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Electronic Supplementary Information for:

A dual-function fluorescent probe for monitoring the degrees of hypoxia in living cells *via* the imaging of nitroreductase and adenosine triphosphate

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1. Reagents and apparatus

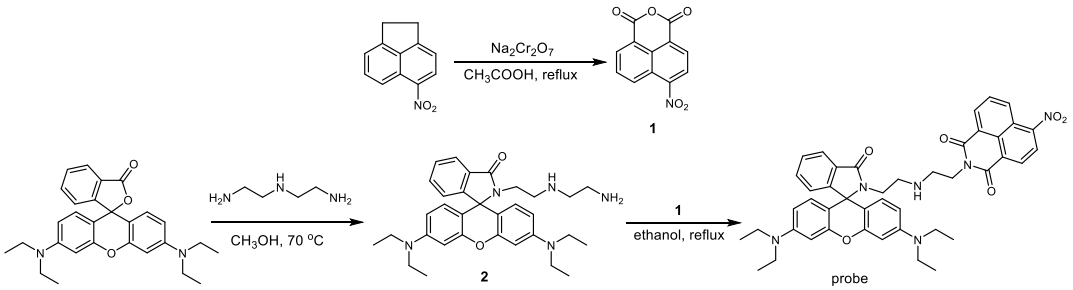
5-Nitroacenaphthene, diethylenetriamine, and adenosine 5'-diphosphate disodium salt hydrate were obtained from Alfa Aesar. Rhodamine B, guanosine 5'-monophosphate disodium salt hydrate, cytidine 5'-monophosphate disodium salt, and uridine 5'-monophosphate disodium salt were purchased from Aladdin. NADH was obtained from Acros. Nitroreductase from *Escherichia coli*, adenosine 5'-triphosphate disodium salt hydrate, adenosine 5'-monophosphate disodium salt, guanosine 5'-triphosphate sodium salt hydrate, uridine 5'-triphosphate trisodium salt hydrate, apyrase from potatoes, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*- tetrazolium bromide (MTT) and rhodamine 123 were purchased from Sigma-Aldrich. Uridine 5'-diphosphate disodium salt hydrate, and cytidine 5'-diphosphocholine sodium salt dihydrate were obtained from Ark. Dicoumarin was obtained from TCI Co. Fetal bovine serum (FBS) was obtained from Invitrogen Corporation. The cell line (HeLa), and Dulbecco's modified Eagle's media (DMEM) were obtained from KeyGEN BioTECH Co., LTD, Nanjing, China. The stock solution (1 mM) of the probe was prepared in DMSO. Ultrapure water (over 18 MΩ·cm) was produced from a Milli-Q reference system (Millipore).

^{1}H-NMR and ^{13}C-NMR spectra were performed on Bruker Fourier-300 spectrometers. Electrospray ionization mass spectra (ESI-MS) were recorded with a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on an APEX IVFTMS instrument (Bruker, Daltonics). UV-vis absorption spectra were made by a TU-1900 spectrophotometer (Beijing, China) in 1-cm quartz cells. Fluorescence spectra were measured on a Hitachi F-2500 spectrophotometer in 1×1 cm quartz cells with a 400 V PMT voltage and both excitation and emission slit widths of 10 nm. The incubations were conducted on a shaker bath (SKY-100C, Shanghai Sukun Industry & Commerce Company). Confocal fluorescence images were made on an FV 1200-IX83 confocal laser scanning microscope (Olympus, Japan) and image processing was performed with Olympus software (FV10-ASW). The absorbance measurements in MTT analysis were measured on a microplate reader (BIO-TEK Synergy HT, USA).

