

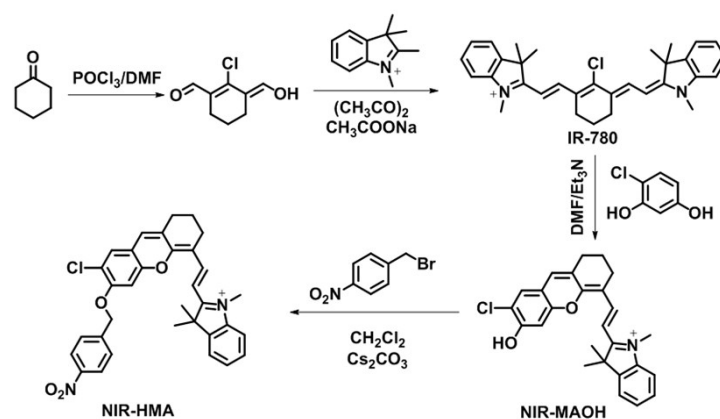
Supporting Information for

Engineering of a Near-infrared Fluorescent Probe for Real-time Simultaneous Visualization of Intracellular Hypoxia and the Induced Mitophagy

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Reagents and Apparatus. All chemicals were purchased from commercial suppliers and used without further purification. The LysoTracker Green, MitoTracker Green, and ERTracker Red, LC3 antibody and adenovirus Ad-GFP-LC3 were purchased from Beyotime Biotechnology. Rapamycin and Hydroxychloroquine were obtained from J&K Scientific. EBSS - Earle's Balanced Salt Solution was purchased from Thermo Fisher Scientific. Leucine aminopeptidase (LAP), glutamyl transpeptidase (GGT), glutathione transferase (GST), nitroreductase (NTR) and NADH were purchased from Sigma-Aldrich. All the enzymes (LAP, GGT, GST, NTR) were dissolved into pure water to form aqueous solution and were divided into several parts for daily experiments. To keep the enzyme activity, all these solutions were stored at -80°C before using. An appropriate amount of probe NIR-HMA and NIR-MAOH was dissolved into DMSO to prepare a stock solution (1 mM). Water was purified and doubly distilled by a Milli-Q system (Millipore, USA). The fluorescence measurements were conducted at room temperature on a Fluoromax-4 spectrofluorometer (HORIBA JobinYvon, Edison, NJ). Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as an internal standard. Thin layer chromatography (TLC) was conducted using silica gel 60 F254 and column chromatography was carried out over silica gel (200-300 mesh), which were obtained from Qingdao Ocean Chemicals (Qingdao, China). The pH was measured with a Mettler-Toledo Delta 320 pH meter. Fluorescence images of cells were obtained from Olympus FV1000-MPE laser scanning confocal microscope (Japan).



Scheme S1 The synthetic route of NIR-HMA and NIR-MAOH.

Synthesis of Compound NIR-MAOH. IR-780 was synthesized according to reported procedures.^{9b} 4-chloro resorcin (1.34g, 9.3 mmol) and triethylamine (3 mL) were placed in a flask containing DMF (20 mL), and the mixture was stirred at room temperature under nitrogen atmosphere for 10 min. IR-780 (2.00 g, 3.1 mmol) in DMF (20 mL) was introduced to the mixture via a syringe, and the reaction mixture was heated at 60°C for 5 h. The solution was cooled to room temperature and then extracted with CH_2Cl_2 . The organic phase was collected, washed with brine, and dried with anhydrous Na_2SO_4 . The solvent in the filtrate was removed under reduced pressure and the solid residue was purified by flash chromatography column using $\text{CH}_2\text{Cl}_2/\text{EtOH}$ (60:1) as eluent to give compound NIR-MAOH as a green solid (576 mg, yield 33.0 %). $\text{C}_{26}\text{H}_{25}\text{NO}_2\text{Cl}$ ^1H NMR (400 MHz, CDCl_3) δ 8.07-8.11 (d, $J = 13.6$ Hz, 1H), 7.41 (s, 1H), 7.29-7.31 (3H), 7.05-7.08 (t, $J = 7.2$ Hz, 1H), 6.84-6.86 (d, $J = 7.6$ Hz, 1H), 6.70 (s, 1H), 5.58-5.61 (d, $J = 13.6$

Hz, 1H), 3.36 (s, 3H), 2.67-2.70 (t, $J = 5.8$ Hz, 2H), 2.61-2.63 (t, $J = 5.6$ Hz, 2H), 1.89-1.92 (t, $J = 5.6$ Hz, 2H), 1.67 (s, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 173.42, 167.23, 160.79, 157.33, 143.22, 139.86, 139.27, 134.83, 130.66, 128.26, 127.27, 122.92, 122.11, 117.90, 115.68, 114.86, 108.71, 103.72, 95.45, 47.92, 29.69, 28.66, 28.06, 24.36, 21.13. MS (EI) m/z calcd for $\text{C}_{26}\text{H}_{25}\text{NO}_2\text{Cl}$: 419.1. Found: 418.1.

Synthesis of Compound NIR-HMA. Compound NIR-MAOH (100 mg, 0.239 mmol), 4-Nitrobenzyl-bromide (206 mg, 0.956 mmol) and Cs_2CO_3 (312 mg, 0.956 mmol) were dissolved in CH_2Cl_2 . The mixture was stirred at 40 °C under nitrogen atmosphere. After overnight reaction, the solvent was removed under reduced pressure. And the residue was subjected to silica gel chromatography, eluted with petroleum ether/ethyl acetate (v/v, 1:1), to afford compound NIR-HMA as a blue solid (60.6 mg, yield 45.9 %). $\text{C}_{33}\text{H}_{30}\text{N}_2\text{O}_4\text{Cl}$ ^1H NMR (400 MHz, CDCl_3) δ 8.69-8.72 (d, $J = 15.0$ Hz, 1H), 8.18-8.20 (d, $J = 8.3$ Hz, 2H), 7.88-7.90 (d, $J = 8.3$ Hz, 2H), 7.74 (s, 1H), 7.53-7.54 (d, $J = 7.3$ Hz, 1H), 7.43 – 7.51 (m, 2H), 7.42 (s, 2H), 7.12 (d, $J = 13.3$ Hz, 1H), 6.30-6.34 (d, $J = 14.9$ Hz, 1H), 5.74 (s, 2H), 4.15 (s, 3H), 2.65 – 2.71 (m, 4H), 1.93 – 1.86 (m, 2H), 1.79 (s, 6H). ^{13}C NMR (101 MHz, CDCl_3) 177.29, 159.89, 157.91, 156.84, 152.53, 145.00, 141.31, 140.77, 130.71, 130.01, 128.41, 128.20, 127.84, 127.64, 126.82, 121.78, 115.66, 114.43, 113.79, 112.04, 108.63, 104.33, 100.75, 69.69, 50.08, 28.45, 28.20, 27.50, 23.95, 21.11. HRMS (ESI) m/z calcd for $\text{C}_{33}\text{H}_{30}\text{N}_2\text{O}_4\text{Cl}$: 553.2015. Found: 553.1900.

Spectral Measurements. The fluorescence measurement experiments were measured in PBS (10 mM) with DMSO as co-solvent solution (PBS/DMSO = 19:1, v/v, 10 mM, pH = 7.4). For the probe respond to nitroreductase, a volume of 10 μL of NIR-HMA stock solution (0.1 mM), 20 μL of NADH solution (5 mM), NTR sample solution, and PBS buffer solution were added into a tube to make the final volume 200 μL . After incubation at 37 °C for 40 min, the reaction solution was transferred into a quartz cell to measure the absorbance or fluorescence spectra, with both excitation and emission slits set at 5 nm. Different pH values of PBS solution from 3. 0 to 8.0 was adjusted by adding minimal volumes of HCl solution or NaOH solution. Procedure of calibration measurements with probe in the buffer with different pH followed: 100 μL stock probe solution and 100 μL PBS buffer with different pH were combined to afford a test solution, which contained 5 μM of probe. The solutions of various testing species were prepared preliminary by using twice-distilled water with the final interference concentration: NaCl (100 μM), KCl (100 μM), CaCl_2 (100 μM), NaHS (100 μM), HClO (100 μM), H_2O_2 (100 μM), glutathione (10 mM), Glucose (10 mM), Vitamin C (1 mM), tyrosine (1 mM), cysteine (1 mM), and glutamate (1 mM), leucine aminopeptidase (100 U/L), glutamyltranspeptidase (10 U/L), glutathione transferase (100 U/L), nitroreductase (10 $\mu\text{g/mL}$). The fluorescence spectra were measured with excitation wavelength at 543 nm.

