Electronic Supplementary Information

Clicking of Organelle-Enriched Probes for Fluorogenic Imaging of

Autophagic and Endocytic Fluxes

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1. Experimental Procedures

1.1 Materials and Instruments. Rabbit antibodies to LC3-I, LC3-II, β-actin, horse radish peroxidase (HRP)-labelled anti-rabbit IgG, rapamycin, liensinine, chloroquine (CQ), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), chlorpromazine, methyl-β-cyclodextrin (Me-β-CD), amiloride, nystatin, epidermal growth factor (EGF), thapsigargin, prochlorperazine, bafilomycin A1, IFN-γ and anti-human MHC-I antibody (H1650) were purchased from Sigma-Aldrich (MO, USA). MitoTracker Green, LysoTracker Red and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) were obtained from Invitrogen (MD, USA). MitoTracker Blue and LysoTracker NIR were purchased from AAT Bioquest (USA). Alexa Fluor 700 anti-human MHC-I (56-9983-42), Alexa Fluor 700 anti-human PD-L1 (56-5983-42), anti-human PD-L1 antibody (PA5-18337) and Alexa Fluor Plus 647 labeled anti-mouse IgG secondary antibody (A32728), Earle's Balanced Salt Solution (EBSS) medium, Dulbecco's Modified Eagle Medium (DMEM) high glucose medium, penicillin, streptomycin and heat-inactivated fetal bovine serum (FBS) were obtained from Thermo Fisher (MA, USA). Plasmid eBFP-tagged LC3 (eBFP-LC3) was purchased from Genscript (China). HeLa cells, MCF-7 cells, HepG2 cells, MDA-MB-231 cells, A549 cells, Hup-T3 cells were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China). Ultrapure water was produced by Master Touch-S30UF (Hhitech, Shanghai, China). Organic solvents were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other reagents were of analytical grade and obtained from Adamas-beta® (Shanghai, China).

¹H and ¹³C NMR spectra were recorded on a Bruker DRX-400 spectrometer (Bruker, USA). The chemical shifts (δ) were reported in ppm (relative to TMS as internal standard) and coupling constants (*J*) are given in Hz. Signal multiplicities were represented by s (singlet), d (doublet), t (triplet), dd (double doublet), and m (multiplet). Electrospray ionization mass spectrometry (ESI-MS) was determined using Finnigan LCQ Advantage MAX (Thermo Finnigan, USA). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was recorded on an ultrafleXtreme instrument (Bruker Daltonics, USA). Thin-layer chromatography (TLC) was performed on silica gel aluminum sheets with an F-254 indicator. The column chromatography was conducted using 200-300 mesh SiO₂. Ultraviolet-visible absorption spectra were measured on UV2450 (Shimadzu, Japan). Fluorescence spectra were collected on an FS5 spectrofluorometer (Edinburgh, UK).

1.2 Synthesis of TCO and tetrazine probes

Lyso-BODIPY-TCO, Lyso-TCO and Mito-TCO were synthesized according to the procedures in Scheme S1.

Scheme S1 Synthetic routes for Lyso-BODIPY-TCO (A), Lyso-TCO (B) and Mito-TCO (C).



A solution of meta-chloroperoxybenzoic acid (mCPBA, 24.4 g, 120 mmol) in CH₂Cl₂ (50 mL) was slowly added to a mixture of (1Z, 5Z)-cycloocta-1,5-diene (10.8 g, 100 mmol) and CH₂Cl₂ (50 mL) at 0 °C. Then the solution was stirred at room temperature overnight and added with saturated Na₂S₂O₃ solution (150 mL). The solution was extracted with CH₂Cl₂ (300 mL) and the organic layer was dried with anhydrous Na₂SO₄ and evaporated. The residue was purified by column chromatography using petroleum ether-ethyl acetate 20:1 (v/v) as the eluent to yield Compound 1 as a colorless oil (10.2 g, 82% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 5.54-5.61 (2H, m, CH=CH), 3.02-3.06 (2H, m, CH₂), 2.42-2.48 (2H, m, CH₂), 1.99-2.18 (6H, m, 2 × CH₂ and 2 × CH). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 128.82, 56.68, 28.10, 23.66.

Syntheis of (Z)-cyclooct-4-en-1-ol (Compound 2)



A solution of lithium aluminium hydride (LAH, 21 mL, 50 mmol) was slowly added to a solution of Compound 1 (6.2 g, 50 mmol) in tetrahydrofuran (THF, 30 mL) at -20 °C under N₂ atmosphere, and the mixture was stirred at room temperature for 6 h. Then saturated NaHCO₃ solution (100 mL) was added and the solution was extracted with CH₂Cl₂ (200 mL). The organic layer was dried with anhydrous Na₂SO₄ and evaporated. The residue was purified by column chromatography using petroleum ether-ethyl acetate 6:1 (v/v) as the eluent to yield Compound 2 as a colorless oil (4.1 g, 65% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 5.58-5.75 (2H, m, CH=CH), 3.84-3.88 (1H, m, CH), 2.27-2.36 (1H, m, CH), 2.08-2.19 (3H, m, CH and CH₂), 1.84-2.09 (2H, m, CH₂), 1.49-1.76 (4H, m, 2 × CH₂). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 130.16, 129.56, 72.77, 37.76, 36.28, 25.68, 24.87, 22.79.

Synthesis of (E)-cyclooct-4-en-1-ol (Compound 3)



Compound 2 (4 g, 32 mmol) was added to a mixture of methyl benzoate (4.4 g, 32 mmol), diethyl ether (450 mL) and n-hexane (50 mL). Then the solution was irradiated with UV light for 12 h while it was constantly flowed through a column containing silica (100 g) treated with AgNO₃ (10 g). The silica was added to a solution of ammonium hydroxide (100 mL) and stirred at room temperature for 10 min. Then the solution was extracted with CH_2Cl_2 (200 mL), dried over Na₂SO₄, filtered and evaporated. The crude product was purified by column chromatography using petroleum ether and ethyl acetate (10:1) to give the Compound 3 as a colorless oil (0.65 g, 32% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 5.57-5.61 (2H, m, CH=CH), 4.03-4.07 (1H, m, CH), 2.37-2.44 (1H, m, CH), 2.07-2.27 (4H, m, 2 × CH₂), 1.76-1.90 (3H, m, CH₂ and CH), 1.62-1.70 (1H, m, CH₂), 1.24-1.31 (1H, m, CH₂). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 134.30, 132.99, 67.37, 42.98, 34.09, 29.29, 27.66.

Syntheis of (E)-cyclooct-4-en-1-yl (4-nitrophenyl) carbonate (Compound 4)



A solution of 4-nitrophenyl chloroformate (0.25 g, 1.25 mmol) in CH₂Cl₂ (5 mL) was slowly added to a mixture of Compound 3 (0.13 g, 1 mmol), triethylamine (TEA, 0.21 mL, 1.5 mmol) and CH₂Cl₂ (10 mL), and the solution was stirred at room temperature overnight. Then the solution was washed with 1 M HCl solution (20 mL), brine (20 mL), and dried with anhydrous Na2SO4. The solvent was evaporated, and the residue was purified by column chromatography using petroleum ether-ethyl acetate 12:1 (v/v) as the eluent to yield Compound 4 as a colorless solid (0.22 g, 76% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 8.29-8.33 (2H, m, Ar-H), 7.39-7.43 (2H, m, Ar-H), 5.58-5.66 (2H, m, CH=CH), 5.00-5.04 (1H, m, CH), 2.31-2.49 (4H, m, 2 × CH₂), 2.18-2.22 (1H, m, CH), 1.89-1.95 (2H, m, CH₂), 1.77-1.82 (1H, m,

CH), 1.27-1.39 (3H, m, CH₂ and CH). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 155.75, 151.97, 145.34, 135.43, 131.45, 125.27, 121.88, 75.98, 53.45, 40.62, 34.12, 32.12, 29.75, 27.99.

