Supporting Information

Fluorescence monitoring of refluxed tyrosinase by endoplasmic reticulum-localized enzymatic activity sensing

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Contents

1. Experimental section

1.1 Chemicals and materials

All chemicals were obtained from commercial suppliers and used without further purification. Water used in all experiments was doubly distilled and purified via a Milli-Q water system (Millipore, USA). Stock solution (500 μM) of the probe was prepared by dissolving ER-Nap-TYR in DMSO. Tyrosinase (from mushroom) and its inhibitors were purchased from Sigma Aldrich and Energy Chemical, respectively. Tyrosinase, was dissolved in water at a concentration of 5 KU/mL. Stock solutions of metal ions and anions were prepared from the corresponding inorganic salts.

1.2 Preparation of ROS

Peroxynitrite ion ($ONOO^-$): $NaNO_2$ (0.6 M), HCl (0.6 M) and H_2O_2 (0.7 M) were added simultaneously to NaOH (3 M) solution at 0 °C. The absorbance value of the solution at 302 nm was then measured in a UV-Vis spectrophotometer and the concentration of $ONOO^-$ in the solution was calculated by using Lambert-Beer law (the molar absorption coefficient was 1670 $M^{-1}cm^{-1}$).

Nitric oxide (NO): NO was obtained from the stock solution prepared from sodium nitroprusside.

Superoxide anion ($O_2^{\cdot-}$): Potassium superoxide (KO_2) was taken and dissolved in dry DMSO under ultrasound, the absorbance value of the solution at 250 nm was measured in a UV-Vis spectrophotometer and the concentration of superoxide anion in the solution was calculated using the Lambert-Beer law (the molar absorption coefficient was 2682 $M^{-1}cm^{-1}$).

Hydroxyl radicals (·OH): Hydroxyl radicals are prepared by the Fenton reaction principle. A solution containing hydroxyl radicals is obtained by mixing and reacting H_2O_2 (200 μM) with $FeCl_2$ (1.0 mM).

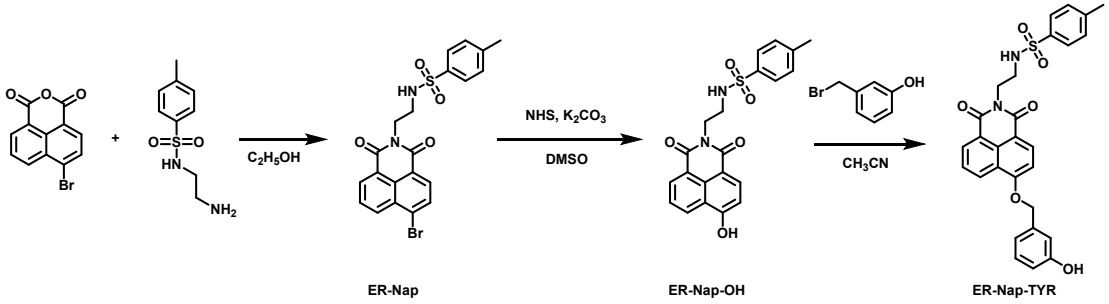
Hypochlorite ion (ClO^-): A solution containing a certain concentration of ClO^- can be obtained by diluting NaClO at the desired concentration.

Hydrogen peroxide (H_2O_2): Hydrogen peroxide solution is made by diluting 30% hydrogen peroxide, determining the absorbance value of the solution at 240 nm in a UV-visible spectrophotometer and calculating the concentration of hydrogen peroxide in the solution using Lambert-Beer's law with a molar absorption coefficient of 43.6 $M^{-1}cm^{-1}$.

1.3 Instruments

UV-vis absorption spectra were measured by using a UV-2700 spectrophotometer (Shimadzu, Japan). NMR spectra were recorded on a JNM-ECZS spectrometer (JEOL, Japan). Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). Fluorescence emission spectra were recorded on a F7000 fluorescence System (Japan) with excitation and emission slit set at 10 nm. The pH was measured with a PHS-3C pH meter (Shanghai INESA Scientific Instrument Co., Ltd., China). Fluorescence images were acquired by a FV3000 confocal laser scanning microscope (Olympus, Japan).

1.4 Synthesis



Scheme S1. Synthetic routes of the probe **ER-Nap-TYR**.