Cell Culture and Imaging. HeLa, HepG-2, MCF-7 and HL-7702 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin at 37 °C in humidified atmosphere containing 20% O_2 and 5% CO_2 as the normoxic condition. Hypoxic microenvironment ($\sim 0.1\%$ O_2 , 5% CO_2) of cell culture in this study was maintained by using the AnaeroPack system for cell culture (Mitsubishi Gas Chemical Co., Tokyo, Japan). In particular, cells were cultured under normoxia conditions unless noticed otherwise. When the cell density reached 90% of confluence, a subculture was done and the medium was changed approximately every day. Cells were first seeded in a 20 mm glass-bottom dish plated and grown to around 80% confluency for 24 h before the experiment. Fluorescence imaging of cells was carried out using an Olympus FV1000 MPE laser scanning microscope (Japan) with a 60 \times oil immersion objective lens. The fluorescence signal of cells incubated with NIR-HMA and NIR-MAOH was collected in Green channel (625-680 nm) and Red channel (705-760 nm) by using semiconductor laser at 543 nm as excitation resource.

Western Blot Analysis. Prior to further analyses, endogenous LC3 expression at the protein level was confirmed by western blotting. The protein was extracted from HeLa cells sampled when the confluence reached 70%. Briefly, the attached cells were cultured in hypoxic microenvironment for different times and then washed with ice-cold DPBS three times. After removal of the DPBS, a radioimmuno precipitation assay (RIPA) lysis buffer containing protease inhibitors provided by the manufacturer (Pierce, Rock ford, IL, USA) was added into the culture dish, and then lysed on ice for 20 minutes to obtain protein lysates. Protein concentrations were measured with protein detector, and then protein (30 $\mu\text{g}/\text{lane}/\text{cell line}$) was loaded onto a sodium dodecyl sulfate poly-acrylamide gel electrophoresis gel (SDS-PAGE) for separating the proteins into bands. The separated protein bands were transferred to PVDF membranes (Beyotime Biotechnology, China). After the membranes were blocked in 5% skimmed milk in Tris-buffered saline Tween-20 (TBS-T) containing 0.05% Tween 20 for 2 h at RT, they were incubated with GAPDH antibodies (KangChen Bio-tech Inc,

Shanghai, China) at 1/10000 or LC3 antibodies (Beyotime Biotechnology, China) at a 1/1000 dilution overnight at 4°C. The resulting membranes were rinsed with TBS-T three times, and incubated with the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. To detect immunoreactive protein bands, chemiluminescence detection kit (Pierce, Thermo Fisher Scientific) were used according to the manufacturer's instructions. For quantifying protein expression, open source image analysis software (ImageJ, NIH) was used.

Cytotoxicity Assay. Cytotoxicity assays were carried out using HeLa cells. Cell viability was determined using CCK-8 assay. 5000 cells per well were seeded in a 96-well plate and incubated for 12 h in a humidified incubator for adherence. NIR-HMA and NIR-MAOH dissolved in DMSO was added to cells at the final concentration of 0, 2, 4, 6, 8, 10 μM and incubated for 24 h. CCK-8 reagent diluted by RPMI-1640 (FBS free) medium (10%) was added to each well after the removal of culture media and incubated for 0.5 h. Following that, the absorbance was measured at 450 nm on a plate reader Synergy 2 Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT).

Transient Transfection of Adenovirus Ad-GFP-LC3. The transfection of adenovirus Ad-GFP-LC3 was conducted according to the manufacturer's instructions. Briefly, HeLa cells were maintained in RPMI-1640 medium with 5% fetal bovine serum (FBS, GIBCO) and without penicillin-streptomycin which will be more conducive for viral infection at 37°C to reach approximately 50% confluence. The culture medium was then replaced with a fresh medium, and transfection was performed at 40 multiplicity of infection (MOI) of adenovirus Ad-GFP-LC3, which is thawed on ice in advance and cultured under normoxic condition for 48 h. Replaced with fresh medium, incubated with NIR-HMA and then cultured in hypoxic microenvironment for 6 h. In order to observe the fluorescence changes of GFP-LC3 and NIR-HMA, confocal microscopy was used for imaging. GFP was excited at 488 nm with the emission collected at 500-550 nm. NIR-HMA was excited at 543 nm with the emission collected at 705-760 nm and 625-680 nm, respectively.

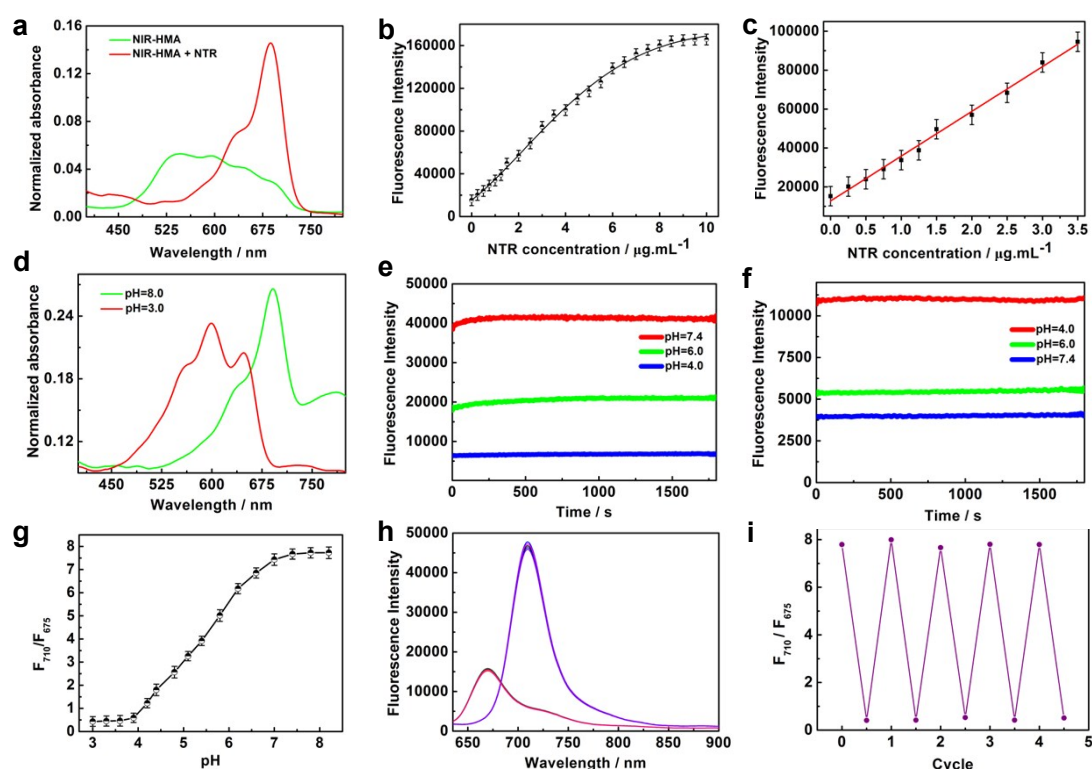


Fig. S1 (a) Absorption spectra of NIR-HMA (5 μM) before (green line) and after (red line) reaction with NTR (10 $\mu\text{g/mL}$). (b) Calibration curve of NIR-HMA to NTR, the curve was plotted with the fluorescence intensity at 710 nm vs NTR concentration after incubation of them for 30 min. (c) The linear responses at low NTR concentrations. $\lambda_{\text{ex}} = 670 \text{ nm}$. (d) Absorption spectra of NIR-MAOH in buffer solution at different pH values (3.0, 8.0). Time scan of fluorescence intensity of NIR-MAOH (5 μM) collected at (e) 710 nm and (f) 675 nm at pH 4.0, 6.0 and 7.4, respectively. (g) Plots of F_{710} / F_{675} versus pH for NIR-MAOH. (h) (i) pH reversibility study of NIR-MAOH between pH 4.0 and 8.0. $\lambda_{\text{ex}} = 570 \text{ nm}$. Data are expressed as the mean of three separate measurements \pm standard deviation (SD).