Synthesis of Methyl 4-(ethyl(phenyl)amino)butanoate (Compound 5)



Methyl 4-bromobutyrate (10.8 g, 60 mmol) was added to a mixture of N-ethylaniline (3.6 g, 30 mmol), K₂CO₃ (8.28 g, 60 mmol) and CH₃CN (100 mL), and the solution was stirred at 80 °C for 24 h. After filtration, the solid was washed with CH₃CN (100 mL) and the combined organic layer was concentrated in vacuum. The residue was purified by column chromatography using petroleum ether-ethyl acetate 20:1 (v/v) as the eluent to yield Compound 5 as a faint yellow oil (4.05 g, 61% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.22-7.28 (2H, m, Ar-H), 6.67-6.74 (3H, m, Ar-H), 3.71 (3H, s, CH₃), 3.32-3.42 (4H, m, 2 × CH₂), 2.40 (2H, t, *J* = 7.2 Hz, CH₂), 1.92-1.99 (2H, m, CH₂), 1.18 (3H, t, *J* = 6.8 Hz, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 134.30, 132.99, 67.37, 42.98, 34.09, 29.29, 27.66. MS (ESI) m/z: [M+H]⁺ calcd 222.15, found 222.11.

Synthesis of Methyl 4-(ethyl(4-formylphenyl)amino)butanoate (Compound 6)



POCl₃ (6.0 mL, 66 mmol) was slowly added to DMF (20 mL) at 0 °C under N₂ atmosphere, and this mixture was stirred at room temperature for 2 h. Then a solution of Compound 5 in DMF (15 mL) was slowly added and the reaction mixture was stirred at 50 °C overnight. After cooling to room temperature, the solution was poured into cold water (200 mL) and extracted with CH₂Cl₂ (300 mL), dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using petroleum ether-ethyl acetate 5:1 (v/v) as the eluent to yield Compound 6 as a faint yellow oil (2.74 g, 73% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 9.73 (1H, s, CHO), 7.73 (2H, d, *J* = 9.2 Hz, Ar-H), 6.72 (2H, d, *J* = 8.8 Hz, Ar-H), 3.72 (3H, s, CH₃), 3.41-3.50 (4H, m, 2 × CH₂), 2.41 (2H, t, *J* = 7.2 Hz, CH₂), 1.94-2.01 (2H, m, CH₂), 1.23 (3H, t, *J* = 6.8 Hz, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 190.04, 173.35, 152.33, 132.27, 124.98, 110.82, 51.72, 49.50, 45.21, 30.97, 22.48, 12.20. MS (ESI) m/z: [M+H]⁺ calcd 250.14, found 250.21.

Synthesis of Methyl 4-((4-(5,5-difluoro-1,3,7,9-tetramethyl-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)phenyl)(ethyl)amino)butanoate (Compound 7)



Trifluoroacetic acid (TFA, 0.5 mL) was added to a mixture of Compound 6 (1.25 g, 5 mmol), 2,4-dimethylpyrrole (1.05 g, 11 mmol) and CH₂Cl₂ (50 mL) at 0 °C under N₂ atmosphere, and this solution was stirred at room temperature for 24 h. Then p-chloranil (1.23 g, 5 mmol) was added and the solution was stirred at room temperature for another 1 h. After addition of TEA (15 mL) and BF₃OEt₂ (15 mL), the reaction was stirred at room temperature overnight. Then CH₂Cl₂ (150 mL) was added and the organic layer was washed with brine (200 mL). The organic layer was dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using petroleum ether-ethyl acetate 6:1 (v/v) as the eluent to yield Compound 7 as a red solid (0.82 g, 35% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.04 (2H, d, *J* = 8.8 Hz, Ar-H), 6.77 (2H, d, *J* = 8.8 Hz, Ar-H), 5.99 (2H, s, 2 × CH=), 3.72 (3H, s, CH₃), 3.31-3.46 (4H, m, 2 × CH₂), 2.57 (6H, s, 2 × CH₃), 2.41 (2H, t, *J* = 6.8 Hz, CH₂), 1.94-2.02 (2H, m, CH₂), 1.53 (6H, s, 2 × CH₃), 1.21 (3H, t, *J* = 6.8 Hz, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 172.89, 154.59, 148.29, 143.40, 143.24, 132.22, 128.83, 121.42, 120.81, 112.06, 49.63, 44.88, 41.35, 38.59, 31.58, 23.16, 22.60, 14.69, 12.02. MS (ESI) m/z: [M+Na]⁺ calcd 490.25, found 490.32.

Synthesis of 4-((4-(5,5-difluoro-1,3,7,9-tetramethyl-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)phenyl)(ethyl)amino)butanoic acid (Compound 8).



LiOH (0.48 g, 20 mmol) was added to a mixture of Compound 7 (1.87 g, 4 mmol), H₂O (20 mL) and ethanol (30 mL), and the solution was stirred at room temperature for 3 h. The pH of the solution was adjusted to 5 with 1 M HCl, then the solution was extracted with ethyl acetate (100 mL) and washed with brine (100 mL). The organic layer was dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using CH₂Cl₂-MeOH 20:1 (v/v) as the eluent to yield Compound 8 as a red solid (1.25 g, 69% yield). ¹H NMR (400 MHz, CD₃OD) δ (ppm): 7.05 (2H, d, *J* = 8.8 Hz, Ar-H), 6.90 (2H, d, *J* = 8.8 Hz, Ar-H), 6.05 (2H, s, 2 × CH=), 3.40-3.49 (4H, m, 2 × CH₂), 2.49 (6H, s, 2 × CH₃), 2.39 (2H, t, *J* = 7.2 Hz, CH₂), 1.91-1.97 (2H, m, CH₂), 1.55 (6H, s, 2 × CH₃), 1.20 (3H, t, *J* = 6.8 Hz, CH₃). ¹³C NMR (100 MHz, CD₃OD) δ (ppm): 175.73, 154.38, 148.55, 143.78, 143.26, 131.98, 128.75, 121.05, 120.40, 111.98, 49.09, 44.51, 30.59, 30.41, 29.38, 22.29, 13.48, 13.11, 10.94. MS (ESI) m/z: [M-H]⁻ calcd 452.23, found 452.68.

Synthesis of Tert-butyl (2-(4-((4-((5,5-difluoro-1,3,7,9-tetramethyl-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'f][1,3,2]diazaborinin-10-yl)phenyl)(ethyl)amino)butanoyl)pi-perazin-1-yl)ethyl)carbamate (Compound 9)



N,N-diisopropylethylamine (DIPEA, 0.33 mL, 2 mmol) and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 0.57 g, 1.5 mmol) were added to a mixture of

Compound 8 (0.45 g, 1 mmol), 4-(2-boc-aminoethyl)piperidine (0.4 g, 1.5 mmol) and CH₂Cl₂ (20 mL), and the solution was stirred at room temperature overnight. Then the solution was washed with water (20 mL) and brine (20 mL), dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using petroleum ether-ethyl acetate 1:2 (v/v) as the eluent to yield Compound 9 as a red solid (0.31 g, 47% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.04 (2H, d, *J* = 8.8 Hz, Ar-H), 6.79 (2H, d, *J* = 8.8 Hz, Ar-H), 5.99 (2H, s, 2 × CH=), 3.67 (2H, t, *J* = 4.4 Hz, CH₂), 3.34-3.49 (6H, m, 3 × CH₂), 3.23-3.29 (2H, m, CH₂), 2.57 (6H, s, 2 × CH₃), 2.43-2.51 (6H, m, 3 × CH₂), 2.39 (2H, t, *J* = 6.8 Hz, CH₂), 1.95-2.03 (2H, m, CH₂), 1.53 (6H, s, 2 × CH₃), 1.48 (9H, s, 3 × CH₃), 1.21 (3H, t, *J* = 6.8 Hz, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 181.47, 170.63, 154.63, 147.84, 143.37, 142.15, 132.71, 128.96, 121.39, 120.26, 111.98, 58.15, 57.75, 53.42, 53.02, 52.71, 49.74, 45.37, 44.94, 38.61, 30.15, 28.44, 22.67, 14.72, 12.10. MS (ESI) m/z: [M+H]⁺ cad 665.42, found 665.12.