2. Synthesis of the probe

The probe was synthesized according to the route shown in Scheme S1 through 3 steps. First, compound **1** was prepared according to the literature.[1] Then, compound **2** was synthesized following the reported procedure[2] with some modifications. In brief, under nitrogen atmosphere, diethylenetriamine (5 mL, 46 mmol) was added to a stirred solution of rhodamine B (1 g, 2.4 mmol) dissolved in 10 mL CH_3OH. The mixture was refluxed for 24 h, cooled to the room temperature, and partitioned between H_2O and CH_2Cl_2 by adding CH_2Cl_2. Then the organic phase was dried over anhydrous Na_2SO_4, and evaporated by a rotary evaporator. The residue was subjected to silica-gel column chromatography, eluted

with CH_2Cl_2/CH_3OH (10:1, v/v), affording compound **2** as a milk white solid (950 mg, yield 41%). Finally, compound **1** (528 mg, 1 mmol) and compound **2** (243 mg, 1 mmol) were dissolved in ethanol (10 mL), and the mixture was refluxed for 8 h. After removing the solvent under reduced pressure, the crude product was purified with silica-gel column chromatography (CH_2Cl_2/CH_3OH, 20:1, v/v), affording the probe (585 mg, yield 78%) as a brown solid. ^{1}H-NMR and ^{13}C-NMR spectra of the probe are shown in Fig. S1 and Fig. S2, respectively. ^{1}H-NMR (400 MHz, 298 K, $CDCl_3$): δ 8.83 (d, 1H, J=8.4 Hz), 8.67 (d, 1H, J=7.2 Hz), 8.62 (d, 1H, J=8.0 HZ), 8.36 (d, 1H, J=8.0 Hz), 7.95 (t, 1H, J=8.4 Hz), 7.56 (d, 1H, J=6.8 Hz), 7.37 (m, 2H), 7.02 (d, 1H, J=7.6 Hz), 6.40 (s, 1H), 6.38 (s, 1H), 6.35 (d, 2H, J=2.4 Hz), 6.27 (d, 1H, J=2.4 Hz), 6.25 (d, 1H, J=2.4 Hz), 4.21 (t, 2H, J=6.0 Hz), 3.32 (m, 8H, 7.2 Hz), 3.24 (t, 2H, J=6.4 Hz), 2.83 (t, 2H, J=5.6 Hz), 2.40 (t, 2H, J=6.0 Hz), 1.16 (t, 12H, J=6.8 Hz) . ^{13}C-NMR (100 MHz, 298 K, $CDCl_3$): δ 168.5, 163.4, 162.6, 153.6, 153.2, 149.4, 148.8, 132.3, 132.3, 130.9, 129.8, 129.7, 129.2, 129.1, 128.7, 127.8, 127.2, 123.8, 123.7, 123.6, 123.2, 122.5, 108.1, 105.5, 97.7, 64.8, 47.5, 46.8, 44.3, 40.2, 39.6, 12.6. HR-ESI-MS: *m/z* calcd. for the probe ($C_{44}H_{45}N_6O_6$, $[M]^+$), 753.3322; found, 753.3396.



Scheme S1. Synthetic route of the probe.

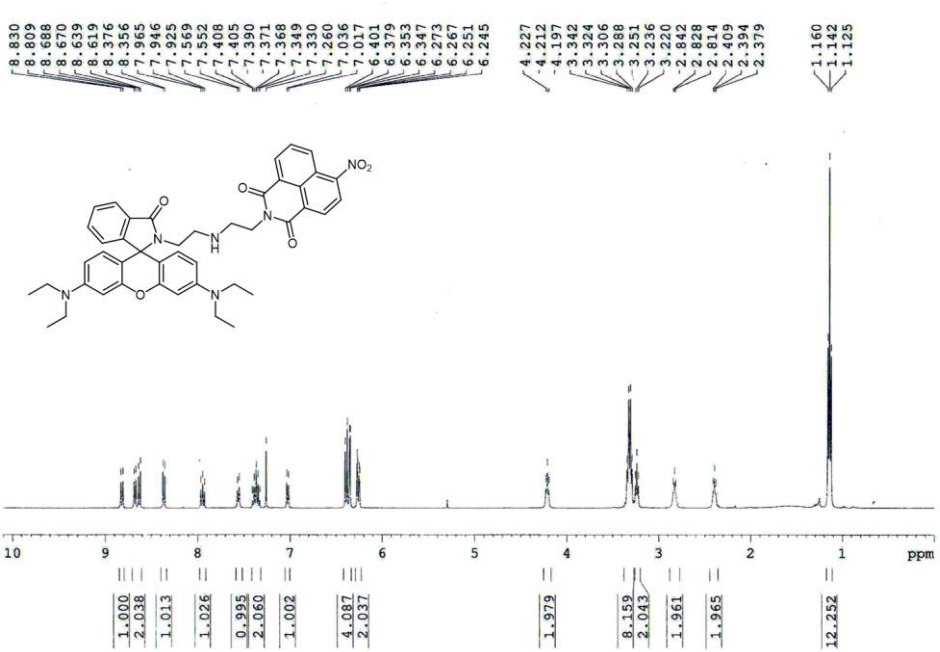


Fig. S1 ^{1}H-NMR spectrum of the probe (400 MHz, 298 K, $CDCl_3$).