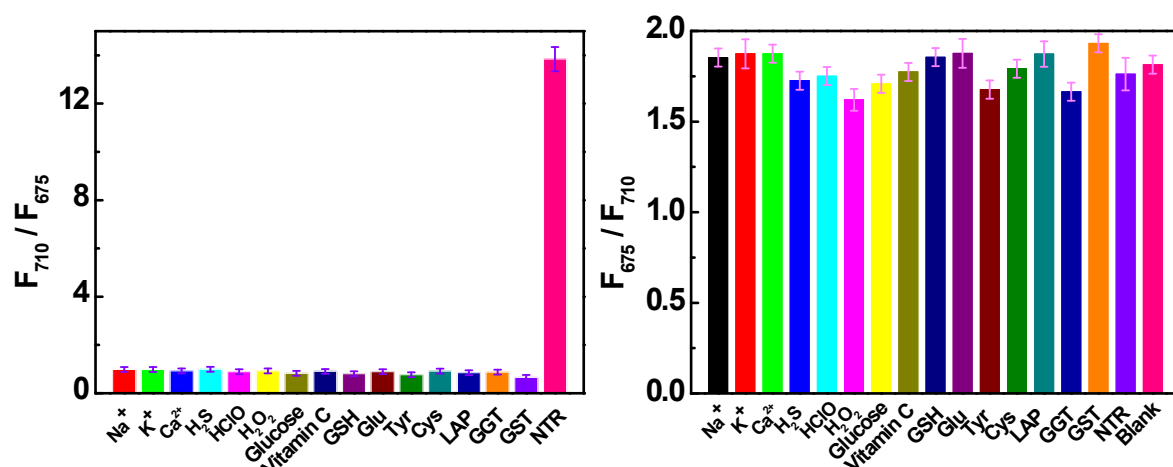


Fig. S2 Fluorescence response (F_{710}/F_{675}) of (a) NIR-HMA or (b) NIR-MAOH (5.0 μ M) in the presence of diverse ions (100 μ M for Na⁺, K⁺, Ca²⁺, H₂S, HClO, H₂O₂), bioactive small molecules (10 mM for GSH, Glucose, 1 mM for Vitamin C, Glu, Tyr and Cys) and different enzymes (100 U/L for LAP, 10 U/L for GGT, 100 U/L for GST, 10 μ g/mL for NTR) in buffer solution (pH 7.4).

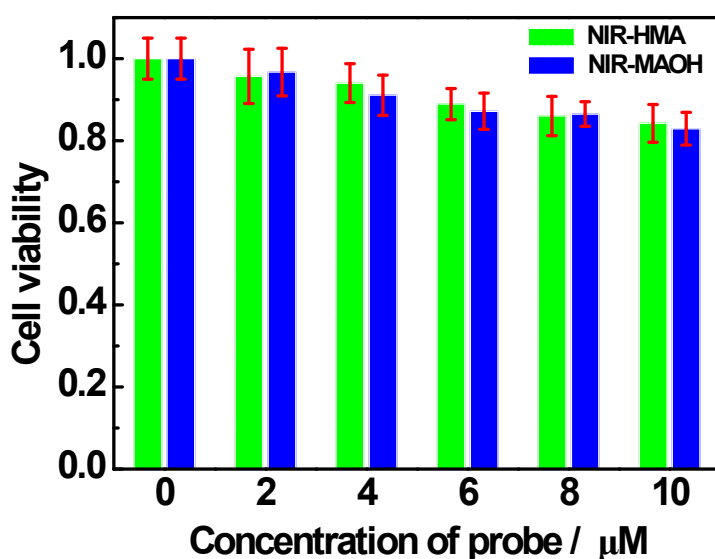


Fig. S3 Cell viability of HeLa cells treated with different concentrations of NIR-HMA (green column) or NIR-MAOH (blue column) for 24 h in fresh medium. The results are the mean standard deviation of five separate measurements.

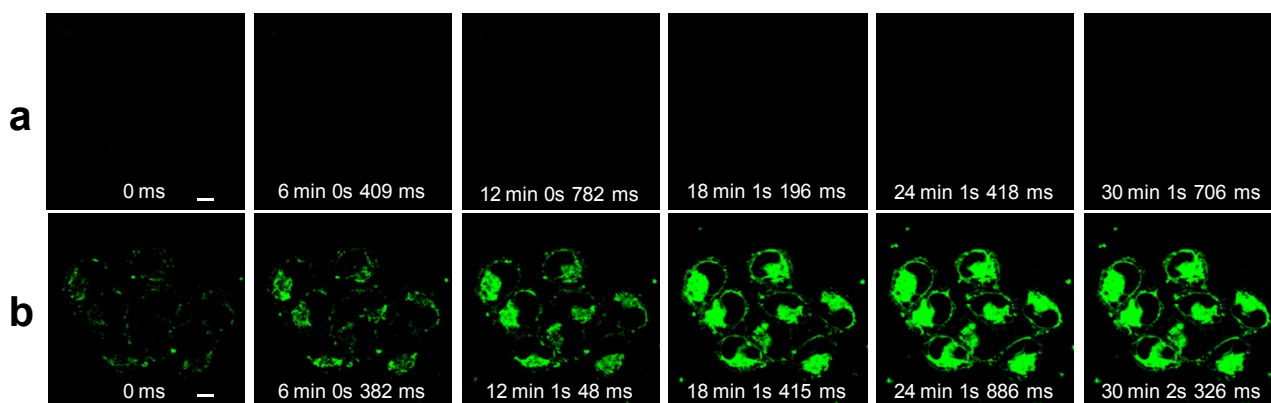


Fig. S4 Real-time imaging of HeLa cells incubated with (a) None. (b) NIR-HMA (5 μ M) (HeLa cells exposed under hypoxia condition for 6h and then incubated with NIR-HMA). λ_{ex} = 543 nm, λ_{em} = 650–750 nm. Scale bar: 20 μ m.

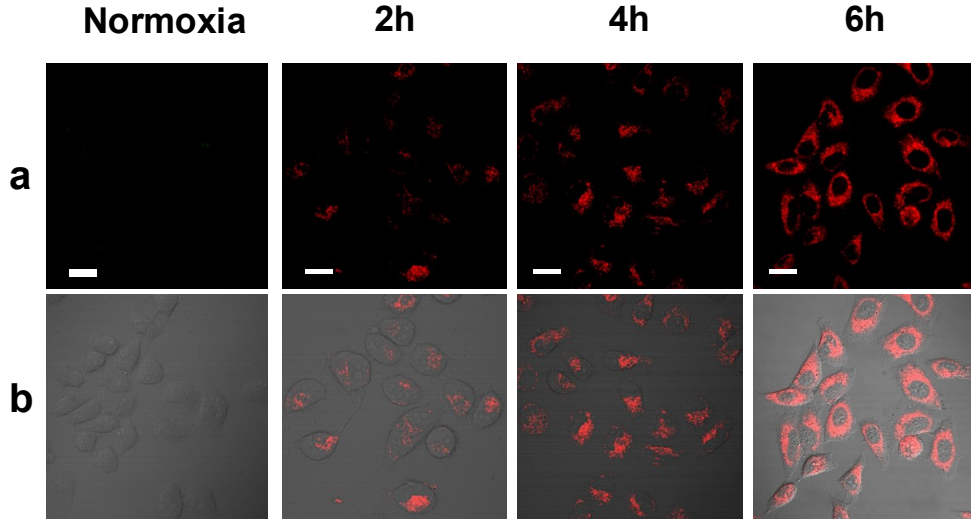


Fig. S5 Confocal fluorescence microscopy imaging of HeLa cells incubated with NIR-HMA (5 μ M) under hypoxic microenvironment (0.1% O_2) for different hours. (a) The fluorescence imaging was collected at red channel (λ_{ex} = 543 nm, λ_{em} = 705–760 nm). (b) The merged fluorescence imaging and bright filed imaging. Scale bar = 20 μ m.