Synthesis of Compound ER-Nap. N-(2-aminoethyl)-4-methyl-benzenesulfonamide (385.7 mg, 1.8 mmol) and 4-Bromo-1,8-naphthalic anhydride (498.7 mg, 1.8 mmol) a were dissolved in 10 mL ethanol, and the mixture was vigorously stirred at 80 ℃ for 2 h. after the reaction, the mixture was washed multiple times with ethanol, and then the precipitated solid was filtered, dried under a vacuum, and afforded **ER-Nap** (681.6 mg, yield:80%).

Synthesis of Compound ER-Nap-OH. Compound **ER-Nap** (118.4 mg, 0.25 mmol), NHS (31.8 mg, 0.275 mmol) and K_2CO_3 (114.0 mg, 0.825 mmol) were dissolved in 10 mL DMSO, and the mixture was vigorously stirred at 100 ℃ for 2.5 h. After the reaction, the mixture was cooled to room temperature and poured into 10 mL cold water, and then acidified by HCl until PH=6 and extracted with CH_2Cl_2. The organic phase was washed with brine water and dried over anhydrous $MgSO_4$. After concentration by rotary evaporation, silica-gel column chromatography (CH_2Cl_2: MeOH = 100:1 to 20:1) afforded **ER-Nap-OH** (46 mg, yield:47%).

Synthesis of ER-Nap-TYR. To a solution of **ER-Nap-OH** (410.5 mg, 1.0 mmol) in 30 mL CH_3CN, NaH (79.2 mg, 3.3 mmol) was added with stirring for 10 min under N_2 atmosphere. Then, a solution of 3-(bromomethyl)phenol (374.1 mg, 2 mmol) in CH_3CN (5 mL) was added dropwise. The resulting mixture was stirred at 60 ℃ for 12 h, and then the solvent was removed by the evaporation under the reduced pressure. CH_2Cl_2 (50 mL) and water (50 mL) were added. The organic layer was collected and dried with anhydrous Na_2SO_4. After the layer was evaporated, the crude compound was purified by silica gel column chromatography using the eluent (CH_2Cl_2/MeOH, v/v, 30:1) to yield **ER-Nap-TYR** as a yellow (87.7 mg, 17 %).^{1}H NMR (400 MHz, DMSO) δ 9.54 (s, 1H), 8.51 (s, 1H), 8.45 (s, 2H), 7.80 (s, 2H), 7.60 (s, 2H), 7.36 (d, *J* = 8.4 Hz, 1H), 7.25 (s, 3H), 6.99 (d, *J* = 7.2 Hz, 2H), 6.78 (d, *J* = 7.7 Hz, 1H), 5.36 (d, *J* = 33.7 Hz, 2H), 4.08 (s, 2H), 3.08 (d, *J* = 6.2 Hz, 2H), 2.25 (s, 3H).^{13}C NMR (101 MHz, DMSO) δ 164.09, 163.40, 159.64, 158.04, 142.87, 138.05, 137.86, 133.47, 131.44, 130.15, 129.90, 129.16, 128.70, 126.80, 123.34, 118.52,

115.57, 114.89, 114.72, 107.83, 70.71, 49.05, 40.84, 39.93, 21.30. HR-MS (MALDI-TOF-MS): m/z = 516.57, calcd for $(C_{28}H_{24}N_2O_6SNa)^+$= 539.127($[M+Na]^+$)

1.5 UV/Vis absorption and fluorescence spectroscopic methods

All spectra were recorded in PBS/DMSO solution (v:v = 99:1, 10 mM, pH = 7.4). Test solutions were prepared by mixturing ER-Nap-TYR and appropriate analyte stock into a tube and then diluting the solution to 500 μL with PBS buffer. After incubation at 37 ℃ for 4h, the absorption or fluorescence spectra measurements were then performed. The fluorescence spectra were recorded at emission wavelength range from 430 to 650 nm with excitation wavelength of 408 nm with all excitation slit widths 10 nm.