Synthesis of 1-(4-(2-aminoethyl)piperazin-1-yl)-4-((4-(5,5-difluoro-1,3,7,9-tetramethyl-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)phenyl)(ethyl)amino)butan-1-one (Compound 10)



TFA (2 mL) was added to a mixture of Compound 9 (0.33 g, 0.5 mmol) and CH₂Cl₂ (10 mL), and the solution was stirred at room temperature for 2 h. Then the solution was washed with saturated NaHCO₃ and brine, dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using CH₂Cl₂-MeOH 15:1 (v/v) as the eluent to yield Compound 10 as a red solid (0.15 g, 53% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.03 (2H, d, *J* = 8.8 Hz, Ar-H), 6.79 (2H, d, *J* = 8.8 Hz, Ar-H), 5.99 (2H, s, 2 × CH=), 3.66-3.75 (6H, m, 3 × CH₂), 3.51 (2H, t, *J* = 4.8 Hz, CH₂), 3.38-3.47 (4H, m, 2 × CH₂), 3.17-3.23 (2H, m, CH₂), 2.61 (2H, t, *J* = 5.2 Hz, CH₂), 2.56 (6H, s, 2 × CH₃), 2.40 (2H, t, *J* = 6.8 Hz, CH₂), 1.95-2.03 (2H, m, CH₂), 1.52 (6H, s, 2 × CH₃), 1.21 (3H, t, *J* = 7.2 Hz, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 170.76, 154.60, 148.33, 143.42, 143.23, 132.22, 128.95, 121.33, 120.81, 111.99, 59.30, 57.75, 55.80, 53.43, 53.04, 52.60, 49.73, 45.33, 44.97, 43.71, 41.55, 30.15, 22.69, 14.72, 14.54, 12.57, 12.09. MS (ESI) m/z: [M+H]⁺ calcd 565.36, found 565.46.

Synthesis of Lyso-BODIPY-TCO



A solution of Compound 4 (0.12 g, 0.04 mmol) in CH_2Cl_2 (10 mL) was added to a mixture of Compound 10 (0.14 g, 0.25 mmol), TEA (0.07 mL, 0.5 mmol) and CH_2Cl_2 (10 mL), and the solution was stirred at room temperature overnight. Then the solution was washed with saturated NaHCO₃ (20 mL) and brine (20 mL), dried over Na₂SO₄, filtered

and evaporated. The residue was purified by column chromatography using CH₂Cl₂-MeOH 30:1 (v/v) as the eluent to yield probe Lyso-BODIPY-TCO as a red oil (0.11 g, 61% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.03 (2H, d, *J* = 8.4 Hz, Ar-H), 6.79 (2H, d, *J* = 8.8 Hz, Ar-H), 5.98 (2H, s, 2 × CH=), 5.56-5.62 (2H, m, CH=CH), 4.89-4.95 (1H, m, CH), 3.66-3.75 (6H, m, 3 × CH₂), 3.32-3.47 (6H, m, 3 × CH₂), 3.12-3.17 (2H, m, CH₂), 2.56 (6H, s, 2 × CH₃), 2.40 (2H, t, *J* = 7.2 Hz, CH₂), 2.25-2.35 (4H, m, 2 × CH₂), 1.96-2.03 (2H, m, CH₂), 1.86-1.92 (2H, m, CH₂), 1.68-1.78 (6H, m, 3 × CH₂), 1.52 (6H, s, 2 × CH₃), 1.21 (3H, t, *J* = 7.2 Hz, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 170.73, 154.63, 148.31, 143.21, 135.30, 134.90, 133.99, 133.58, 128.96, 120.84, 120.80, 112.01, 54.30, 53.40, 50.79, 44.98, 42.45, 41.35, 36.08, 34.66, 31.58, 29.05, 27.66, 26.91, 22.64, 22.59, 20.68, 14.09, 11.40. MS (ESI) m/z: [M+H]⁺ calcd 717.45, found 717.32.

Syhtnesis of Lyso-TCO



A solution of Compound 4 (0.12 g, 0.04 mmol) in CH₂Cl₂ (10 mL) was added to a mixture of N-(2-aminoethyl)morpholine (0.033g, 0.25 mmol), TEA (0.07 mL, 0.5 mmol) and CH₂Cl₂ (10 mL), and the solution was stirred at room temperature overnight. Then the solution was washed with saturated NaHCO₃ solution (20 mL) and brine (20 mL), dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using CH₂Cl₂-MeOH 40:1 (v/v) as the eluent to yield Lyso-TCO as a colorless oil (0.052 g, 73% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 5.51-5.61 (2H, m, CH=CH), 5.12-5.15 (1H, m, CH), 3.68 (4H, t, *J* = 4.8 Hz, 2 × CH₂), 3.24-3.29 (2H, m, CH₂), 2.41-2.49 (6H, m, 3 × CH₂), 2.08-2.37 (5H, m, 2 × CH₂ and CH), 1.53-1.88 (5H, m, 2 × CH₂ and CH). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 156.23, 135.27, 131.76, 129.60, 70.12, 66.88, 57.57, 53.36, 41.07, 37.37, 35.85, 34.29, 27.99, 25.54, 22.38. MS (ESI) m/z: [M+H]⁺ calcd 299.19, found 299.07.

Syntnesis of Mito-TCO



A mixture of DCC (0.041 g, 0.2 mmol) and DMAP (0.003 g, 0.02 mmol) was added to a mixture of Compound 3 (0.013g, 0.01 mmol), 2-Carboxyethyl)triphenylphosphonium chloride (0.056 g, 0.15 mmol) and DMF (3 mL), and the solution was stirred at room temperature overnight. Then the solution was evaporated, and the residue was purified by column chromatography using CH₂Cl₂-MeOH 40:1 (v/v) as the eluent to yield Mito-TCO as a colorless oil (0.029 g, 61% yield). ¹H NMR (400 MHz, CD₃OD) δ (ppm): 7.78-7.95 (15H, m, Ar-H), 5.48-5.66 (2H, m, CH=CH), 4.01-4.04 (1H, m, CH), 3.75 (2H, t, *J* = 4.8 Hz, CH₂), 2.77 (2H, t, *J* = 4.4 Hz, CH₂), 2.35-2.44 (1H, m, CH), 2.17-2.23 (2H, m, CH₂), 1.96-2.09 (2H, m, CH₂), 1.63-1.92 (5H, m, 2 × CH₂ and CH). MS (ESI) m/z: [M-CI]⁺ calcd 443.21, found 443.18.

Synthesis of Mito-Rh-Tz. Mito-Rh-Tz was synthesized according to the procedures in Scheme S2. **Scheme S2** Synthetic routes for Mito-Rh-Tz.



Synthesis of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (Compound 11)



A solution of hydrazine hydrate (30 mL) was added to a solution of 4-(hydroxymethyl)benzonitrile (1.45 g, 11 mmol) and zinc trifluoromethanesulfonate (2.2 g, 5.5 mmol) in acetonitrile (25 mL) at 0 °C under N₂ atmosphere and the mixture was stirred at 80 °C for 24 h. Then sodium nitrite (34.5 g, 500 mmol) in water (200 mL) and concentrated hydrochloric acid (30 mL) were sequentially added to the mixture slowly. The solution was extracted with ethyl acetate (300 mL), and the organic layer was dried with anhydrous Na₂SO₄ and evaporated. The crude product was purified by column chromatography using petroleum ether-ethyl acetate 2:1 (v/v) as the eluent to afford Compound 11 as a pink solid (0.58 g, 26% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 8.58 (2H, d, *J* = 8.4 Hz, Ar-H), 7.59 (2H, d, *J* = 8.0 Hz, Ar-H), 4.85 (2H, s, CH₂), 3.11 (3H, s, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 167.21, 163.94, 145.66, 128.12, 127.38, 64.72, 31.59.

Synthesis of 4-(6-methyl-1,2,4,5-tetrazin-3-yl) benzaldehyde (Compound 12)



A solution of Dess-Martin periodinane (1.26 g, 3 mmol) in CH₂Cl₂ (5 mL) was slowly added to a solution of Compound 11 (0.4 g, 2 mmol) in CH₂Cl₂ (10 mL) at 0 °C. The mixture was stirred at room temperature for 2 h under N2 atmosphere and diluted with CH₂Cl₂ (40 mL). The organic layer was washed with saturated NaHCO₃ solution (80 mL), dried with anhydrous Na₂SO₄ and concentrated in vacuum. The crude product was purified by column chromatography using petroleum ether-ethyl acetate 4:1 (v/v) as the eluent to yield Compound 12 as a pink solid (0.29 g, 73% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 10.18 (1H, s, CHO), 8.80 (2H, d, *J* = 8.0 Hz, Ar-H), 8.13 (2H, d, *J* = 8.0 Hz, Ar-H), 3.17 (3H, s, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 191.59, 167.74, 163.50, 138.92, 137.01, 130.29, 128.48, 21.27.

