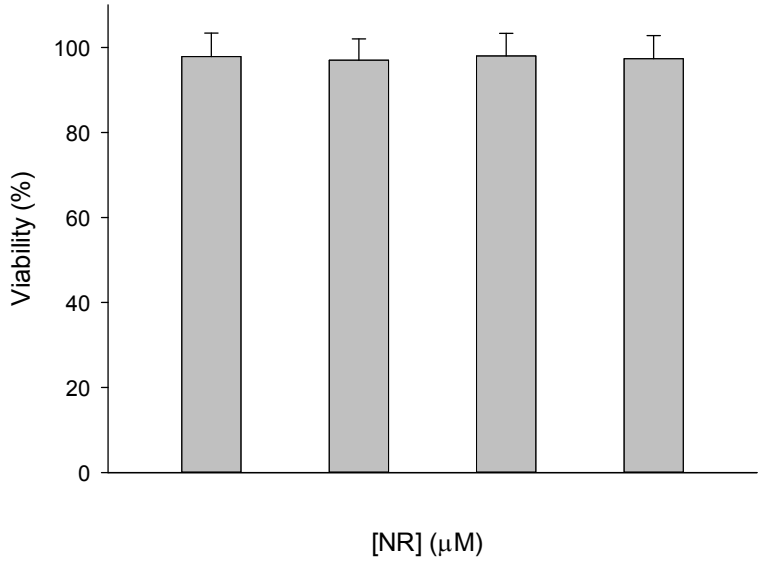


Fig. S23 Cell viability values (%) estimated by MTT proliferation test versus incubation concentrations of NR. HeLa cells were cultured in the presence of 0-10 μM NR.

16. Cell images

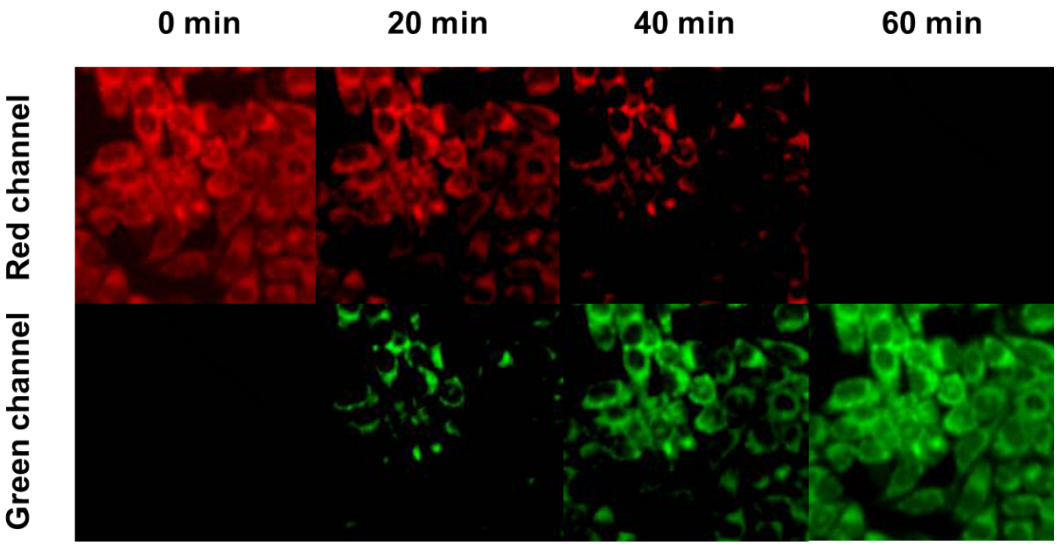


Fig. S24 Images of Hela cells treated with apyrase from 0 to 60 min and then incubated with NR (10 μM) for further 30 min at 37 °C.

References

S1. B. Valeur, Molecular Fluorescence: Principles and Applications, Wiley-VCH Verlag GmbH, 2001.