

An Endoplasmic Reticulum Targeting Green Fluorescent Protein Chromophore-based Probe for the Detection of Viscosity

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1. Experimental section

1.1 Materials and equipment

All reagents are commercially available and used without any purification unless specifically noted. ER-Tracker Red, dexamethasone, acetic acid, potassium carbonate, *p*-hydroxybenzaldehyde, piperidine, 2-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU), *N,N*-diisopropylethylamine (DIPEA), sodium acetate trihydrate, *N*-acetylglycine, an ethanol solution of methylamine (33 wt. %), were purchased from Energy Chemical. Acetonitrile, 2,3,4,5,6-pentafluorobenzoic acid, 4-(methylthio)benzaldehyde, toluene, glycerin, 2-bromoethylamin hydrobromide, *N,N*-dimethylformamide (DMF) were obtained from Shanghai Macklin Biochemical Co., Ltd. RIPA lysate was purchased from Beyotime, BCA protein quantitative kit, ECL chemiluminescence detection reagent and pre-dyed egg marker were purchased from Thermo Fisher Scientific, BSA was purchased from Sigma, polyvinylidene fluoride(PVDF) membranes were purchased from Millipore, horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Lianke Biotech Co., Ltd. Anti-LC3 antibodies were purchased from Novus Biologicals, Inc. The pH of the solutions was adjusted with sodium hydroxide solution (0.1 M) and hydrochloric acid (0.1 M), and measured using a pH meter (Leici PHS-3E). ^1H and ^{13}C NMR spectra were collected on a Bruker AV-400 spectrometer. The ^1H NMR spectra were calibrated using an internal standard (TMS: δ 0.0 ppm) or solvent (CDCl_3 : δ 7.26 ppm, CD_3OD : δ 3.31 ppm, $\text{DMSO-}d_6$: δ 2.50 ppm) and ^{13}C NMR spectra were calibrated using the solvent (CDCl_3 : δ 77.16 ppm, $\text{DMSO-}d_6$: δ 39.52 ppm). Chemical shifts (δ) are reported in parts per million (ppm) relative to the standard or residual solvent and the coupling constants (J) are reported in hertz (Hz). MALDI-TOF mass spectrometry was performed using an AB SCIEX 5800 MALDI-TOF/TOF™. All absorption spectra and fluorescence spectra were recorded using a Hitachi U-

3900 UV-Vis spectrometer and a Hitachi fluorescence spectrophotometer F-7000, respectively.

1.2 Optical measurements

In a typical optical test, **GE-Y** (10 μM) solutions were obtained by diluting the probe stock solution (1 mM) into a PBS/glycerin system of different viscosities (from 0.89 cP to 438.40 cP at 25°C). The volume ratio of PBS buffer (10 mM, pH 7.4) and glycerin of different viscosity solutions is shown in **Table S1**. All the absorption and emission spectra were recorded using standard 1-cm path length quartz fluorescence cuvettes at 25°C. The slit width of excitation and emission were set to 10 nm and excitation wavelength was set at 430 nm for the fluorescence spectroscopy.

1.3 Cell culture and cell viability

The human breast cancer (MCF-7) cells, human cervical cancer cells (HeLa), human alveolar epithelial cells (A549), and Chinese hamster ovary cells (CHO) were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were incubated in medium (high glucose DMEM for MCF-7 cells and HeLa cells, F-12 for A549 cells and CHO cells) supplemented with 10% FBS (fetal bovine serum) and placed in a 37°C constant temperature incubator containing 5% carbon dioxide. The toxicity of **GE-Y** towards MCF-7 cells was evaluated by the standard MTT method. Firstly, the cells were seeded in a 96-well cell culture plate at a density of 8000 cells/well with medium volume of 200 μL . Then, the plate was placed in a 37°C constant temperature incubator under 5% CO₂ for 12 h. Subsequently, probe **GE-Y** of different concentrations (0, 4, 8, 12, 16, 20 μM) were added to the wells and five parallel groups were set. After the culture medium was removed, 100 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) reagent (1 mg/mL) was loaded in to each well after culturing the cell for 12 h and 24 h, respectively, followed by incubation at 37 °C for another 4 h. Then the MTT reagent was removed and 100 μL DMSO was added to each well. The plate was placed on an oscillator and