Synthesis of Mito-Rh-Tz



Tosic acid (0.035 g, 0.2 mmol) was added to a solution of Compound 12 (0.20 g, 1 mmol) and 3-(dimethylamino) phenol (0.44 g, 2.2 mmol) in acetic acid (10 mL), and the reaction mixture was stirred at 70 °C for 8 h. After removal of the solvent under vacuum, the residue was dissolved with CH₂Cl₂ (20 mL). P-chloranil (0.12 g, 0.5 mmol) was added and the solution was stirred at room temperature for 2 h. The solution was concentrated in vacuum, and the crude product was purified by column chromatography using CH₂Cl₂-MeOH 20:1 (v/v) as the eluent to yield Mito-Rh-Tz as a purple solid (0.12 g, 27% yield). ¹H NMR (400 MHz, CD₃CN) δ (ppm): 8.75 (2H, d, *J* = 4.0 Hz, Ar-H), 7.86 (2H, d, *J* = 8.8 Hz, Ar-H), 7.38 (2H, m, Ar-H), 7.01 (2H, d, *J* = 8.0 Hz, Ar-H), 6.87 (2H, d, *J* = 6.8 Hz, Ar-H), 3.29 (12H, s, 4 × CH₃), 3.05 (3H, s, CH₃). ¹³C NMR (100 MHz, CD₃CN) δ (ppm): 169.43, 168.66, 157.82, 157.46, 132.02, 131.30, 128.22, 125.68, 124.04, 114.42, 113.33, 96.29, 40.34, 21.35. MS (ESI) m/z: [M]⁺ calcd 437.20, found 437.19.

Synthesis of Mem-Rh-Tz. Mem-Rh-Tz was synthesized according to the procedures in Scheme S3. **Scheme S3.** Synthetic routes for Mem-Rh-Tz.



2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-3-yl (4-nitrophenyl) carbonate (Compound 13)



A solution of 4-nitrophenyl chloroformate (2.53 g, 12.5 mmol) in THF (20 mL) was slowly added to a mixture of cholesterol (3.86 g, 10 mmol), pyridine (1.6 mL, 20 mmol) and THF (20 mL) at 0 °C. After reacting at room temperature overnight, the solution was washed with 1 M HCl solution (30 mL), brine (30 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated, and the residue was purified by column chromatography using petroleum ether-ethyl acetate 10:1 (v/v) as the eluent to yield Compound 13 as a colorless solid (3.47 g, 63% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 8.28-8.32 (2H, m, Ar-H), 7.39-7.43 (2H, m, Ar-H), 5.44-5.47 (1H, m, = CH), 4.59-4.67 (1H, m, CH), 2.48-2.54 (2H, m, CH₂), 1.71-2.08 (6H, m, alicyclic-H), 0.87-1.56 (31H, m, alicyclic-H), 0.71 (3H, s, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 155.65, 151.77, 145.29, 138.85, 125.26, 123.52, 121.78, 79.78, 56.69, 56.15, 49.99, 42.33, 39.71, 39.52, 37.87, 36.80, 36.56, 36.19, 35.79, 31.92, 31.84, 28.22, 28.02, 27.60, 24.29, 23.84, 22.82, 22.56, 21.07, 19.28, 18.73, 11.87.

Synthesisof(8R,9S,10R,13R,14R,17S)-10,13-dimethyl-17-(6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl(2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (Compound 14)



A solution of Compound 13 (2.75 g, 5 mmol) in CH2Cl2 (15 mL) was added to a mixture of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethan-1-amine) (1.48 g, 10 mmol), TEA (1.38 mL, 10 mmol) and CH₂Cl₂ (20 mL), and the solution was stirred at room temperature overnight. Then the solution was washed with brine (30 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated, and the residue was purified by column chromatography using CH₂Cl₂-MeOH 25:1 (v/v) as the eluent to yield Compound 14 as a colorless oil (1.97 g, 71% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 5.34-5.39 (1H, m, = CH), 4.43-4.53 (1H, m, CH), 3.52-3.64 (8H, m, 4 × CH₂), 3.32-3.39 (4H, m, 2 × CH₂), 2.89 (2H, t, *J* = 5.2 Hz, CH₂), 2.14-2.37 (4H, m, CH₂), 1.78-2.01 (6H, m, alicyclic-H), 0.83-1.62 (36H, m, alicyclic-H), 0.67 (3H, s, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 156.23, 139.82, 122.41, 74.25, 73.04, 70.13, 56.68, 56.13, 50.01, 42.29, 41.58, 40.66, 39.73, 39.50, 38.59, 36.99, 36.54, 36.17, 35.78, 34.65, 31.89, 31.86, 31.57, 29.04, 28.21, 28.18, 27.98, 26.89, 25.26, 24.27, 23.82, 22.79, 22.63, 22.54, 21.03, 20.68, 19.31, 18.69, 14.29, 14.09, 11.83, 11.40.

Synthsis of methyl 4-((3-hydroxyphenyl)(methyl)amino)butanoate (Compound 15)



Methyl 4-bromobutyrate (1.8 g, 10 mmol) was added to a mixture of 3-aminophenol (1.09 g, 10 mmol), DIPEA (1.74 mL, 10 mmol) and DMF (30 mL), and the solution was stirred at 100 °C for 2 h. After addition of iodomethane (0.63 ml, 10 mmol) and DIPEA (1.74 mL, 10 mmol), the reaction was stirred at 80 °C for 2 h and then poured into H₂O (200 ml). The solution was extracted with ethyl acetate (200 mL) and washed with brine (200 mL), dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using petroleum ether-ethyl acetate 3:1 (v/v) as the eluent to yield Compound 15 as a colorless oil (0.65 g, 29% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.09 (1H, t, *J* = 8.0 Hz, Ar-H), 6.29-6.33 (1H, m, Ar-H), 6.18-6.24 (2H, m, Ar-H), 5.09 (1H, s, OH), 3.70 (3H, s, CH₃), 3.35 (2H, t, *J* = 7.6 Hz, CH₂), 2.93 (3H, s, CH₃), 2.38 (2H, t, *J* = 7.2 Hz, CH₂), 1.89-1.97 (2H, m, CH₂). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 173.89, 156.76, 150.78, 130.08, 104.99, 103.30, 99.24, 51.84, 51.67, 38.36, 31.24, 22.10.

Synthsis of 4-((3-hydroxyphenyl)(methyl)amino)butanoic acid (Compound 16)



LiOH (0.48 g, 20 mmol) was added to a mixture of Compound 15 (0.89 g, 4 mmol), H₂O (15 mL) and ethanol (25 mL), and the mixture was stirred at room temperature for 3 h. The pH was adjusted to 5 with 1 M HCl solution, and the resulting mixture was extracted with ethyl acetate (100 mL) and washed with brine (100 mL). The organic layer was dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using CH₂Cl₂-MeOH 30:1 (v/v) as the eluent to yield Compound 16 as a gray oil (0.49 g, 58% yield). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.08 (1H, t, *J* = 8.0 Hz, Ar-H), 6.30-6.34 (2H, m, Ar-H), 6.22-6.25 (1H, m, Ar-H), 3.73 (2H, t, *J* = 6.0 Hz, CH₂), 3.41 (2H, t, *J* = 6.8 Hz, CH₂), 2.97 (1H, s, OH), 2.87 (3H, s, CH₃), 1.79-1.86 (2H, m, CH₂). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 162.94, 157.07, 151.13, 130.11, 105.20, 104.03, 100.09, 60.85, 49.97, 38.38, 29.48.

Syntheiss of (Z)-3-carboxy-N-(6-((3-carboxypropyl)(methyl)amino)-9-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3H-xanthen-3-ylidene)-N-methylpropan-1-aminium (Compound 17).



Tosic acid (0.035 g, 0.2 mmol) was added to a solution of Compound 12 (0.20 g, 1 mmol) and Compound 16 (0.46 g, 2.2 mmol) in acetic acid (10 mL), and the reaction mixture was stirred at 70 °C for 8 h. After removal of the solvent under vacuum, the residue was dissolved in THF (20 mL). P-chloranil (0.12 g, 0.5 mmol) was added and the solution

was stirred at room temperature for 2 h. Then the solution was concentrated in vacuum, and the crude product was purified by column chromatography using CH_2Cl_2 -MeOH-CH₃COOH 50:10:2 (v/v) as the eluent to yield Compound 17 as a purple solid. Then Compound 17 was used without further purfication.