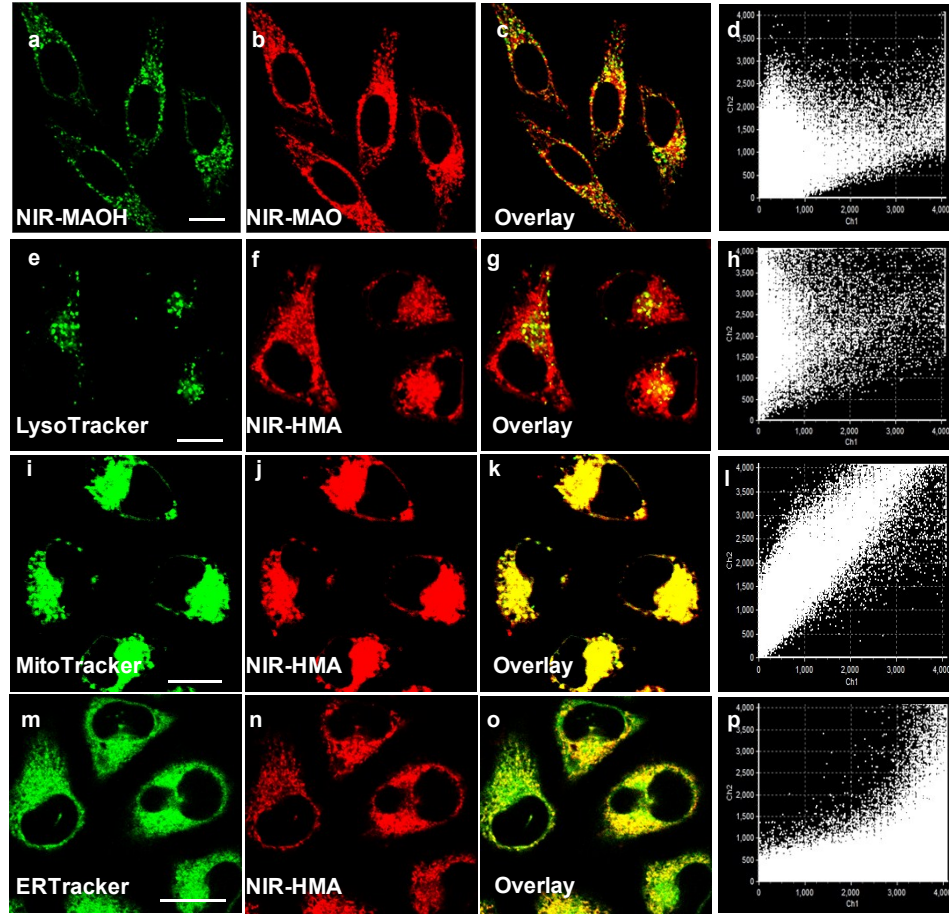


Fig. S6 Co-localization experiments of HeLa cells cultured in hypoxic microenvironment of 0.1% O_2 . Fluorescence image of cells incubated with (a-d) NIR-HMA (5 μ M) for 40 min and then (e-h) LysoTracker Green (100 nM) or (i-l) MitoTracker Green (100 nM) or (m-p) ERTracker Red (100 nM). NIR-MAOH channel: 625–680 nm, NIR-MAO channel: 705–760 nm, λ_{ex} = 543 nm. NIR-HMA channel: λ_{ex} = 543 nm, λ_{em} = 650–750 nm. LysoTracker Green channel: λ_{ex} = 405 nm, λ_{em} = 425–525 nm. MitoTracker Green channel: λ_{ex} = 405 nm, λ_{em} = 450–530 nm. ERTracker Red channel: λ_{ex} = 543 nm, λ_{em} = 580–620 nm. Scale bar: 20 μ m.

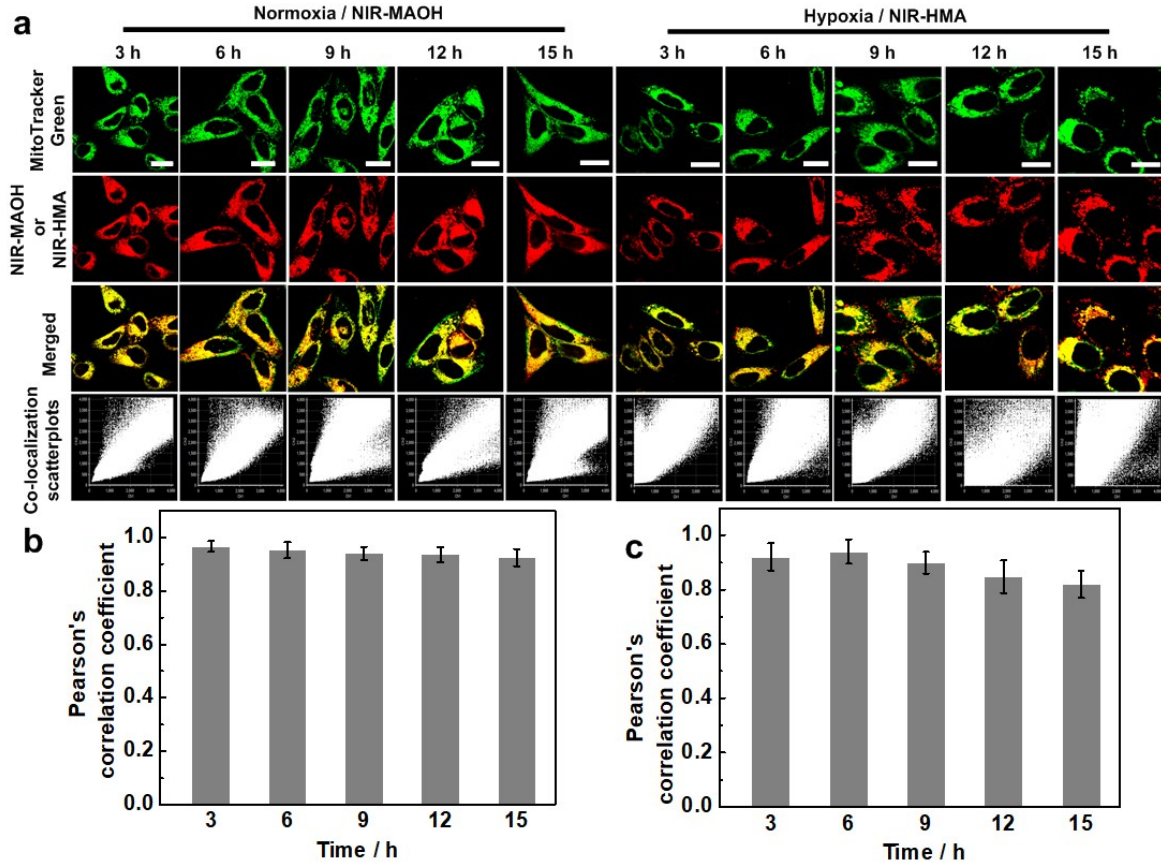


Fig. S7 Real-time co-localization experiments of HeLa cells cultured under hypoxia (0.1% O₂) and normoxia (21% O₂). (a) Fluorescence images of HeLa cells incubated with NIR-MAOH under normoxia or NIR-HMA under hypoxia for different times. MitoTracker Green channel: $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 450\text{--}530 \text{ nm}$. NIR-MAOH or NIR-HMA channel: $\lambda_{\text{ex}} = 543 \text{ nm}$, $\lambda_{\text{em}} = 650\text{--}750 \text{ nm}$. Scale bar: 20 μm . Pearson's correlation coefficient of the colocalization scatterplots in Fig.a which the HeLa cells incubated with NIR-MAOH under (b) normoxia or NIR-HMA under (c) hypoxia for different times.