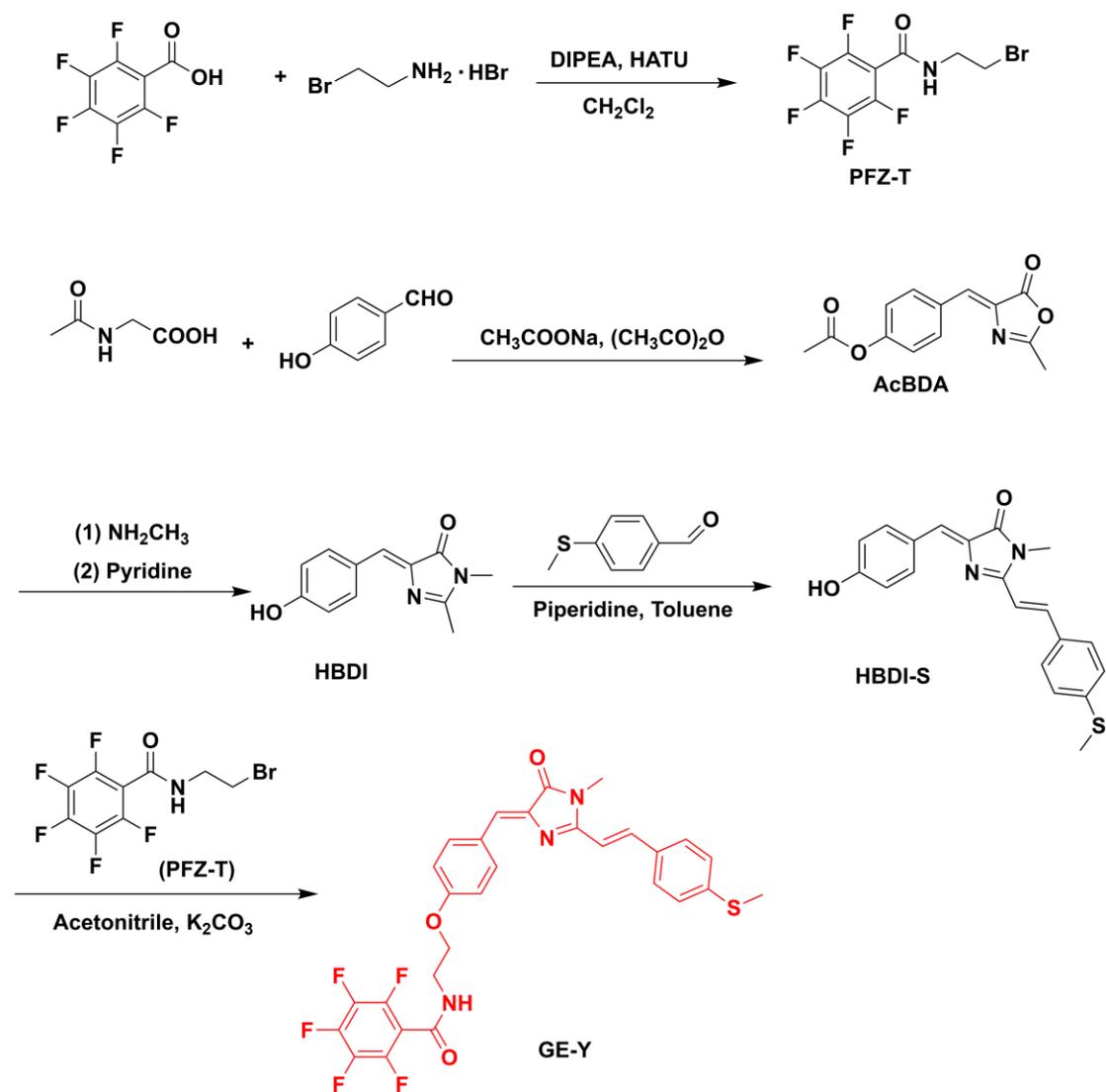
gently shaken for 10 minutes to dissolve the blue crystals, and the optical density value was measured using a Thermo scientific multiskan spectrometer.