Synthesis of Mem-Rh-Tz.



DIPEA (0.17 mL, 1 mmol) and HATU (0.29 g, 0.75 mmol) were added to a mixture of Compound 17 (0.14 g, 0.25 mmol), Compound 14 (0.42 g, 0.75 mmol) and DMF (6 mL), and the solution was stirred at room temperature overnight. Then the solution was concentrated in vacuum and the residue was purified by column chromatography using petroleum CH₂Cl₂-MeOH 12:1 (v/v) as the eluent to yield probe Mem-Rh-Tz as a purple oil (0.15 g, 35% yield). ¹H NMR (400 MHz, DMSO- d_6) δ (ppm): 7.48 (1H, d, J = 7.2 Hz, Ar-H), 7.39 (2H, d, J = 8.0 Hz, Ar-H), 7.21-7.26 (1H, m, Ar-H), 7.06 (1H, t, J = 6.4 Hz, Ar-H), 6.94-6.98 (2H, d, J = 7.6 Hz, Ar-H), 6.37 (1H, d, J = 8.0 Hz, Ar-H), 6.24 (1H, s, Ar-H), 5.29-5.33 (2H, m, =CH), 4.24-4.28 (2H, m, CH), 3.27-3.73 (60H, m, alicyclic-H), 3.08 (4H, t, J = 5.2 Hz, $2 \times$ CH₂), 2.84 (2H, t, J = 5.6 Hz, CH₂), 2.16-2.28 (4H, m, $2 \times$ CH₂), 1.72-1.97 (8H, m, $4 \times$ CH₂), 0.82-1.56 (64H, m, alicyclic-H), 0.64 (6H, s, $2 \times$ CH₃). ¹³C NMR (100 MHz, DMSO- d_6) δ (ppm): 168.15, 156.21, 152.62, 147.79, 140.21, 138.15, 130.96, 129.53, 129.39, 128.52, 125.96, 122.31, 119.30, 112.58, 108.14, 97.32, 96.54, 73.43, 70.05, 69.89, 69.58, 69.16, 58.96, 56.60, 49.95, 44.07, 42.32, 38.77, 37.05, 36.53, 36.13, 35.67, 31.85, 31.81, 28.33, 28.25, 27.86, 24.32, 23.68, 23.12, 22.85, 21.24, 21.04, 19.46, 19.01, 12.96, 12.13. MS (ESI) m/z: [M]⁺ calcd 1666.14, found 1666.27.

1.3 Experimental procedures for *in vitro* and cellular assays

In vitro Chacterizations of TCO and tetrazine probes. Fluorescence emission spectrum for Lyso-BODIPY-TCO (10 μ M) in PBS buffer (20 mM, pH 7.4) was recorded in the range from 500 nm to 650 nm with an excitation wavelength of 480 nm. The excitation and emission slit widths were 5 nm. Fluorescence emission spectra for Mito-Rh-Tz (10 μ M) or Mem-Rh-Tz (10 μ M) in citric acid-sodium citrate buffer (20 mM, pH 4.5) were recorded in the range from 560 to 710 nm with an excitation wavelength of 550 nm. All buffers used were supplemented with 5% DMF as the co-solvent.

To investigate the effect of pH on the fluorescence of Lyso-BODIPY-TCO, fluorescence emission spectra of Lyso-BODIPY-TCO (10 μ M) in different pH buffers (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.4) were recorded in the range from 500 nm to 650 nm with an excitation wavelength of 480 nm. Buffers with pH values of 3.0-6.0 were prepared using citrate buffers (20 mM). Buffers with pH values of 6.5-7.4 were prepared using phosphate buffers (20 mM).

To examine fluorescence response of the tetrazine probes toward TCO probe, Mito-Rh-Tz (10 μ M) or Mem-Rh-Tz

(10 μ M) was mixed with Lyso-BODIPY-TCO (100 μ M) in citric acid-sodium citrate buffer (20 mM, pH 4.5) and incubated at 37 °C for 30 min. To examine the fluorescent spectrum of tetrazine probes, different concentrations of tetrazine probes were added in PBS buffer and incubated at 37 °C for 30 min. The fluorescence spectra were recorded in the range from 560 to 710 nm with an excitation wavelength of 550 nm.

To inspect real-time fluorescence responses of the tetrazine probes toward TCO probe, Mito-Rh-Tz (10 μ M) or Mem-Rh-Tz (10 μ M) was mixed quickly with Lyso-BODIPY-TCO (100 μ M) in citric acid-sodium citrate buffer (20 mM, pH 4.5) or PBS buffer (20 mM, pH 7.4) at 37 °C. The fluorescence intensity at 585 nm was recorded immediately for 90 s with an interval of 1 s using an excitation wavelength of 550 nm.

Cell culture and transfection. HeLa cells, MCF-7 cells, HepG2 cells, A549 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C in a humidified atmosphere incubator containing 5% CO₂. MDA-MB-231 cells were cultures in L-15 medium supplemented with 15% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C and Hup-T3 cells were cultures in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C. For plasmid transfection, HeLa cells were plated on sterilized glass coverslips in 35-mm plates with 14-mm wells and grown to 50-70% confluency. The cells were transfected with 1.5 μ g of the plasmid BFP-LC3 using lipofectamine 3000 according to the manufacture's protocol.

Colocalization assay. To determine the cellular localization of the paired probes, cells were incubated with 5 μ M of Mito-Rh-Tz, 0.5 μ M of Lyso-BODIPY-TCO or 5 μ M of Mem-Rh-Tz in the serum-supplemented DMEM medium at 37 °C for 1 h. After washing with PBS for three times, the cells were incubated with 0.1 μ M of Mito-Tracker Green, 0.1 μ M of LysoTracker Red or 10 μ M of DiO in the serum-supplemented DMEM medium at 37 °C for 1 h. To further investigate the cellular localization of low concentrations of tetrazine probes with TCO activation, cells were incubated with 0.01 μ M of Mito-Rh-Tz or Mem-Rh-Tz for 1 h, followed by the treatment with 0.1 μ M of Mito-TCO or TCO. After washing with PBS for three times, the cells were incubated with 0.1 μ M of Mito-Tracker Green or 10 μ M of DiO

in the serum-supplemented DMEM medium at 37 °C for 1 h. The cells were washed with PBS for three times before confocal laser scanning microscopy fluorescence (CLSM) imaging. The fluorescence of MitoTracker Green and DiO was excited with a 488 nm laser with emission collected at 500-550 nm. The fluorescence of LysoTracker Red was excited with a 560 nm laser with emission collected at 570-620 nm.

Live cell imaging of autophagic and endocytic fluxes. Cells including HeLa cells, MCF-7 cells and HepG2 cell were grown plated on a 35-mm confocal dish with a 10-mm bottom well in a serum-supplemented (10% FBS) DMEM medium for 24 h cells to confluency of 50-70%. To inspect autophagic flux, the cells were incubated with 0.01 μ M of Mito-Rh-Tz at 37 °C for 1h in the serum-supplemented DMEM medium. After washing with pH 7.4 PBS for three times, cells were then incubated with 0.1 μ M of Lyso-BODIPY-TCO at 37 °C for 1 h in the serum-supplemented DMEM medium followed by washing with pH 7.4 PBS for three times. Subsequently, the cells were incubated in the given medium to induce or inhibit autophagic flux. Fluorescence imaging was performed after washing the cells twice with PBS. To investigate endocytic flux, the cells were incubated with 0.5 μ M of Lyso-BODIPY-TCO at 37 °C for 1 h in the serum-supplemented DMEM medium. After washing with pH 7.4 PBS for three times, cells were then incubated with 0.5 μ M of Lyso-BODIPY-TCO at 37 °C for 1 h in the serum-supplemented DMEM medium. After washing with pH 7.4 PBS for three times, cells were then incubated with 0.05 μ M of Mem-Rh-Tz at 37 °C for 0.5 h in the serum-supplemented DMEM medium followed by washing with pH 7.4 PBS for three times. Then, the cells were incubated in the given medium to induce or inhibit endocytic flux. Fluorescence imaging was performed after washing with pH 7.4 PBS for three times. Then, the cells were incubated in the given medium to induce or inhibit endocytic flux. Fluorescence imaging was performed after washing with pH 7.4 PBS for three times. Then, the cells were incubated in the given medium to induce or inhibit endocytic flux. Fluorescence imaging was performed after washing the cells twice with PBS. CLSM images were acquired on a Nikon A1+ confocal microscope. The emission for Lyso-BODIPY-TCO was collected in the range of 500 nm to 550 nm with

an excitation wavelength of 488 nm. The emission for Mito-Rh-Tz and Mem-Rh-Tz was collected in the range of 570 nm to 620 nm with an excitation wavelength of 560 nm. The fluorescence of Mito-Tracker Blue was excited with a 405 nm laser with emission collected at 425-475 nm. The fluorescence of LysoTracker NIR was excited with a 640 nm laser with emission collected at 670-720 nm.