1.6 Enzyme kinetics

Time dependences of fluorescence response of various concentrations of ER-Nap-TYR (1–9 μM) toward tyrosinase (200 U/mL) were determined at 550 nm, with a time interval of 15 min for 4 h. All measurements were recorded in PBS/DMSO solution (v:v = 99:1, 5 μM, pH = 7.4) at 37 ℃ incubation. λ_{ex} = 405 nm. K_m and V_{max} values were obtained by plotting of velocity ($\mu Ms^{-1)}$ vs. ER-Nap-TYR (μM) for Michelis-Menten kinetics.

$$1/V=K_m+[S]/V_{max}[S]$$

1.7 Density functional theory (DFT) calculation

The ground state structure of compounds ER-Nap-TYR and ER-Nap-OH were optimized using DFT with B3LYP function and 6-31G basis set. And the excitation energies of ER-Nap-TYR and ER-Nap-OH in quantum chemistry were calculated by the most popular method time dependent density functional theory (TD-DFT). All of these calculations were performed with Gaussian 09 program package.

1.8 Cell Incubation and Cytotoxicity Assay

B16 and HeLa cells were cultured using high-glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 1% penicillin-streptomycin (10,000 U/mL, 10,000 μg mL^{-1}, Invitrogen) and 10% fatal bovine serum (GIBCO) in an atmosphere of 5% CO_2 and 95% air at 37°C.

The cellular cytotoxicity of ER-Nap-TYR towards B16 cells as the model was evaluated using the standard cell viability assay - the MTT assay. B16 cells were seeded into a 96-well plate at a concentration of 5×10^3 cells well-1 in 100 μL of DMEM medium with 10% FBS. Plates were maintained at 37 °C in a 5% CO_2 95% air incubator for 24 h. After the original medium was removed, the B16 cells were incubated with different concentrations of ER-Nap-TYR (0-25 μM). The cells incubated with the culture medium only were served as the controls. The cells were washed with PBS for three times and then 100 μL MTT solution (0.5 mg mL^{-1} in PBS) was added to each well.

After addition of DMSO (200 μL $well^{-1}$), the assay plate was allowed to shake at room temperature for 10 min. The spectrophotometrical absorbance of the samples was measured by using a Tecan microplate (ELISA) reader. The cell viability was calculated based on measuring the UV-vis absorption at 490 nm using the following equation, where OD490 represents the optical density.

$$\text{Cell viability} = [OD_{490(sample)} - OD_{490(blank)}] / [OD_{490(control)} - OD_{490(blank)}]$$

1.9 Ratiometric Imaging of TYR in Living Cells

(1) Colocalization experiments: For the colocalization experiment, the cells were treated with ER-Nap-TYR (5.0 μM) for 4 h and ER-Tracker Red (1.0 μM) simultaneously stained the B16 cells in the incubator at 37 °C with 5 % CO_2 for 15 min and then rinsed for three times with PBS. Fluorescence images were acquired with FV3000 confocal laser scanning microscope with a 40× objective lens.

(2) Fluorescence imaging of endogenous tyrosinase: (A)The images were detected at different incubation time points (0 h to 5 h) upon the treatment of 5 μM ER-Nap-TYR in the melanoma B16 cells, rinsed for three times with PBS, and then the cells were imaged; (B) B16 cells pretreated with 200 μM Kojic Acid for 4 h then treated with 5 μM ER-Nap-TYR for 4 h, rinsed for three times with PBS, and then the cells were imaged; (C) HeLa cells pretreated with 5 μM ER-Nap-TYR for 4 h, rinsed for three times with PBS, and then the cells were imaged. Fluorescence images were acquired with FV3000 confocal laser scanning microscope with a 40× objective lens.

(3) Fluorescence imaging of tyrosinase level changes during H_2O_2 treatment: The B16 cells were treated with H_2O_2(0-150 μM) for 4 h, and washed three times with PBS then treated with 5 μM ER-Nap-TYR for 4 h, rinsed for three times with PBS, and then the cells were imaged. Fluorescence images were acquired with FV3000 confocal laser scanning microscope with a 40× objective lens.