1.4 Western blot assay

The treated cells were washed twice with PBS buffer. After incubating with 100 μ L RIPA lysis buffer at 4°C for 10 min, the supernatant was collected by centrifuging at 12 000 rpm for 5 minutes. Total protein of supernatant was determined by BCA protein quantitative kit. 30 μ g total protein was separated by 12% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked with 5% BSA at 25°C for 2h and incubated with primary rabbit polyclonal LC3 (1:1000) at 4°C overnight with gentle shaking. Washing membranes three times with TBST buffer, and incubated with HRP-conjugated secondary antibodies (1:2000) for 1 hour at 25 °C. After washing with TBST buffer three times, the membranes were incubated with ECL chemiluminescence detection reagent and exposed to X-ray film.

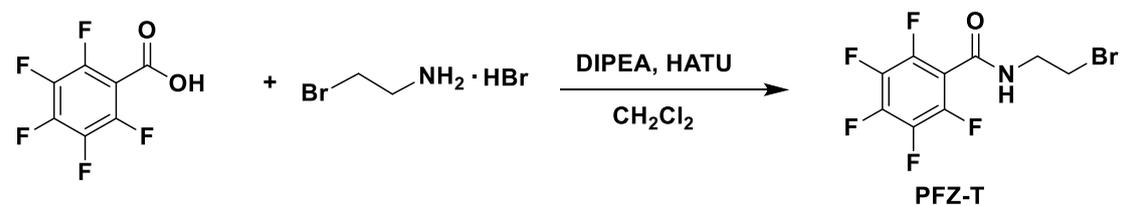
Protein light chain 3(LC3) is a biomarker of autophagy.¹ When autophagy occurs, LC3 precursor molecules are cleaved to form cytoplasmic LC3-I by removing the c-terminal 5-peptide. Subsequently, LC3-I was coupled with phosphatidylethanolamine to form membrane-bound LC3-II, so the ratio of LC3-II/LC3-I could be used to evaluate the degree of autophagy. We monitored the ratio of LC3-II/LC3-I by western blot to ensure autophagy occurred in the experimental group.

1.5 Synthetic procedures



Scheme S1. Synthetic route for preparing **GE-Y**.

Synthesis of PFZ-T

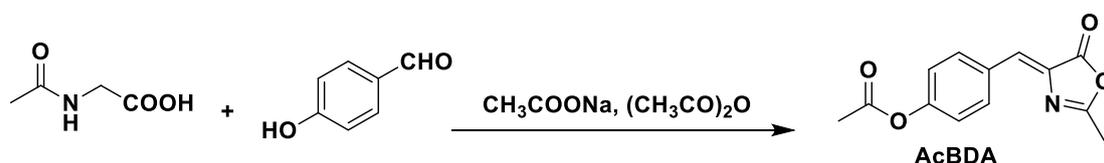


2-bromoethan-1-amine hydrobromide (410 mg, 2 mmol), 2,3,4,5,6-pentafluorobenzoic acid (424 mg, 2 mmol), DIPEA (517 mg, 4 mmol) and HATU (1.520 g, 4 mmol) were added to a 100 mL round bottom flask containing dichloromethane. The mixture was

stirred at 23°C under nitrogen overnight. Then, the mixture was diluted with dichloromethane, and washed with water and brine. The organic layer was separated and dried with anhydrous Na₂SO₄. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography column using petroleum ether/ethyl acetate (v/v, 5/1) as eluent to give PFZ-T as a white solid (210 mg, 33%). ¹H NMR (400 MHz, CD₃OD) δ 3.78 (t, *J* = 8.0 Hz, 2H), 3.58 (t, *J* = 8.0 Hz, 2H), 2.81 (s, 1H).