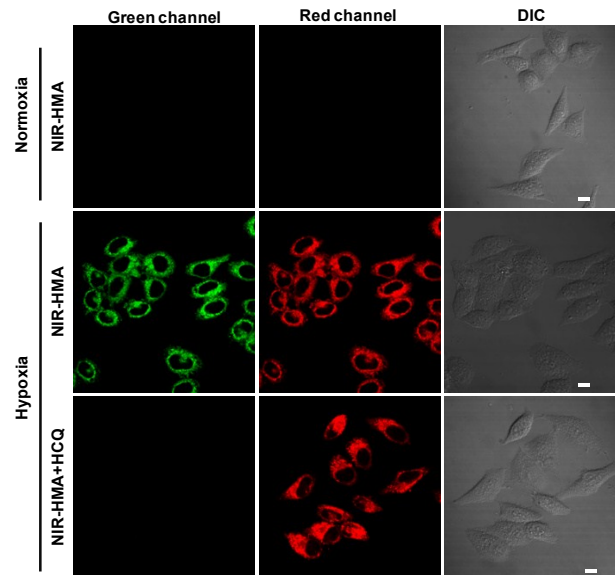


Fig. S8 Confocal images of HeLa cells incubated with NIR-HMA (5 μM) and then exposed under normoxia (21% O₂, first row) or hypoxic microenvironment (0.1% O₂, second row) for 6 h. The third row showed the fluorescence images of HeLa cells pretreated with hydroxychloroquine (HCQ, 50 μM) for 12 h and then incubated with NIR-HMA (5 μM) under hypoxia condition for 6 h. Green channel: 625–680 nm, Red channel: 705–760 nm, $\lambda_{\text{ex}} = 543 \text{ nm}$. Scale bar: 20 μm .

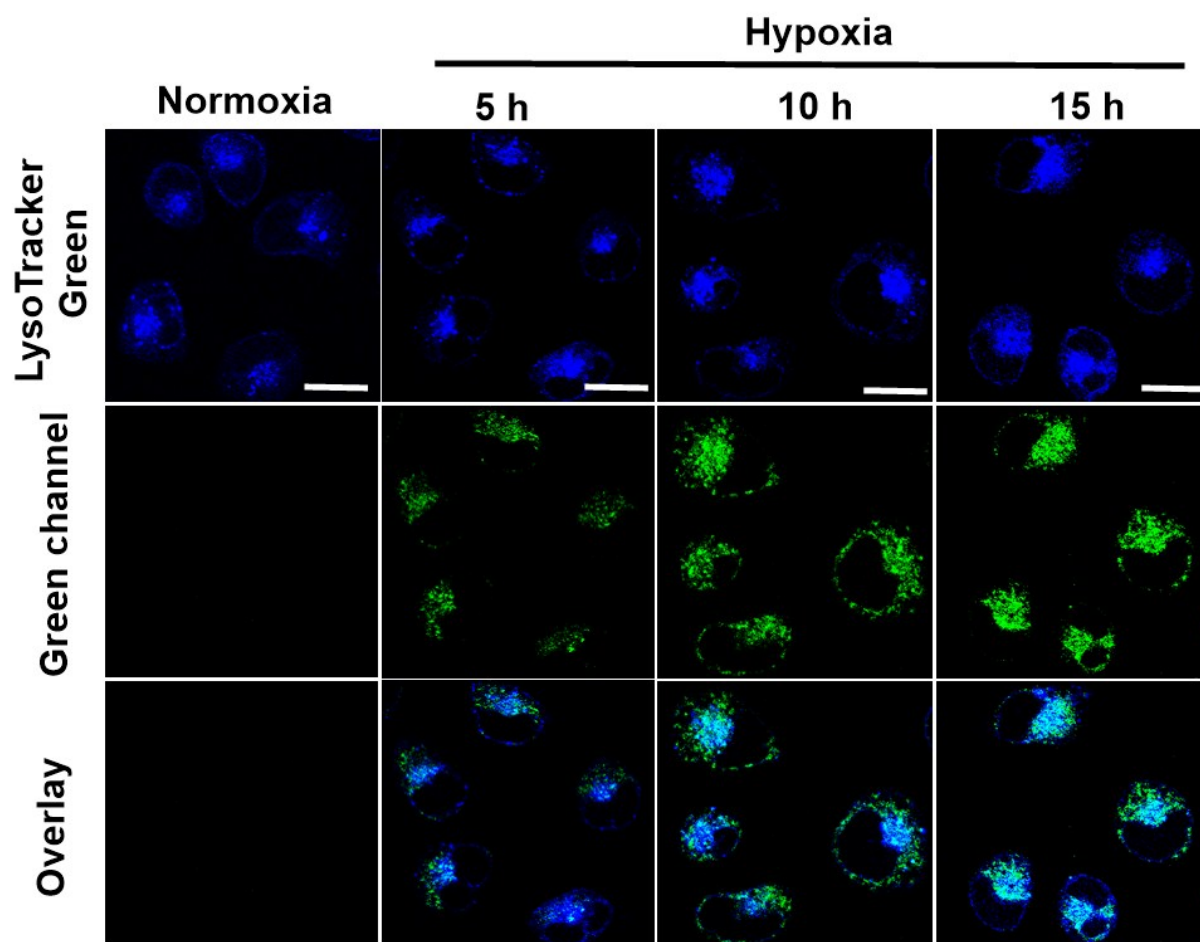


Fig. S9 Confocal images of HeLa cells incubated with NIR-HMA (5 μ M) under hypoxic microenvironment for different times (5 h, 10 h, 15 h) and then LysoTracker Green (100 nM) for 40 min. Blue channel: $\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{em}} = 425\text{-}525$ nm, Green channel: $\lambda_{\text{ex}} = 543$ nm, $\lambda_{\text{em}} = 625\text{-}680$ nm. Scale bar: 20 μ m.

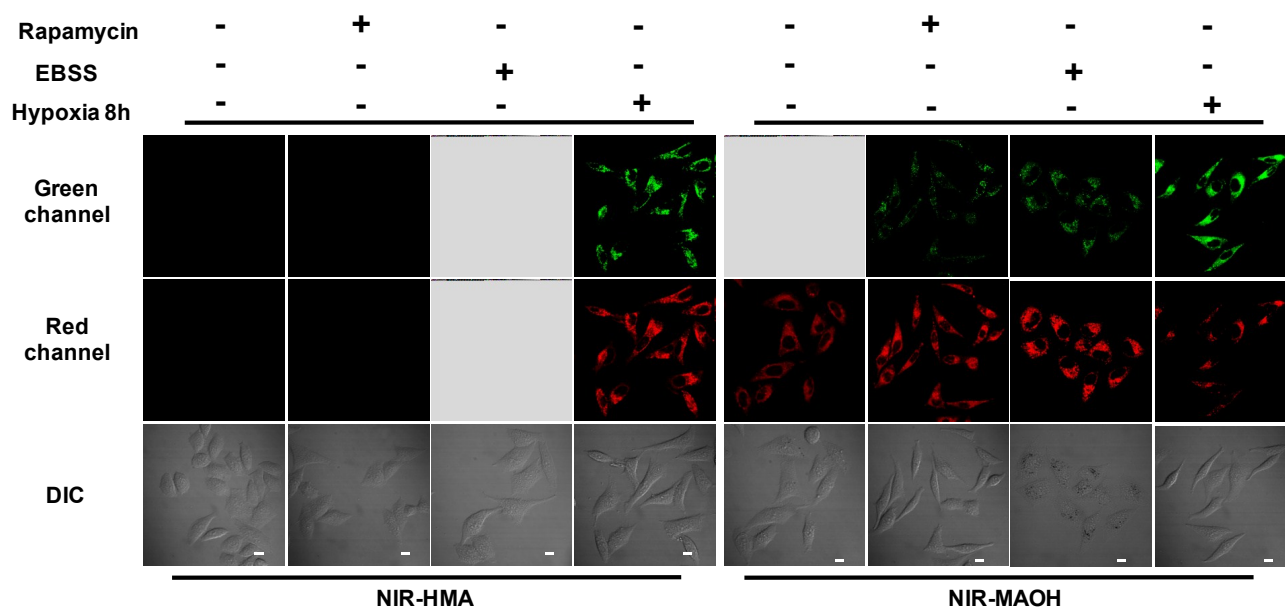


Fig. S10 Specificity of NIR-HMA towards hypoxia induced mitophagy. HeLa cells incubated with rapamycin (100 nM) in full culture medium for 24 h, EBSS for 3 h, and then incubated with NIR-HMA or NIR-MAOH for 40 min. HeLa cells incubated with NIR-

HMA or **NIR-MAOH** under hypoxia (0.1% O₂) condition in full culture medium for 8 h. $\lambda_{\text{ex}} = 543$ nm, Green channel: $\lambda_{\text{em}} = 625\text{--}680$ nm, Red channel: $\lambda_{\text{em}} = 705\text{--}760$ nm. Scale bar: 20 μm .