2.Spectroscopic and Cells Data

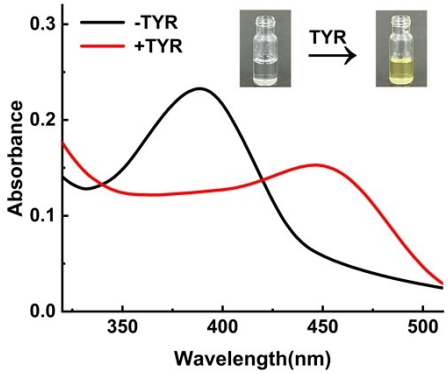


Fig. S1 UV-Vis spectra of 5 μM ER-Nap-TYR in the absence and presence of TYR in PBS (pH = 7.4, 1% DMSO, 10 mM). Inset: Images of the probe in the absence (left) and presence (right) of 300 U/mL TYR.

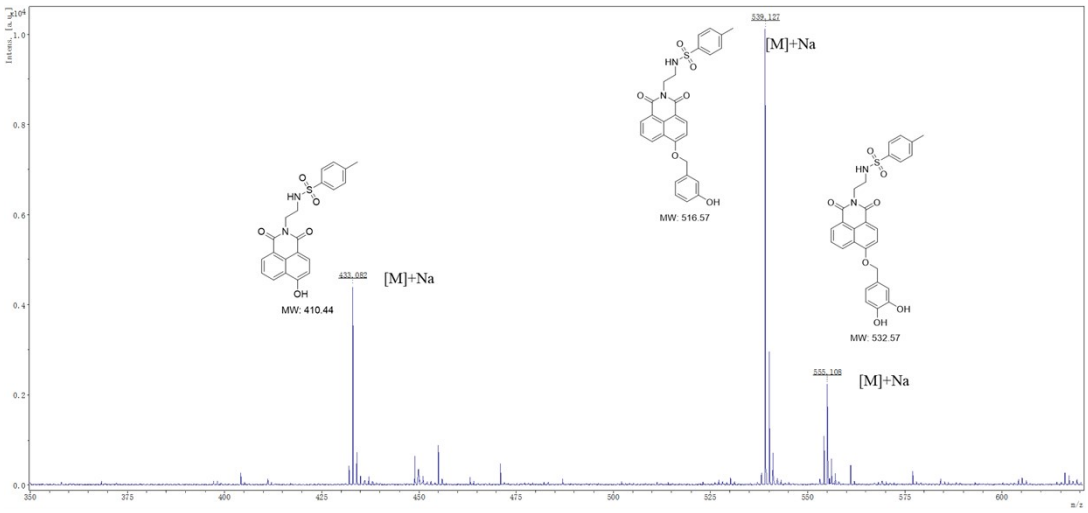
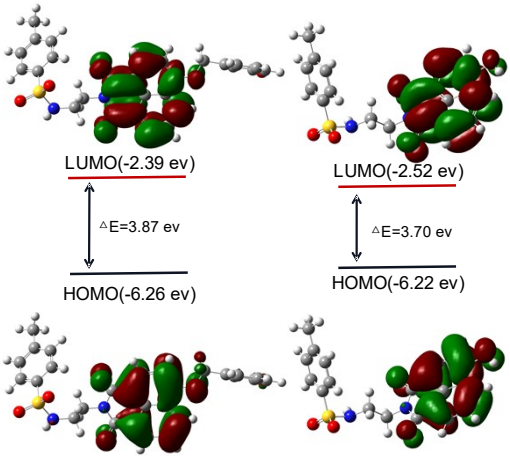


Figure S2 MALDI-TOF-MS spectra showing the analysis of reaction intermediate and product of ER-Nap-TYR (500 μM) with TYR (200 U/mL).