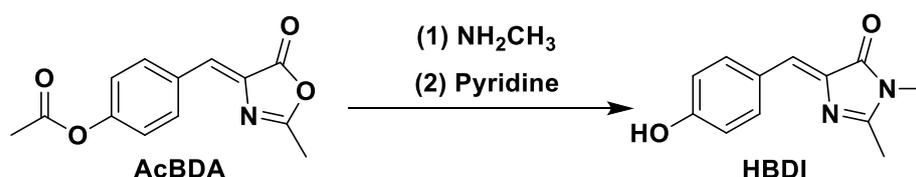
AcBDA and HBDI were synthesized according to our previously reported method with minor modification²

Synthesis of AcBDA



N-acetylglycine (234 mg, 2 mmol), 4-hydroxybenzaldehyde (244 mg, 2 mmol), and sodium acetate (246 mg, 3 mmol) were added to a 50 mL round bottom flask containing acetic anhydride (6 mL), and the mixture was stirred at 110 °C under nitrogen atmosphere for 3-4 hours. Water/ethanol (2/1, v/v) equivalent to five times the volume of acetic anhydride was added after the reaction solution was cooled to 25°C. The solution was stirred at 0°C for 30 minutes and then filtered under vacuum. The filtered product was dried overnight in a vacuum drying oven at 50°C to provide golden solid (260 mg, 53%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.23 (d, *J* = 8.0 Hz, 1H), 7.29-7.24 (m, 4H), 2.39 (s, 3H), 2.38 (s, 3H).

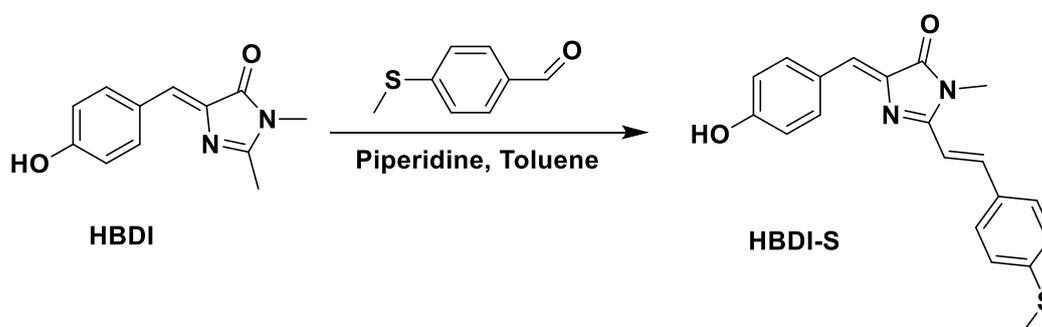
Synthesis of HBDI



AcBDA (100 mg, 0.4 mmol) and methylamino alcohol (4 mL) were added to a 50 mL

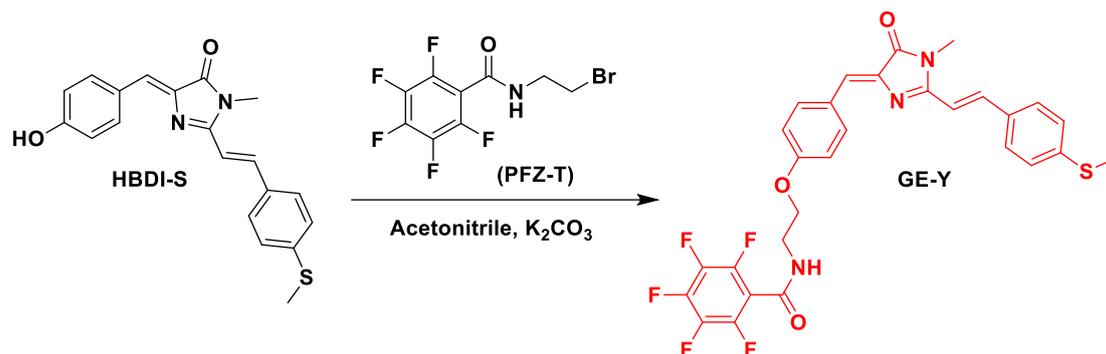
round bottom flask. The mixture was stirred at 25°C under nitrogen for 2-3 hours. Then the solvent was removed under reduced pressure. Pyridine (5 mL) was added to dissolve the residue and the mixture continued to reflux at 110°C under nitrogen for 4-5 hours. After removal of the solvent, the residue was purified by flash chromatography on silica gel using CH₂Cl₂/CH₃OH (v/v, 110/1) as eluent to afford **HBDI** as a yellow solid (71 mg, 81%). ¹H NMR (400 MHz, CD₃OD) δ 7.98 (d, *J* = 8.4 Hz, 2H), 7.01 (s, 1H), 6.84 (d, *J* = 8.4 Hz, 2H), 3.18 (s, 3H), 2.38 (s, 3H).