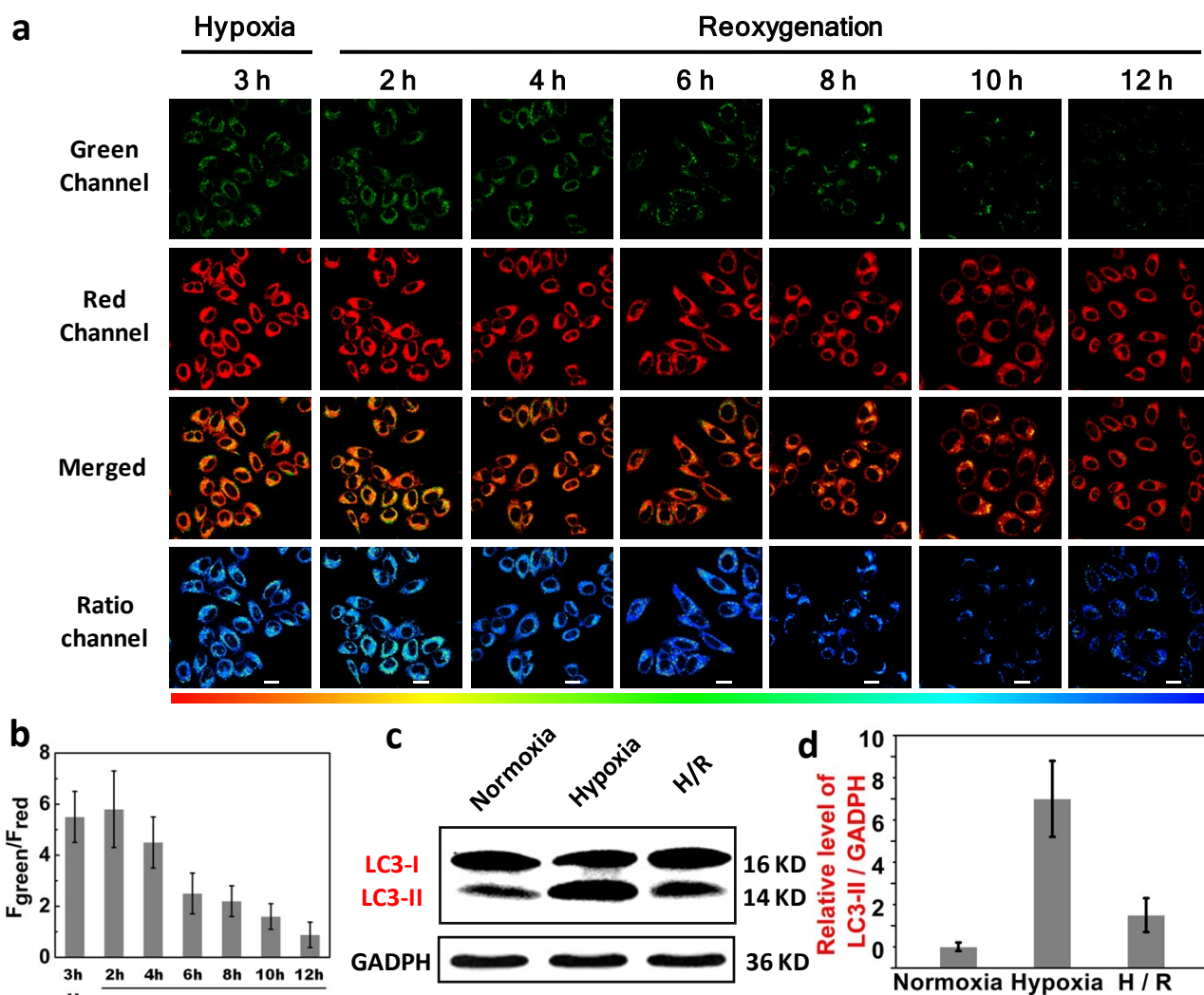


Fig. S11 Real-time imaging of HeLa cells incubated with NIR-HMA. (a) Fluorescence images of HeLa cells incubated with NIR-HMA under hypoxic microenvironment for 3 h and then reoxygenation for different times. (b) Normalized average fluorescence intensity of the ratio channel in (a). H: hypoxia, R: reoxygenation. $\lambda_{\text{ex}} = 543$ nm, Green channel: $\lambda_{\text{em}} = 625\text{--}680$ nm, Red channel: $\lambda_{\text{em}} = 705\text{--}760$ nm. Scale bar: 20 μm . (c) HeLa cells were exposed to normoxia, or hypoxia for 3 h and then reoxygenation under normoxia condition for 12 h. Total cell extracts were analyzed by western blotting with antibodies against LC3. Western blot of GAPDH was used as a loading control. H/R: hypoxia/reoxygenation. (d) ImageJ normalized quantitative analysis of the LC3-II/GAPDH ratios from western blot in (c). H/R: hypoxia/reoxygenation.

MS and ¹H NMR

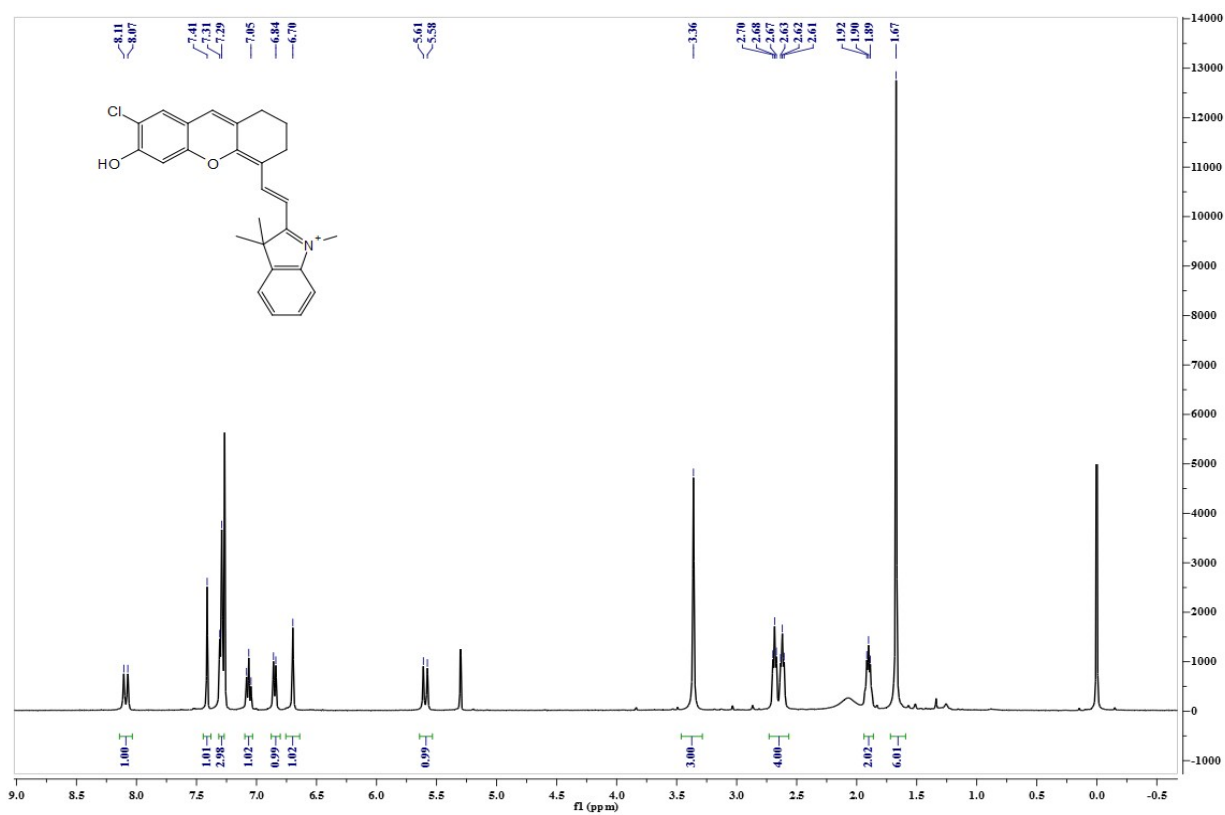


Fig. S12 ¹H NMR spectrum of NIR-MAOH.