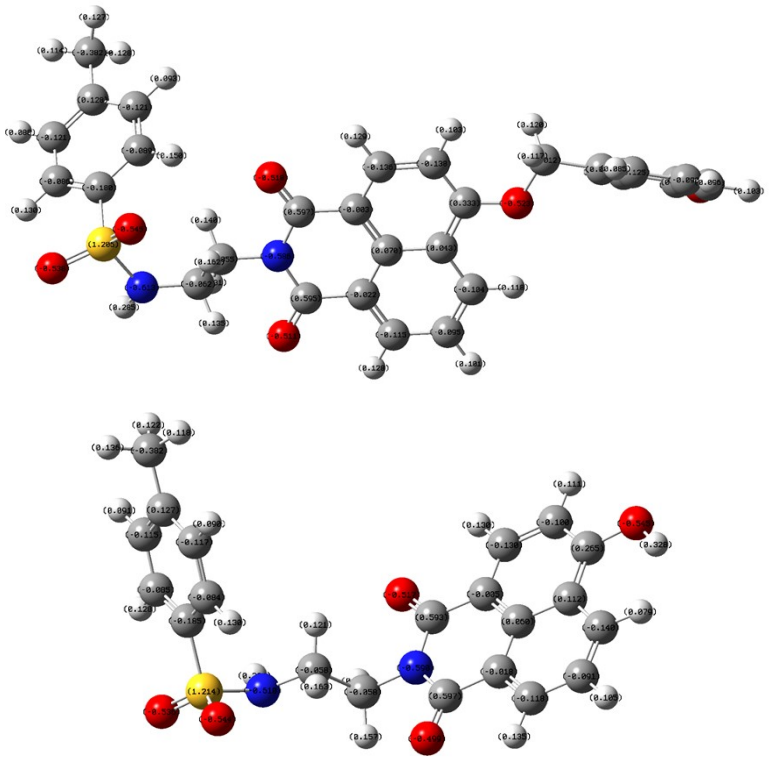


Figure S3 Frontier molecular orbital profiles and the charges of ER-Nap-TYR and ER-Nap-OH in the excited states based on DFT (B3LYP/6-31G*) calculations.

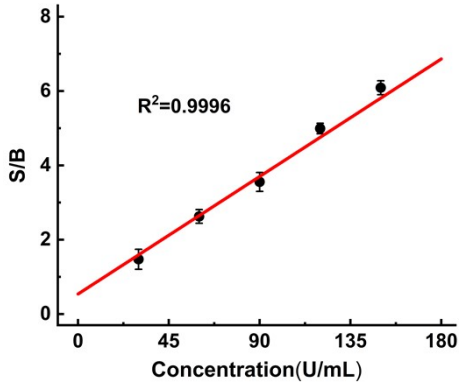


Figure S4 Linear relationship between S/B and TYR concentration in the range 0, 30, 60, 90, 120, 150, 180 U/mL. λ_{ex} = 408 nm.

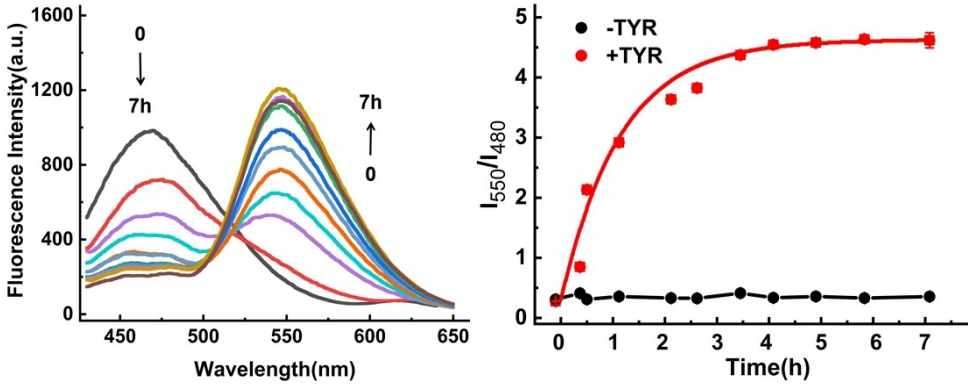


Figure S5 Time-dependent Fluorescence intensity ratios (I_{550}/I_{480}) of ER-Nap-TYR in the absence (black) and presence (red) of 300 U/mL TYR. λ_{ex} = 408 nm.

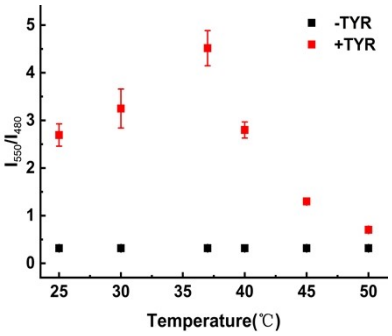


Figure S6 Fluorescence intensity ratios (I_{550}/I_{480}) of ER-Nap-TYR in the absence (black) and presence (red) of 300 U/mL TYR at various temperature conditions. λ_{ex} = 408 nm.