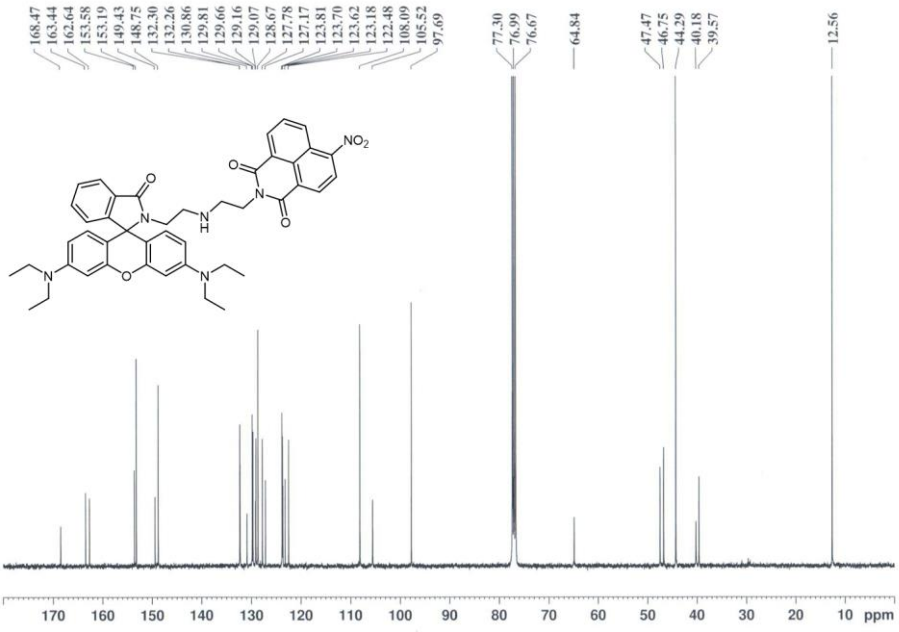


Fig. S2 ^{13}C-NMR spectrum of the probe (100 MHz, 298K, $CDCl_3$).

3. General procedure for spectroscopic analysis and solution preparation

Unless otherwise specified, the spectral measurements were made in 10 mM Tris buffer (pH 7.4) according to the following procedure. In a test tube, 1.5 mL of 10 mM Tris buffer was added, followed by addition of 20 μL probe stock solution (1 mM) and appropriate volume of reactant (ATP, NTR, or other substances) solution, and the final volume was adjusted to 2 mL by 10 mM Tris buffer (note: for all tests about NTR, a final concentration of 500 μM NADH was used). The fluorescence of the reaction solution was measured at 37 °C.

Reactive oxygen species such as H_2O_2 and OCl^- were prepared and determined as described previously.[3]

4. Cell incubation and fluorescence imaging

HeLa cells were cultured on glass-bottom culture dishes (MatTek Co.) in DMEM complemented with FBS (10%, v/v) and penicillin-streptomycin (1%, v/v) at 37 °C in a humidified normoxic atmosphere (20% O_2) of 95% air and 5% CO_2 or different degrees of hypoxic atmosphere: 85% N_2, 10% O_2, 5% CO_2; 90% N_2, 5% O_2, 5% CO_2; 94% N_2, 1% O_2, 5% CO_2. For fluorescence imaging, the adherent cells were washed three times with FBS-free DMEM, then incubated with 10 μM probe for 40 min under the respective atmosphere, and finally washed three times with FBS-free DMEM to eliminate the free probe. The pixel intensity of the cells in the fluorescence image was determined by using ImageJ software (version 1.45s, NIH). The cells were taken as a Region of Interest (ROI) based on their periphery. For comparison, the pixel intensity at least from three cells in each fluorescence image was measured and averaged in this work.