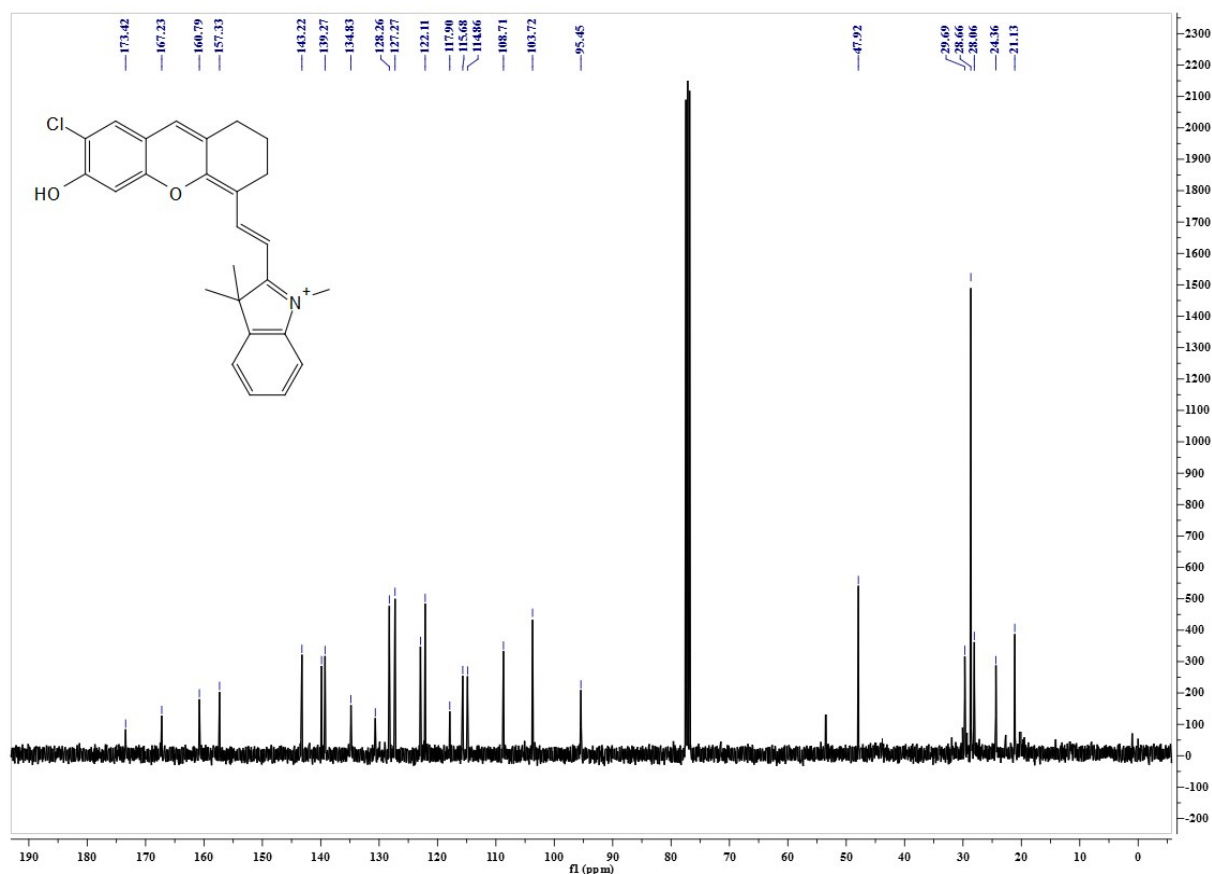


Fig. S13 ^{13}C NMR spectrum of NIR-MAOH.

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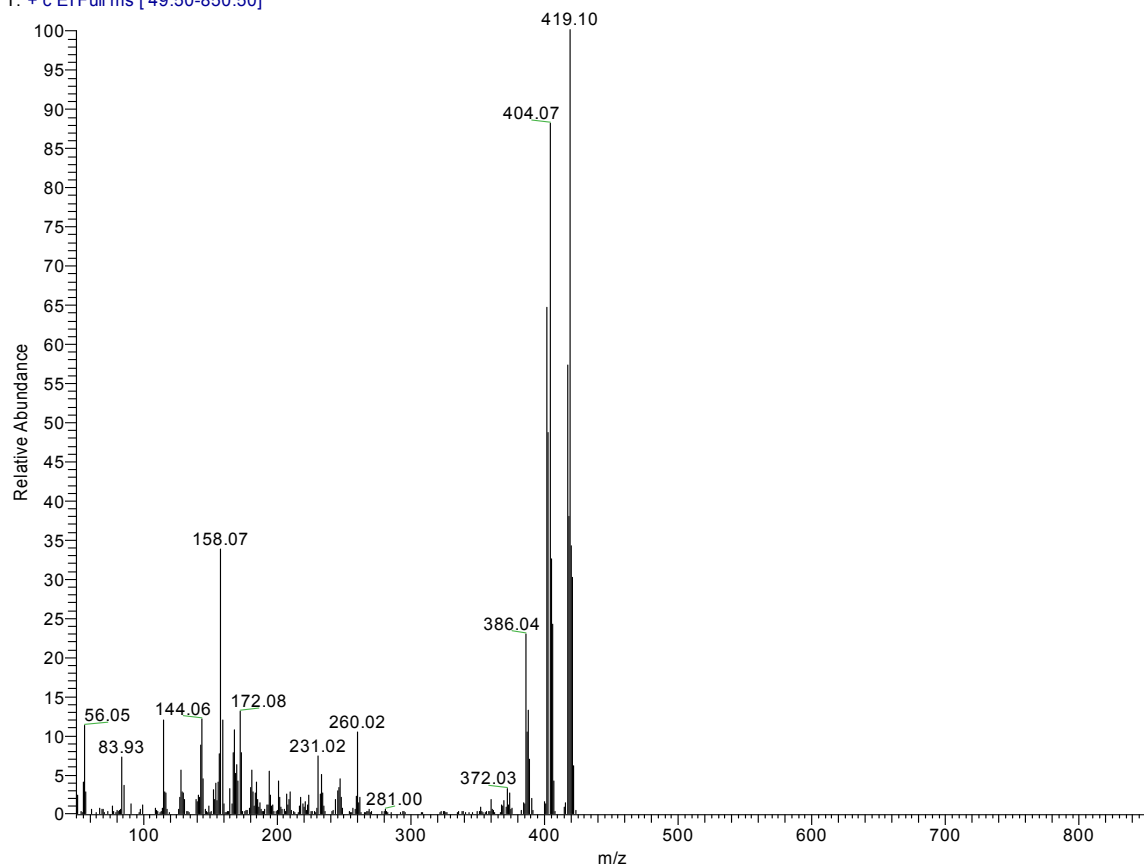


Fig. S14 EI-MS spectrum of NIR-MAOH.