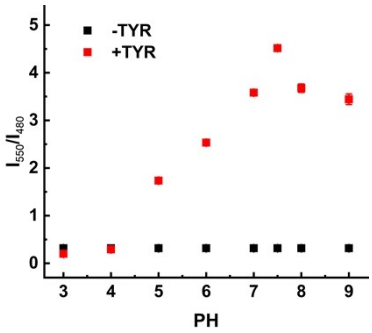


Figure S7 Fluorescence intensity ratios (I_{550}/I_{480}) of ER-Nap-TYR in the absence (black) and presence (red) of 300 U/mL TYR at various PH conditions. λ_{ex} = 408 nm.

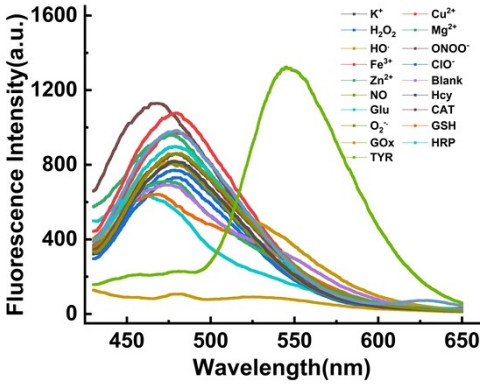


Figure S8 Fluorescence intensity ratios (I_{550}/I_{480}) of probe in the presence of 100 μM metals (K^+, Cu^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}), 100 μM reactive oxygen/nitrogen species (ClO^-, $ONOO^-$, $OH^·$, $O^{2·-}$, H_2O_2, NO), biothiols (100 μM Cys and Hcy, 1 mM GSH), 300 U/mL enzymes (CAT, GOx, HRP, TYR) in PBS/DMSO solution (v:v = 99:1, 5 μM), pH = 7.4). λ_{ex} = 408 nm.

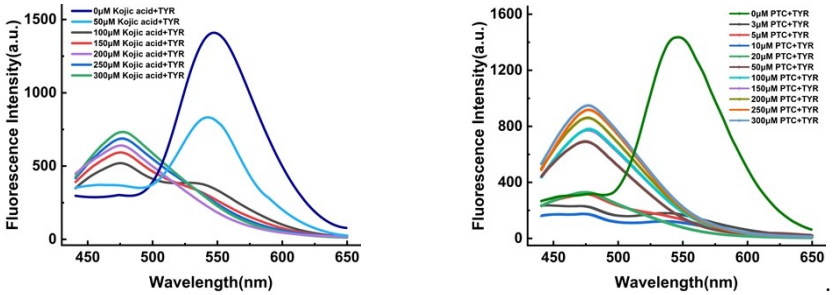


Figure S9 Fluorescence intensity ratio (I_{550}/I_{480}) of ER-Nap-TYR towards various concentrations of tyrosinase inhibitors kojic acid (left) and phenylthiourea (PTC,right).

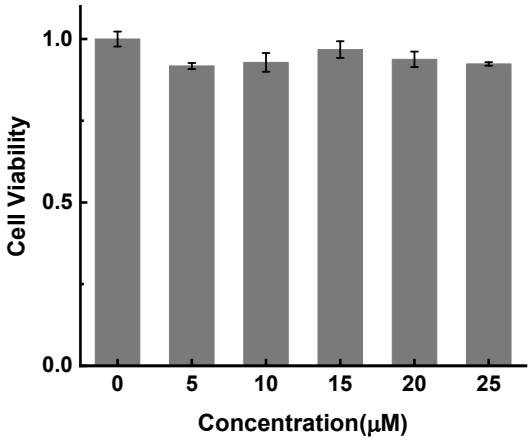


Figure S10 Survival of B16 cells in the presence of ER-Nap-TYR at various concentrations measured using MTT assay.