5. Spectroscopic properties of the probe

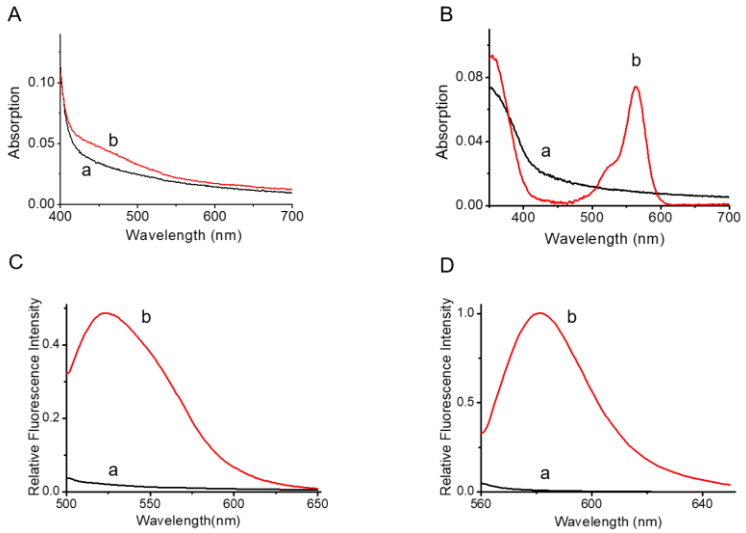


Fig. S3 Absorption (A, B) and fluorescence (C, D) spectra of the probe (10 μM) before (a) and after (b) reaction with (A, C) NTR (10 μg/mL; λ_{ex} = 420 nm) in the presence of 500 μM NADH and (B, D) ATP (4 mM; λ_{ex} = 540 nm) at 37 °C in Tris buffer (pH 7.4).

6. Study on the reaction mechanism of the probe with ATP and NTR

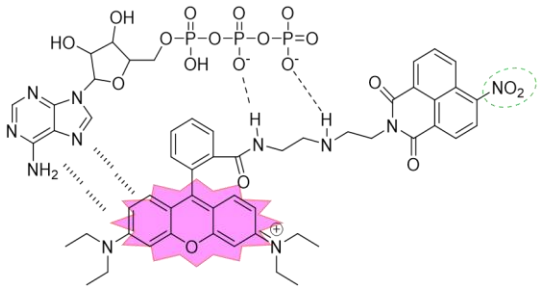


Fig. S4 The reaction mechanism of the probe with ATP.

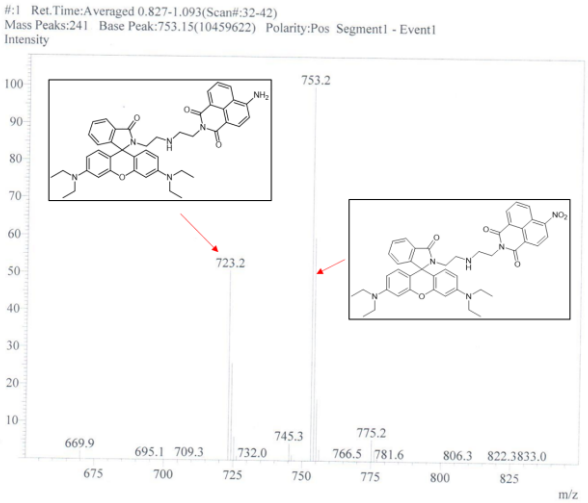


Fig. S5 ESI-MS of reaction solution of the probe (50 μM) with NTR (50 μg/mL). The peak at *m/z* = 723.2 indicates the conversion from nitro to amino.