For induction of autophagic flux, the cells were incubated in a serum-depleted EBSS medium or pretreated with rapamycin (0.2 μ M) or CCCP (10 μ M) in serum-supplemented DMEM medium at 37 °C for 12 h. For inhibition of autophagic flux, the cells were pretreated with liensinine (30 μ M) for 12 h, bafilomycin A1 (0.2 μ M) for 6 h, or CQ (40 μ M) for 4 h in the serum-depleted EBSS medium. For verify-ing the capability of the paired probes to specific imaging of autophagic flux, HeLa cells were transfected with BFP-LC3. For real-time fluorescence imaging of autophagic flux, HeLa cells were treated with 1 mL of the serum-supplemented DMEM culture medium containing 0.01 μ M of Mito-Rh-Tz, 0.1 μ M of Lyso-BODIPY-TCO, MitoTracker Blue and LysoTracker NIR. After washing with PBS for three times, the cells were incubated in the serum-depleted medium and placed into a live cell incubator equipped on the Nikon microscope. CLSM images were then obtained at different time intervals.

For induction of endocytotic flux, the cells were incubated with 0.08 μ g/mL EGF in the serum-supplemented medium at 37 °C for 1 h. For endocytosis inhibition, the cells were incubated with 1 mL of the culture medium containing NaN₃ (0.2%, w/v) at 37 °C for 1 h. To investigate the EGF mediated endocytosis pathway, the cells were incubated with 1 mL of the culture medium containing Nystatin (100 μ g/mL), amiloride (2 mM), chlorpromazine (40 μ g/mL), or Meβ-CD (0.5 mM) at 37 °C for 2 h. For verifying the capability of the probes to specific imaging of endocytic flux, the cells were pretreated with FITC-dextran (0.5 mg/mL) at 37 °C for 2 h.

Correlation of autophagic and endocytic fluxes with expression of cell surface proteins. To correlate autophagic or endocytic flux with the expression of MHC-I, cells including Hup-T3 cells, A549 cells and MCF-7 cells were cultured a serum-supplemented (10% FBS) medium for 24 h cells to confluency of 50-70%. To correlate autophagic or endocytic flux with the expression of PD-L1, cells including MDA-MB-231 cells HeLa cells, and MCF-7 cells were cultured in the medium supplemented with IFN- γ (50 ng/mL) and 10% FBS. To correlate autophagic flux to the expression levels of surface proteins, the cells were incubated with the probes, 0.01 μ M of Mito-Rh-Tz and 0.1 μ M of Lyso-BODIPY-TCO. The cells were then treated with thapsigargin (6 μ M), incubated in the se-rum-depleted medium for 6 h or treated with bafilomycin A1 for 6 h, followed by incubation with Alexa Fluor 700 anti-human MHC-I or PD-L1 for 30 min at 4 °C. To correlate endocytic flux to the expression of MHC-I and PD-L1, the cells were incubated with the probes, 0.01 μ M of Mem-Rh-Tz and 0.1 μ M of Lyso-BODIPY-TCO. The cells were incubated in the se-rum-depleted medium for 6 h or treated with bafilomycin A1 for 6 h, followed by incubation with Alexa Fluor 700 anti-human MHC-I or PD-L1 for 30 min at 4 °C. To correlate endocytic flux to the expression of MHC-I and PD-L1, the cells were incubated with the probes, 0.01 μ M of Mem-Rh-Tz and 0.1 μ M of Lyso-BODIPY-TCO. The cells were treated with prochlorperazine (20 μ M), stimulated with EGF (0.08 μ g/mL) for 6 h, or treated with bafilomycin A1 (0.2 μ M) for 6 h, followed by incubation with Alexa Fluor 700 anti-human MHC-1 or PD-L1 for 30 min at 4 °C. All cells were washed three times with PBS before CLSM imaging. The fluorescence of Alexa Fluor 700 was excited with a 640 nm laser with emission collected at 670-720 nm.

Flow cytometry assay. The cells were seeded on a 35-mm Petri-dish and incubated with the serum-supplemented medium for 24 h. After washing with PBS, the cells were treated according to the before mentioned procedures. Then, the cells were washed with PBS and digested with trypsin (0.25%) for 5 min. After washing with PBS for twice, the cells were suspended in 0.3 mL PBS and analysed using a CytoFLEX FC system (Beck-man Coulter, USA). For analyzing the surface abundance of MHC-I and PD-L1 in different cell lines, the cells treated with the aforementioned procedures were stained with Alexa Fluor 700 labeled anti-human MHC-I or PD-L1 at 4 °C for 30 min. The cells were then analysed using the CytoFLEX FC system.

Cell viability assay. The cytotoxicity of Mito-Rh-Tz, Lyso-BODIPY-TCO, Mem-Rh-Tz, the conjugates of Lyso-BODIPY-TCO and tetrazine probes was examined using HeLa cells through a standard MTT assay. The cells were seeded into a 96-well plate at 5×10^4 cells /well in DMEM, medium containing 10% FBS. The cells were incubated for 24 h at 37 °C under 5 % CO₂. After washing with PBS for three times, the cells were incubated with fresh medium containing various concentrations of FORBIT probes (0 - 100 μ M) for 12 h. Then, 20 μ L of MTT solution (5.0 mg/mL) was added and incubated for another 4 h. The supernatant was removed, and 150 μ L of dimethyl sulfoxide was added to dissolve the formazan crystals. The cell viability was determined by measuring the absorbance at 450 nm on a microplate reader.

Immunofluorescence imaging of fixed cells. Different cell lines were seeded on coverslips in 12-well plates and incubated overnight to reach 80% confluence. Cells under the aforementioned treatments were sequentially washed with cold PBS, fixed in 4% paraformaldehyde/PBS for 20 min, permeablized with 0.1% saponin for 15 min and blocked in 5% (w/v) bovine serum albumin/PBS for 30 min at room temperature. The cells were then incubated with anti-human MHC-I or PD-L1 antibody at 37 °C for 60 min. After washing three times with PBST buffer, the cells were incubated with Alexa Fluor Plus 647 labeled secondary antibody at 37 °C for 30 min. CLSM imaging was obtained after washing with PBS for three times. The fluorescence of Alexa Fluor Plus 647 was excited with a 640 nm laser with emission collected at 670-720 nm.

Western Blotting Analysis. HeLa cells (1×10^5) were seeded onto 6-well plates and grown to 50-70% confluency. For preparing samples for LC3 analysis, cells were cultured in a serum-depleted EBSS medium. The cells were collected at different time intervals. Subsequently, the cells were treated with 50 µL lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mg/mL leupeptin, 1 mM phenylmethyl-sulfonylfluoride). Protein concentrations were determined using the bicinchoninic acid protein assay. Equal amounts of proteins (30 µg) were separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were subsequently blocked with 5% nonfat dry milk in tris-buffered saline (TBS) for 1 h and incubated with anti-LC3-I antibodies (dilution, 1:2000), or anti-LC3-II antibodies (dilution, 1:2000) at 4 °C overnight. After washing three times with TBS containing 0.1% Tween 20, the membranes were incubated with HRP-labelled anti-rabbit IgG (dilution, 1:2500), and signals were developed by luminol chemiluminescence. As an internal reference, β -actin was also analyzed following the same procedure using anti- β -actin antibodies (dilution, 1:1000). Densitometric analysis was performed using a Scion Image software.