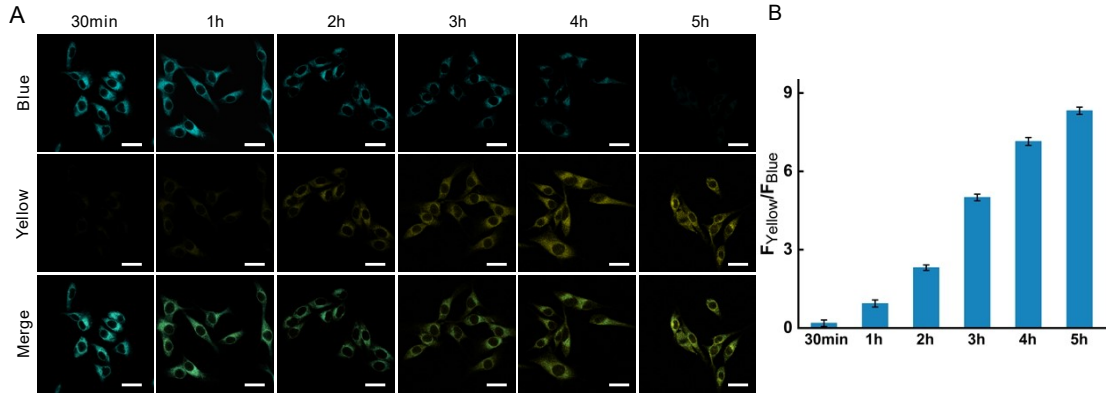


Figure S11 Fluorescence microscopy images of probe ER-Nap-TYR (5 μM) in living cells (B16) for different time. (A) Images were acquired in B16 cells after washing by PBS; (B)The relative ratio value of fluorescence intensity (FYellow/FBlue). Data represent mean standard error (n = 3). Blue channel (450-490 nm); Yellow channel (530-580 nm), λ_{ex}= 405 nm. Scale bar = 25 μm.

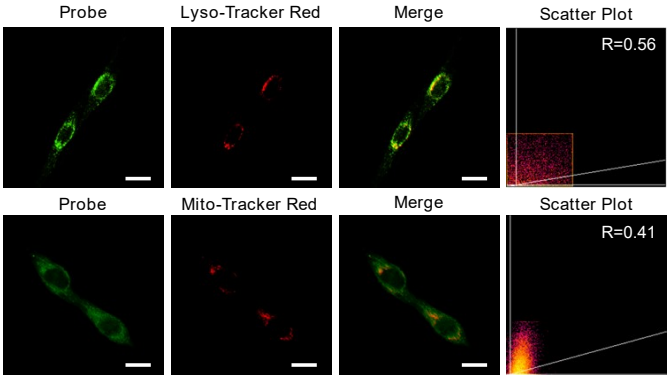
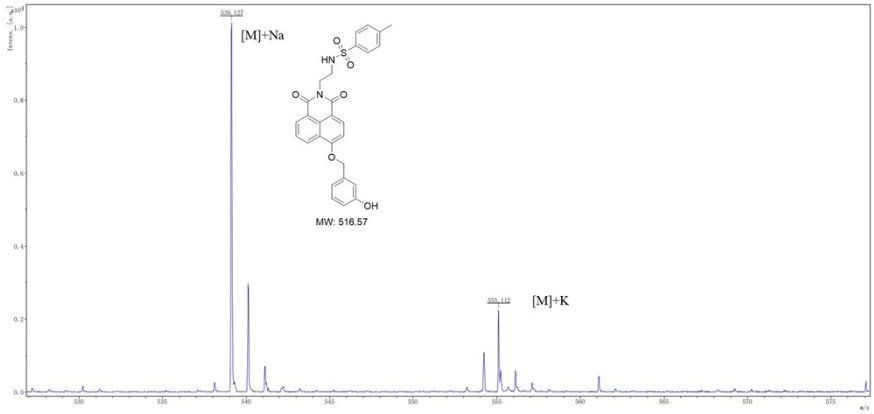


Figure S12 Measuring TYR activity in subcellular organelle with ER-Nap-TYR. Fluorescence of probe ER-Nap-TYR (25 μM): Green channel (530-580 nm) with excitation 405 nm, Red channel: Lyso-Tracker Red (λ_{ex} = 561 nm, λ_{em} = 585-620 nm) and Mito-Tracker Red (λ_{ex} = 561 nm, λ_{em} = 585-620 nm), Scale bar: 25 μm.

3.NMR and Mass Spectra

3.1 MALDI-TOF-MS of ER-Nap-TYR.



3.2 ^{1}H NMR and ^{13}C NMR of ER-Nap-TYR in DMSO-d_6