7. Optimization of experimental conditions

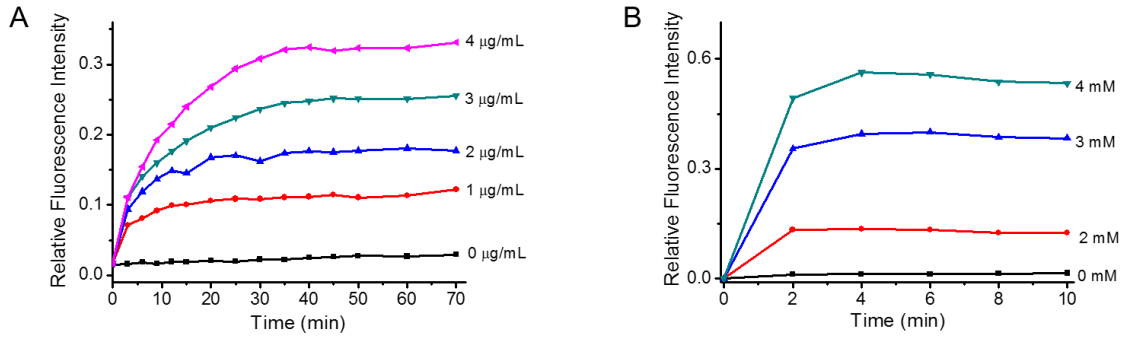


Fig. S6 Plots of fluorescence intensity vs. the reaction time of the probe (10 μM) with varied concentrations of (A) NTR ($\lambda_{ex/em}$= 420/520 nm) and (B) ATP ($\lambda_{ex/em}$= 540/580 nm) in Tris buffer (pH = 7.4).

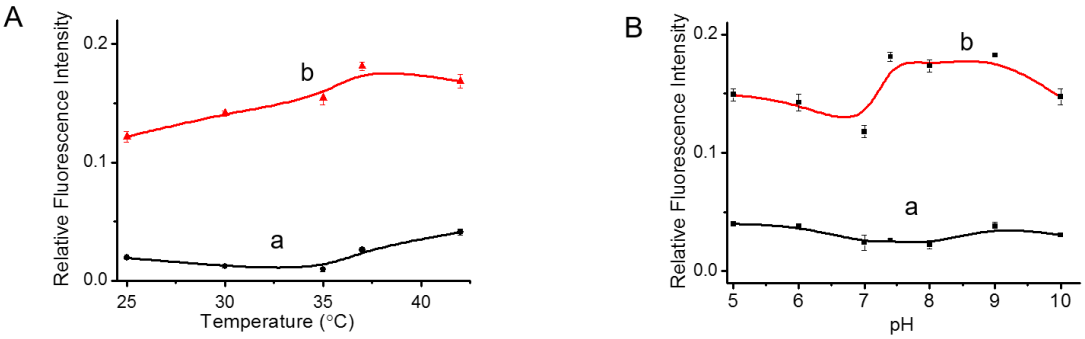


Fig. S7 Effects of (A) temperature and (B) pH on the fluorescence of the probe (10 μM) in the absence (a) and presence (b) of NTR (2 μg/mL) in Tris buffer (pH 7.4). $\lambda_{ex/em}$= 420/520 nm.

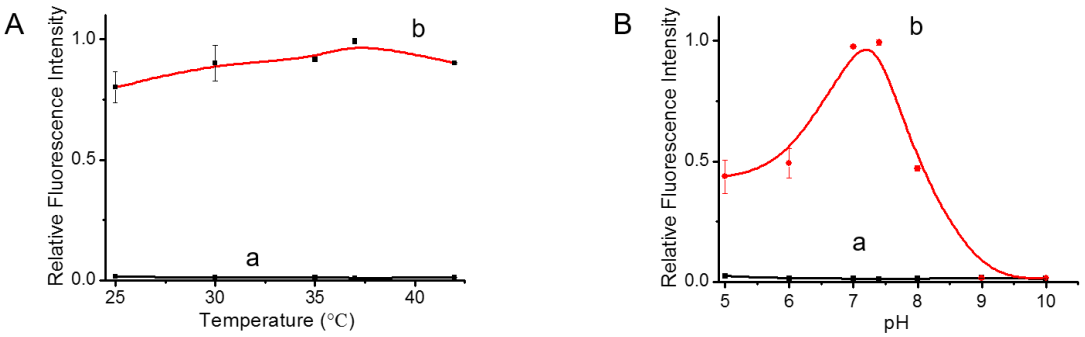


Fig. S8 Effects of (A) temperature and (B) pH on the fluorescence of the probe (10 μM) in the absence (a) and presence (b) of ATP (5 mM). $\lambda_{ex/em}$= 540/580 nm.