Synthesis of **HBDI-S**



HBDI (100 mg, 0.46 mmol), 4-(methylthio) benzaldehyde (105 mg, 0.69 mmol), acetic acid (139 mg, 2.3 mmol), and piperidine (197 mg, 23 mmol) were dissolved in 5 mL toluene at 120°C for 6 h. And a water trap was used to remove the water produced during the reaction. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography column using petroleum ether/ethyl acetate (v/v, 2/1) as eluent to give **HBDI-S** as a dark red solid (87 mg, 54%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.19 (d, *J* = 8.0 Hz, 2H), 7.95 (d, *J* = 16.0 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 2H), 7.32 (d, *J* = 8.0 Hz, 2H), 7.16 (d, *J* = 16.0 Hz, 1H), 6.95 (s, 1H), 6.88 (d, *J* = 8.0 Hz, 2H), 3.26 (s, 3H), 2.53 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 170.54, 160.09, 159.33, 141.65, 139.50, 137.50, 134.94, 132.14, 132.11, 129.19, 126.40, 126.07, 116.31, 113.31, 26.85, 14.69.

Synthesis of GE-Y



HBDI-S (100 mg, 0.29 mmol), **PFZ-T** (136 mg, 0.42 mmol) and K₂CO₃ (193 mg, 1.4 mmol) were mixed in 10 mL acetonitrile, and the resulting mixture was stirred at 65 °C under nitrogen for 4 h. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel using CH₂Cl₂ as eluent. The final pure product **GE-Y** was obtained as a yellow solid (35 mg, 21%).

¹H NMR (400 MHz, CDCl₃) δ 8.25 (d, *J* = 8.0 Hz, 2H), 8.03 (d, *J* = 16.0 Hz, 1H), 7.54 (d, *J* = 8.0 Hz, 2H), 7.24 (s, 2H), 7.09 (s, 1H), 7.05 (d, *J* = 12.0 Hz, 2H), 6.76 (d, *J* = 16.0 Hz, 1H), 4.50 (t, *J* = 8.0 Hz, 2H), 4.16 (t, *J* = 8.0 Hz, 2H), 3.31 (s, 3H), 2.52 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.85, 159.94, 157.74, 142.25, 140.86, 139.23, 134.20, 131.59, 131.05, 128.41, 126.00, 125.31, 115.90, 111.34, 67.97, 55.28, 26.62, 15.12.

MALDI-TOF: calculated [M+H]⁺ 588.1375 m/z, found 588.1377 m/z, M represents C₂₉H₂₂F₅N₃O₃S (chemical formula of **GE-Y**)

3. Procedure for preparing different volume ratios of

PBS/glycerin solution

Table S1. The volume ratio of PBS buffer (10 mM, pH 7.4) and glycerin of different viscosity solutions (25°C).