2. Additional Table and Figures

Ref	Structure	Response Mechanism	Advantages	Disadvantages
20	GFP LC3 RFP LC3∆G	Fluorescent proteins	Standard method for detecting autophagic flux Quantitative detection	Need cell transfection may affect autophagic flux
22		Acidic lysosomes	Ratiometric detection	High susceptibility to cellular pH and polarity
24		ONOO ⁻ and acidic microenvironments	High specificity	High susceptibility to cellular pH variations and co-existing interferents
26		Viscosity	Two-Photon fluorescence lifetime Imaging	High susceptibility to lysosomal and mitochondrial viscosity variations
27	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} $	Supramolecular host- guest recognization	Ratiometric detection	Susceptible to co-existing interferents, limited contrast
This work	$\begin{array}{c} \text{AdA-Cy5} \\ & & \text{H} \\ & $	COP strategy	Dual-color fluorescence imaging Bioorthogonal High specificity High contrast Capable of measuring autophagic flux and endocytic flux	

Table S1. Comparison with reported works



Fig. S1 (A) UV-vis absorption spectrum of Lyso-BODIPY-TCO (20 μ M) in pH 7.4. (B) Fluorescence spectrum of Lyso-BODIPY-TCO (10 μ M) in pH 7.4, λ_{ex} = 485 nm.



Fig. S2 (A) Fluorescence spectra of Lyso-BODIPY-TCO (10 μ M) in different pH buffers (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.5, 7.0, 7.4), $\lambda ex = 485$ nm. (B) Plot of fluorescence intensities at 515 nm versus different pH values.



Fig. S3 UV-vis absorption spectra for Mito-Rh-Tz (20 µM, black) and Mem-Rh-Tz (20 µM, red).



Fig. S4 (A) Fluorescence spectra of different concentrations of Mito-Rh-Tz. (B) Plot of fluorescence intensities at 585 nm versus different concentrations of Mito-Rh-Tz. (C) Fluorescence spectra of different concentrations of Mem-Rh-Tz. (D) Plot of fluorescence intensities at 585 nm versus different concentrations of Mem-Rh-Tz. $\lambda ex = 550$ nm.



Fig. S5 (A) ESI-MS spectrum for the reaction product of Lyso-BODIPY-TCO and Mito-Rh-Tz. (B) MALDI-TOF-MSspectrumforthereactionproductofLyso-BODIPY-TCOandMem-Rh-Tz.



Fig. S6 Kinetic analysis for the reaction between Lyso-BODIPY-TCO (100 μ M) and Mito-Rh-Tz (10 μ M) at pH 4.5 (A) or pH 7.4 (B), $\lambda_{ex/em} = 550/585$ nm. The data were fitted to an exponential equation (y = (y0 - a) e^{-k*t} + a)^{s1} using a GraphPad Prism 6.0 program. The second-order rate constant k_2 was calculated using the equation $k_2 = k/[Lyso-BODIPY-TCO]$.



Fig. S7 (A) Colocalization assay for HeLa cells co-stained with Mito-Rh-Tz (0.01 μ M) upon Mito-TCO activation (0.1 μ M) and MitoTracker Green (0.1 μ M). (B) Colocalization assay for HeLa cells co-stained with Mem-Rh-Tz (0.01 μ M) upon TCO activation (0.1 μ M) and DiO (10 μ M). Scale bar = 10 μ m.



Fig. S8 Colocalization assay in HepG2 cells co-stained with Lyso-BODIPY-TCO (0.5 μ M) plus LysoTracker Red (0.1 μ M), Mito-Rh-Tz (5 μ M) plus MitoTracker Green (0.1 μ M), and Mem-Rh-Tz (5 μ M) plus DiO (10 μ M), respectively. Scale bar = 10 μ m.



Fig. S9 Colocalization assay for MCF-7 cells co-stained with Lyso-BODIPY-TCO (0.5 μ M) plus LysoTracker Red (0.1 μ M), Mito-Rh-Tz (5 μ M) plus MitoTracker Green (0.1 μ M), and Mem-Rh-Tz (5 μ M) plus DiO (10 μ M), respectively. Scale bar = 10 μ m.



Fig. S10 (A) CLSM images of HeLa cells incubated with TCO and tetrazine probes without washing. HeLa cells were incubated with Lyso-BODIPY-TCO (0.5 μ M) for 1 h, and CLSM images were obtained by adjusting the pH of the culture medium to 4.5; HeLa cells were incubated with Mito-Rh-Tz (5 μ M) and Mem-Rh-Tz (5 μ M) for 1 h. Scale bar = 10 μ m. (B) Average fluorescence intensity of TCO and tetrazine probes in ten arbitrary regions in (A).



Fig. S11 Cell viability obtained in MTT assay. (A) HeLa cells incubated with different concentrations of TCO and tetrazine probes, Mito-Rh-Tz (0 - 100 μ M), Lyso-BODIPY-TCO (0 - 100 μ M) or Mem-Rh-Tz (0 - 100 μ M) for 24 h. (B) HeLa cells incubated with different concentrations of conjugates of Lyso-BODIPY-TCO and Mito-Rh-Tz or Mem-Rh-Tz for 24 h. Error bars represent SD of three repetitive experiments.



Fig. S12 (A) CLSM images of different slices along the Z-direction for HeLa cells incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mito-Rh-Tz (0.01 μ M) in serum-depleted medium for 2 h. Scale bar = 10 μ m. (B) Fluorescence intensity profile for Lyso-BODIPY-TCO (green) and Mito-Rh-Tz (red) across the white line.

Lyso-BODIPY-TCO	Mito-Rh-Tz	Overlay	
a1	a2	a3	
b1	b2	b3	
c1	c2	c3	
d1	d2	d3	

Fig. S13 CLSM images for HepG2 cells incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mito-Rh-Tz (0.01 μ M), in serum-supplemented medium (a1-a3) or in serum-depleted medium (b1-b3) for 2 h. (c1-c3) CLSM images for MCF-7 cells pretreated with rapamycin for 12 h and then incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mito-Rh-Tz (0.01 μ M) in serum-supplemented medium for 2 h; (d1-d3) CLSM images for MCF-7 cells pretreated with liensinine for 12 h and then incubated with CLSM images for MCF-7 cells pretreated with liensinine for 12 h and then incubated with Lyso-BODIPY-TCO (0.1 μ M) in serum-depleted medium for 2 h; (d1-d3) CLSM images for MCF-7 cells pretreated with liensinine for 12 h and then incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mito-Rh-Tz (0.01 μ M) in serum-depleted medium for 2 h. Scale bar = 10 μ m.



Fig. S14 CLSM images for MCF-7 cells incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mito-Rh-Tz (0.01 μ M), in serum-supplemented medium (a1-a3) or in serum-depleted medium (b1-b3) for 2 h. (c1-c3) CLSM images for MCF-7 cells pretreated with rapamycin for 12 h and then incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mito-Rh-Tz (0.01 μ M) in serum-supplemented medium for 2 h; (d1-d3) CLSM images for MCF-7 cells pretreated with liensinine for 12 h and then incubated with CSM images for MCF-7 cells pretreated with liensinine for 12 h and then incubated with Lyso-BODIPY-TCO (0.1 μ M) in serum-depleted medium for 2 h; (d1-d3) CLSM images for MCF-7 cells pretreated with liensinine for 12 h and then incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mito-Rh-Tz (0.01 μ M) in serum-depleted medium for 2 h. Scale bar = 10 μ m.



Fig. S15 CLSM images for plasmid BFP-LC3 transfected cells treated with Lyso-BODIPY-TCO and Mito-Rh-Tz. (a) cells incubated in serum-supplemented medium, (b) cells incubated in serum-depleted medium, (c) cells pretreated with prochlorperazine (20 μ M) in serum-depleted medium. Scale bar = 10 μ m



Fig. S16 Overlay 1: merged image for Mito-Rh-Tz and LysoTracker NIR in Fig. 3E in the main text. Overlay 2: merged image for MitoTracker Blue with LysoTracker NIR in Fig. 3E in the main text. Scale bar = $10 \mu m$.



Fig. S17 (A) The quantitative intensities of the band in Fig. 3G using ImageJ software^{2,3}. (B) The ratio of LC3II to β -actin.



Fig. S18 Immunofluorescence images of fixed Hup-T3 cells treated with LysoTracker Green, followed by incubation with MHC-I antibody and Alexa Fluor Plus 647 labeled secondary antibody. (a) Control (b) cells pretreated with thapsigargin (6 μ M), (c) cells incubated in serum-depleted medium, (d) cells pretreated with prochlorperazine (20 μ M), (e) cells stimulated with EGF (0.08 μ g/mL) and (f) cells pretreated with bafilomycin A1 (0.2 μ M). Scale bar = 10 μ m.