8. The linear relationship between ΔF and the low concentration of ATP

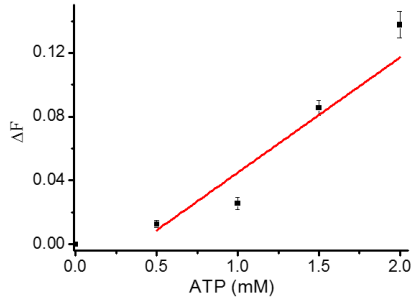


Fig. S9 The linear relationship between ΔF and the low concentrations of ATP (0.5-2 mM).

9. Fluorescence responses of the probe in the presence of both NTR and ATP

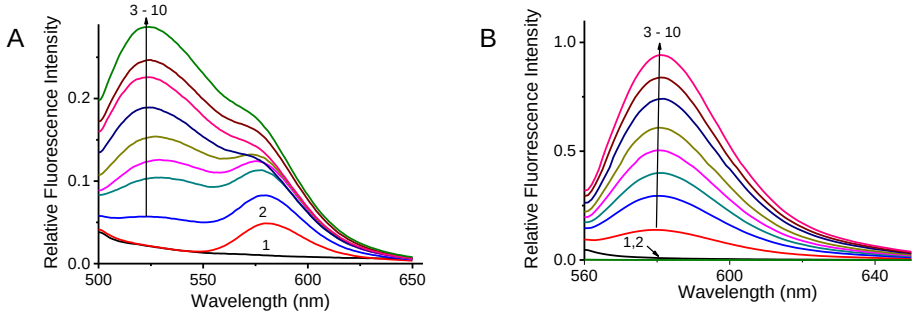


Fig. S10 Fluorescence response of the probe (10 μM) to NTR and ATP in 10 mM Tris buffer (pH 7.4). (A) The probe reacted with different concentrations of NTR (0-5 μg/mL) in the presence of 4 mM ATP (curves 2-10). The fluorescence from the solution of the probe itself without NTR and ATP is also shown as curve 1 (another control); λ_{ex} = 420 nm. (B) The probe reacted with different concentrations of ATP (0-5 mM) in the presence of 5 μg/mL NTR (curves 2-10). The fluorescence from the solution of the probe itself without NTR and ATP is also shown as curve 1 (another control); λ_{ex} = 540 nm.

10. Fluorescence responses of the probe to inorganic or nucleoside phosphates

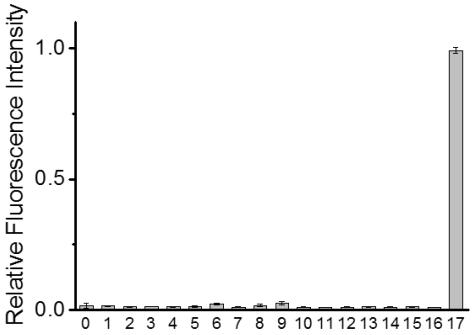


Fig. S11 Fluorescence responses of the probe (10 μM) to 5 mM of various inorganic phosphates or nucleoside polyphosphates: (0) blank; (1) Na_3PO_4; (2) Na_2HPO_4; (3)

NaH_2PO_4; (4) $Na_4P_2O_7$; (5) NaH_2PO_2; (6) adenosine diphosphate; (7) adenosine monophosphate; (8) guanosine triphosphate; (9) cytidine triphosphate; (10) uridine triphosphate; (11) guanosine diphosphate; (12) cytidine diphosphate; (13) uridine diphosphate; (14) guanosine monophosphate; (15) cytidine monophosphate; (16) uridine monophosphate; (17) ATP. $\lambda_{ex/em}$ = 540/580 nm.

11. Cytotoxicity of the probe to cells

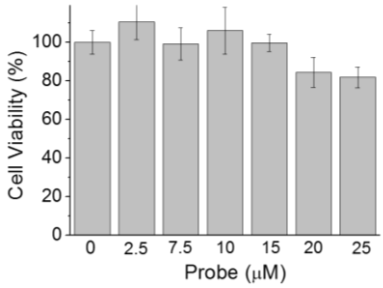


Fig. S12 Effects of the probe at varied concentrations on the viability of HeLa cells for 12 h. The cytotoxicity of the probe to HeLa cells was measured by standard MTT assay.[4] The viability of the cells without the probe is defined as 100%. The results are expressed as the mean ± standard deviation of five separate measurements.