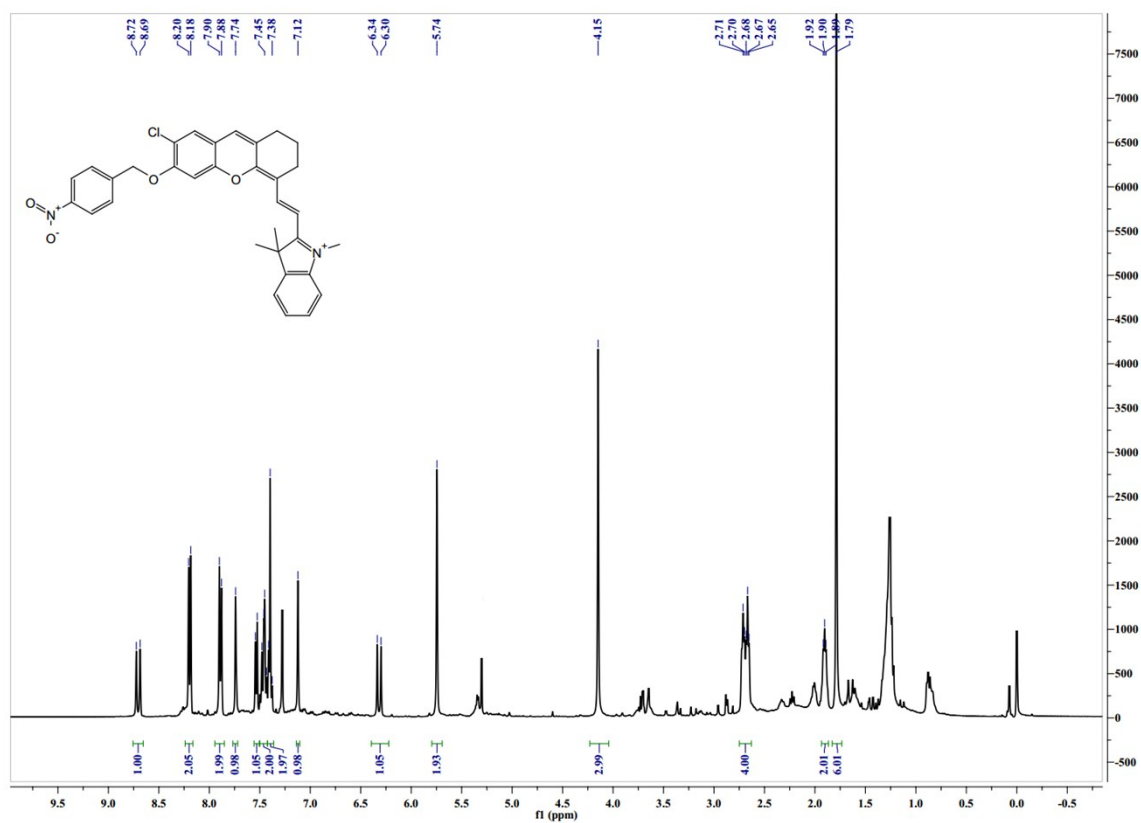


Fig. S15 ¹H NMR spectrum of NIR-HMA.

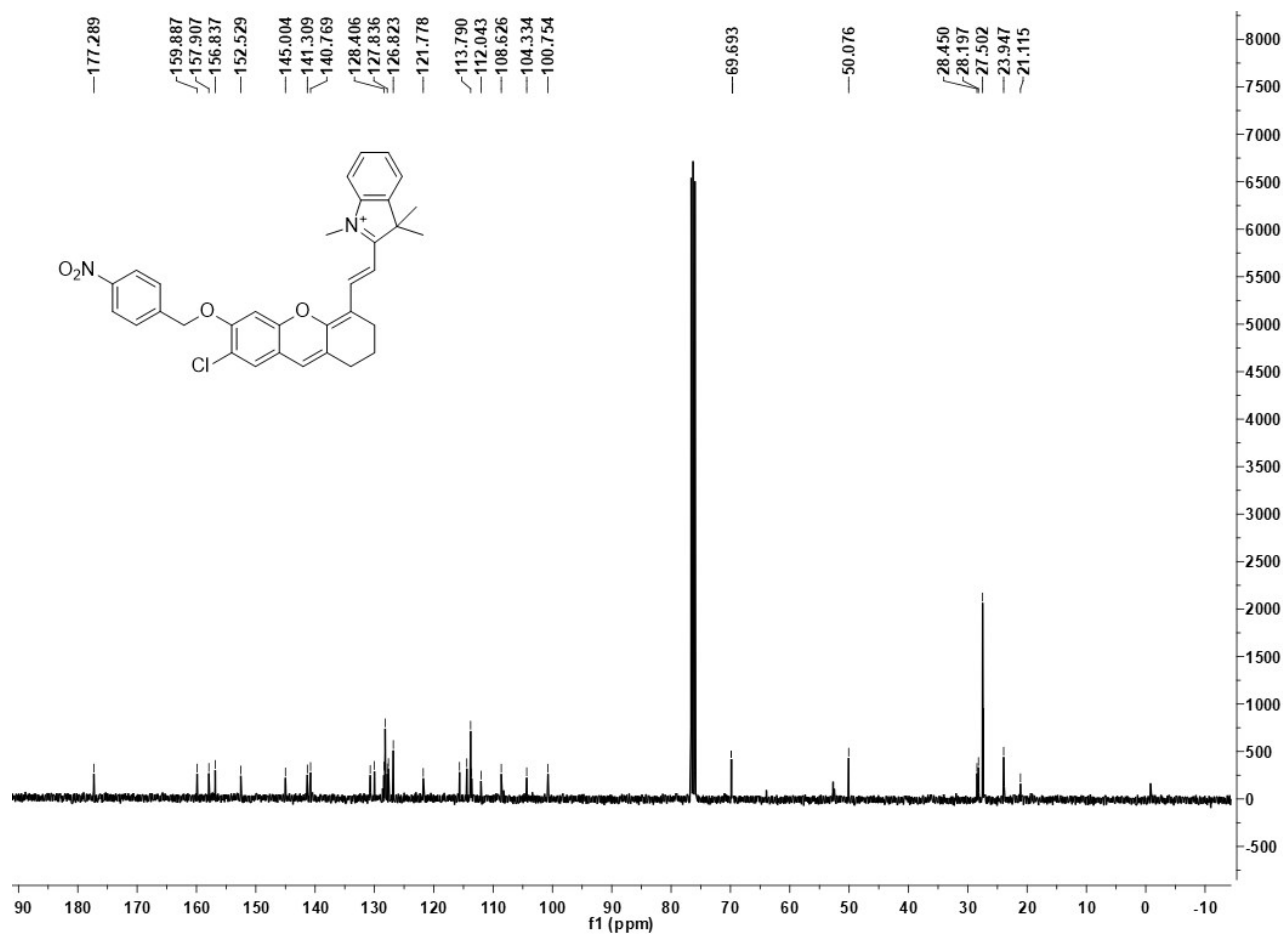


Fig. S16 ^{13}C NMR spectrum of NIR-HMA.

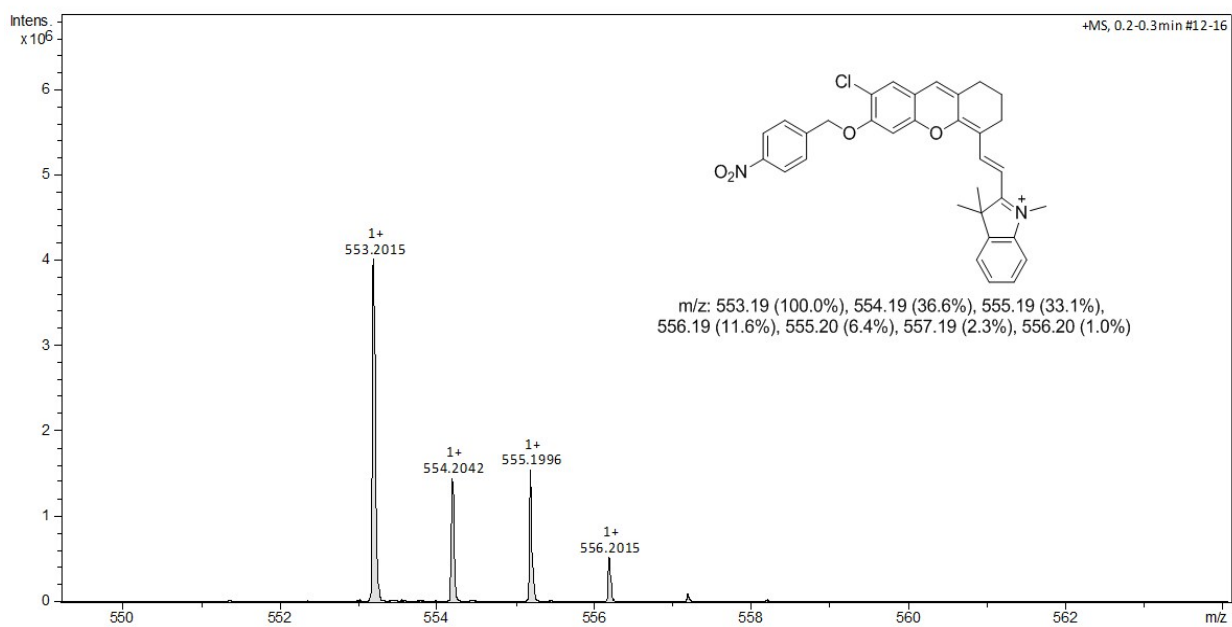


Fig. S17 HR-MS spectrum of NIR-HMA.