Fig. S19 (A) CLSM images for A549 cells incubated with Lyso-BODIPY-TCO (0.1 μ M), Mito-Rh-Tz (0.01 μ M, a-d) or Mem-Rh-Tz (0.01 μ M, e-h), followed by immunostaining. (a) Cells in serum-supplemented medium, (b) cells pretreated with thapsigargin (6 μ M), (c) cells in serum-depleted medium, (d) cells pretreated with bafilomycin A1 (0.2 μ M), (e) cells in serum-supplemented medium, (f) cells pretreated with prochlorperazine (20 μ M), (g) cells stimulated with EGF (0.08 μ g/mL) and (h) cells pretreated with bafilomycin A1 (0.2 μ M), Scale bar = 10 μ m. (B, C) Flow cytometry analysis of immunofluorescence for MHC-I in A549 cells treated under the conditions indicated in (A).



Fig. S20 (A) CLSM images for MCF-7 cells incubated with Lyso-BODIPY-TCO (0.1 μ M), Mito-Rh-Tz (0.01 μ M, a-d) or Mem-Rh-Tz (0.01 μ M, e-h), followed by immunostaining. (a) Cells in serum-supplemented medium, (b) cells pretreated with thapsigargin (6 μ M), (c) cells in serum-depleted medium, (d) cells pretreated with bafilomycin A1 (0.2 μ M), (e) cells in serum-supplemented medium, (f) cells pretreated with prochlorperazine (20 μ M), (g) cells stimulated with EGF (0.08 μ g/mL) and (h) cells pretreated with bafilomycin A1 (0.2 μ M), Scale bar = 10 μ m. (B, C) Flow cytometry analysis of immunofluorescence for MHC-I in MCF-7 cells treated under the conditions indicated in (A).



Fig. S21 (A) CLSM images of MCF-7 cells incubated with Lyso-BODIPY-TCO (0.1 μ M) and Mem-Rh-Tz (0.01 μ M). (a) Cells incubated in serum-supplemented medium, (b) cells incubated in serum-depleted medium (c) cells pretreated with thapsigargin (6 μ M), Scale bar = 10 μ m. (B) Flow cytometry analysis of MCF-7 cells treated under the conditions indicated in (A).



Fig. S22 Immunofluorescence images of fixed MDA-MB-231 cells treated with LysoTracker Green, followed by incubation with PD-L1 antibody and Alexa Fluor Plus 647 labeled secondary antibody. (a) Control, (b) cells pretreated with thapsigargin (6 μ M), (c) cells incubated in serum-depleted medium, (d) cells pretreated with prochlorperazine (20 μ M), (e) cells stimulated with EGF (0.08 μ g/mL) and (f) cells pretreated with bafilomycin A1 (0.2 μ M). Scale bar = 10 μ m.



Fig. S23 (A) CLMS images for HeLa cells incubated with Lyso-BODIPY-TCO (0.1 μ M), Mito-Rh-Tz (0.01 μ M, a-d) or Mem-Rh-Tz (0.01 μ M, e-h), followed by immunostaining. (a) Cells in serum-supplemented medium, (b) cells pretreated with thapsigargin (6 μ M), (c) cells in serum-depleted medium, (d) cells pretreated with bafilomycin A1 (0.2 μ M), (e) cells in serum-supplemented medium, (f) cells pretreated with prochlorperazine (20 μ M), (g) cells stimulated with EGF (0.08 μ g/mL) and (h) cells pretreated with bafilomycin A1 (0.2 μ M), Scale bar = 10 μ m. (B, C) Flow cytometry analysis of immunofluorescence for MHC-I in HeLa cells treated under the conditions indicated in (A).



Fig. S24 (A) Confocal images for MCF-7 cells incubated with Lyso-BODIPY-TCO (0.1 μ M), Mito-Rh-Tz (0.01 μ M, a-d) or Mem-Rh-Tz (0.01 μ M, e-h), followed by immunostaining. (a) Cells in serum-supplemented medium, (b) cells pretreated with thapsigargin (6 μ M), (c) cells in serum-depleted medium, (d) cells pretreated with bafilomycin A1 (0.2 μ M), (e) cells in serum-supplemented medium, (f) cells pretreated with prochlorperazine (20 μ M), (g) cells stimulated with EGF (0.08 μ g/mL) and (h) cells pretreated with bafilomycin A1 (0.2 μ M), Scale bar = 10 μ m. (B, C) Flow cytometry analysis of immunofluorescence for MCF-7 cells in HeLa cells treated under the conditions indicated in (A).



Fig. S25 Immunofluorescence images of fixed A549 cells treated with LysoTracker Green, followed by incubation with MHC-I antibody and Alexa Fluor Plus 647 labeled secondary antibody. (a) Control, (b) cells pretreated with thapsigargin (6 μ M), (c) cells incubated in serum-depleted medium, (d) cells pretreated with prochlorperazine (20 μ M), (e) cells stimulated with EGF (0.08 μ g/mL) and (f) cells pretreated with bafilomycin A1 (0.2 μ M). Scale bar = 10 μ m.



Fig. S26 Immunofluorescence images of fixed MCF-7 cells treated with LysoTracker Green, followed by incubation with MHC-I antibody and Alexa Fluor Plus 647 labeled secondary antibody. (a) Control (b) cells pretreated with thapsigargin (6 μ M), (c) cells incubated in serum-depleted medium, (d) cells pretreated with prochlorperazine (20 μ M), (e) cells stimulated with EGF (0.08 μ g/mL) and (f) cells pretreated with bafilomycin A1 (0.2 μ M). Scale bar = 10 μ m.



Fig. S27 Immunofluorescence images of fixed HeLa cells treated with LysoTracker Green, followed by incubation with PD-L1 antibody and Alexa Fluor Plus 647 labeled secondary antibody. (a) Control, (b) cells pretreated with thapsigargin (6 μ M), (c) cells incubated in serum-depleted medium, (d) cells pretreated with prochlorperazine (20 μ M), (e) cells stimulated with EGF (0.08 μ g/mL) and (f) cells pretreated with bafilomycin A1 (0.2 μ M). Scale bar = 10 μ m.



Fig. S28 Immunofluorescence images of fixed MCF-7 cells treated with LysoTracker Green, followed by incubation with PD-L1 antibody and Alexa Fluor Plus 647 labeled secondary antibody. (a) Control, (b) cells pretreated with thapsigargin (6 μ M), (c) cells incubated in serum-depleted medium, (d) cells pretreated with prochlorperazine (20 μ M), (e) cells stimulated with EGF (0.08 μ g/mL) and (f) cells pretreated with bafilomycin A1 (0.2 μ M). Scale bar = 10 μ m.

3. NMR and MS Spectra



Fig. S29 ¹H NMR spectrum of Compound 1 in CDCl₃.



Fig. S30 ¹³C NMR spectrum of Compound 1 in CDCl₃.

5.732 5.707 5.707 5.707 5.707 5.707 5.707 5.707 5.707 5.707 5.708 5.5635 5.5635 5.5635 5.5635 5.5636 5.5637 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5633 5.5133 5.5133 5.5133 5.5133 5.5141 5.5153 5.5154 5.5154 5.5154 5.5154 5.5154 5.5154 5.5154 5.5154



Fig. S31 ¹H NMR spectrum of Compound 2 in CDCl₃.



Fig. S32 ¹³C NMR spectrum of Compound 2 in CDCl₃.







































7.042 7.020 6.6802 6.6802 6.6802 6.6802 6.6802 6.7805


















Fig. S59 ¹³C NMR spectrum of Lyso-TCO in CDCl₃.



Fig. S60 ESI-MS spectrum of Lyso-TCO.



Fig. S61 ¹H NMR spectrum of Mito-TCO in CD₃OD.



Fig. S62 ESI-MS spectrum of Mito-TCO.



Fig. S63 ¹H NMR spectrum of Compound 11 in CDCl₃.



Fig. S64 ¹³C NMR spectrum of Compound 11 in CDCl₃.



Fig. S65 ¹H NMR spectrum of Compound 12 in CDCl₃.

























7.496 7.409 7.409 7.409 7.409 7.412 7.412 7.412 7.412 7.412 7.412 7.412 7.412 7.412 7.412 7.412 7.412 7.412 7.423







4. Supplemental References

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