12. Fluorescence imaging of NTR and ATP in HeLa cells

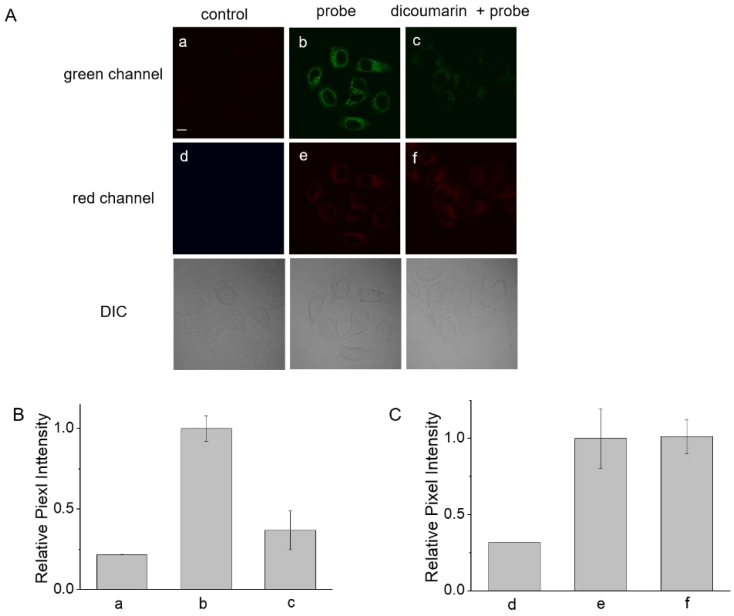


Fig. S13 (A) Fluorescence imaging of NTR in HeLa cells which were cultured under the hypoxia condition of 1% O_2 for 12 h. (a,d) Cells only (control); (b,e) the cells were incubated with the probe (10 μM) for 40 min; (c,f) the cells were pretreated with 0.1 mM dicoumarin for 30 min and then incubated with the probe as (b,e). The differential interference contrast (DIC) images of the corresponding samples are shown at the bottom. Scale bar, 10 μm. (B) Relative pixel intensity (n = 3) of the fluorescence images from the

green channel in panel A (the maximum pixel intensity from image b is defined as 1.0). (C) Relative pixel intensity (n = 3) of the fluorescence images from the red channel in panel A (the maximum pixel intensity from image f is defined as 1.0).

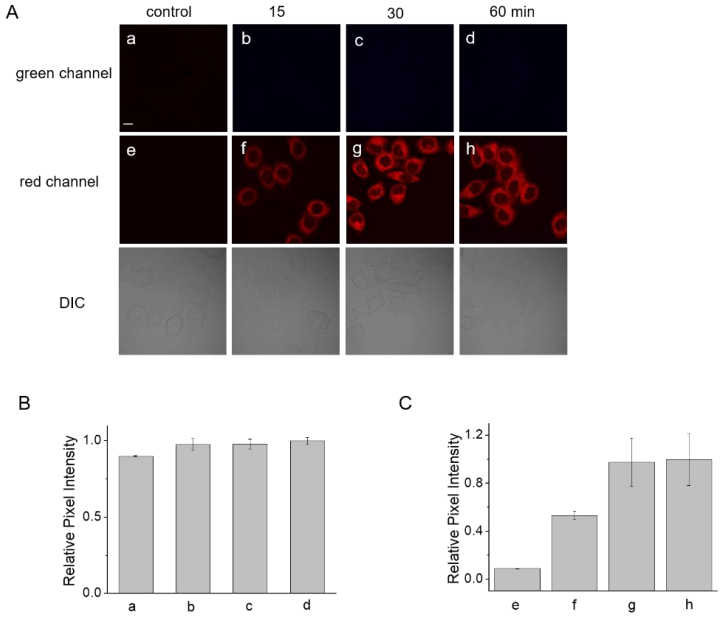


Fig. S14 Effects of time on the fluorescence images of ATP in HeLa cells. (A) (a,e) Cells only (control); (b-d and f-h) the cells incubated with the probe (10 μM) for different periods of time. The DIC images of the corresponding samples are shown at the bottom. Scale bar, 10 μm. (B) Relative pixel intensity (n = 3) of the fluorescence images from the green channel in panel A (the maximum pixel intensity from image d is defined as 1.0). (C) Relative pixel intensity (n = 3) of the fluorescence images from the red channel in panel A (the maximum pixel intensity from image h is defined as 1.0).

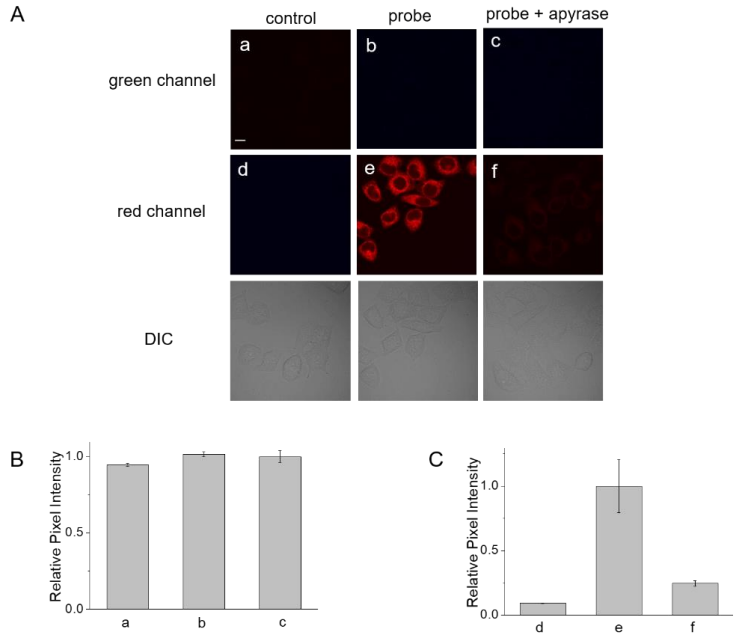


Fig. S15 Effects of apyrase on the fluorescence imaging of ATP in HeLa cells. (A) (a,d)

Cells only (control); (b,e) the cells were incubated with the probe (10 μM) for 40 min; (c,f) the cells were pretreated with apyrase (0.5 U/mL) for 30 min, and then treated as (b,e). The DIC images of the corresponding samples are shown at the bottom. Scale bar, 10 μm. (B) Relative pixel intensity (n = 3) of the fluorescence images from the green channel in panel A (the maximum pixel intensity from image b is defined as 1.0). (C) Relative pixel intensity (n = 3) of the fluorescence images from the red channel in panel A (the maximum pixel intensity from image e is defined as 1.0).

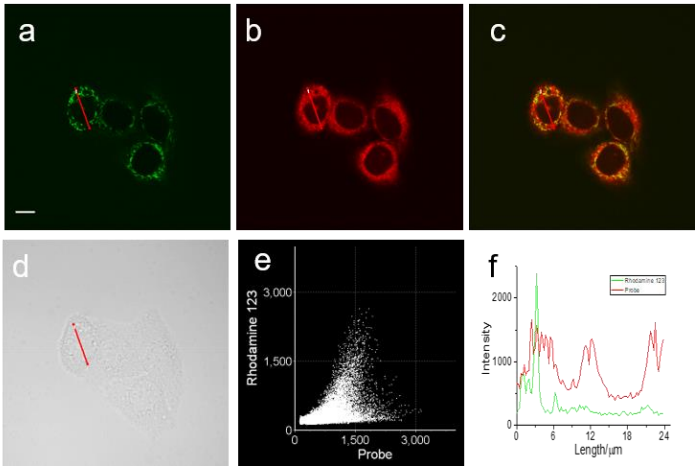


Fig. S16 Colocalization of the probe and rhodamine 123 (a commercial mitochondrial-targeting agent) in HeLa cells. Cells were co-stained with the probe (10 μM) and rhodamine 123 (500 nM) for 30 min. Scale bar, 10 μm. (a) Fluorescence image from rhodamine 123 channel (λ_{ex} = 488 nm, λ_{em} = 500-550 nm); (b) fluorescence image from the probe channel (λ_{ex} = 559 nm, λ_{em} = 570-670 nm); (c) the merged image of images (a) and (b); (d) the DIC image; (e) the intensity correlation plot of the probe and rhodamine 123; (f) intensity profiles of the probe and rhodamine 123 within the linear ROI (red line in images a and b) across the cell.

11. References

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