Solvent Viscosity (cP)	PBS (mL)	Glycerin (mL)	DMSO solution of GE-Y (mL)
0.89	980	0	20
7.90	480	500	20
32.50	280	700	20
78.90	180	800	20
126.80	140	840	20
140.60	130	850	20
175.50	120	860	20
240.70	80	900	20
438.40	30	950	20

4. UV-Vis absorption spectra and fluorescence spectra of GE-Y in different solutions

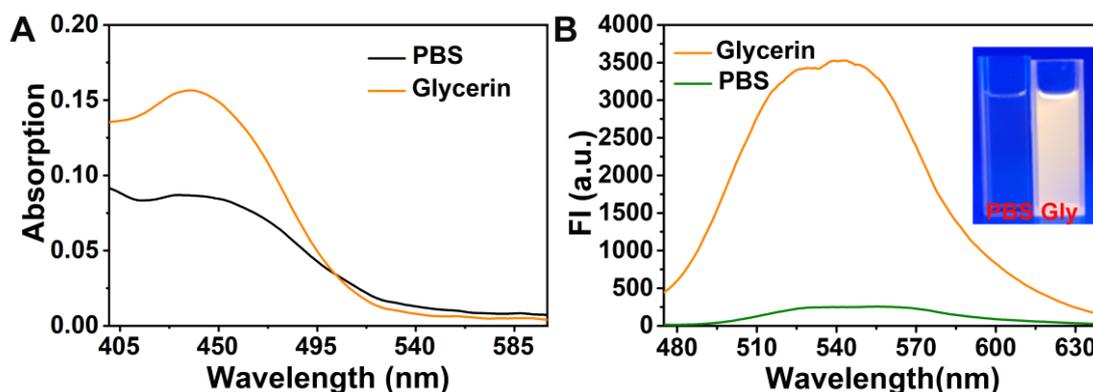


Figure S1. (A) The UV-Vis absorption spectra (B) Fluorescence spectra of **GE-Y** (10 μM) in PBS buffer (10 mM, pH 7.4) and glycerin, respectively. Inset: Fluorescence photographs of **GE-Y** (10 μM) in PBS buffer (10 mM, pH 7.4) and glycerin (Gly) respectively under irradiation by a 365 nm hand-held UV lamp. $\lambda_{\text{ex}} = 430$ nm, $\lambda_{\text{em}} = 550$ nm. Slit widths $\text{ex} = 10$ nm and $\text{em} = 10$ nm; temperature, 25°C.

5. Fluorescence quantum yield for GE-Y

The fluorescence quantum yields of **GE-Y** in solution with low viscosity (0.89 cP) and high viscosity (438.4 cP) were determined using coumarin 153 in absolute ethanol ($\Phi = 0.58$) as a reference.³ The quantum yield of **GE-Y** in PBS/ glycerin solution (10 mM, pH 7.4) at 25°C was then calculated. The relative fluorescence quantum yields (Φ) were measured on optically dilute samples (absorbance < 0.05) which were degassed by bubbling with oxygen-free nitrogen.

$$\Phi_{\text{sample}} = \Phi_{\text{standard}} \left(\frac{\text{Grad}_{\text{sample}}}{\text{Grad}_{\text{standard}}} \right) \left(\frac{\eta_{\text{sample}}^2}{\eta_{\text{standard}}^2} \right)$$

Where Φ (sample) is the relative fluorescence quantum yield of **GE-Y** in a buffer with low and high viscosity, respectively. And Φ (standard) is the fluorescence quantum yield of coumarin 153 in absolute ethanol ($\Phi = 0.58$)⁴. Grad (standard) and Grad (sample) are the gradients from the plot of integrated fluorescence intensity vs absorbance (absorbance < 0.05). Grad (standard) was calculated to be 348145 and Grad

(samples) were calculated to be 6645 and 65838 for solutions with low viscosity (0.89 cP) and high viscosity (438.4 cP), respectively. The η (standard) and η (sample) are the refractive indexes of the solvents. Where, η (standard) is 1.362, and η (sample) is 1.333 (for PBS) and 1.466 (for PBS/ glycerol solution with viscosity of 438.4 cP). The refractive index of the solvents was determined using an Abbe refractometer (WYA-2S, Shanghai INESA) at 25°C

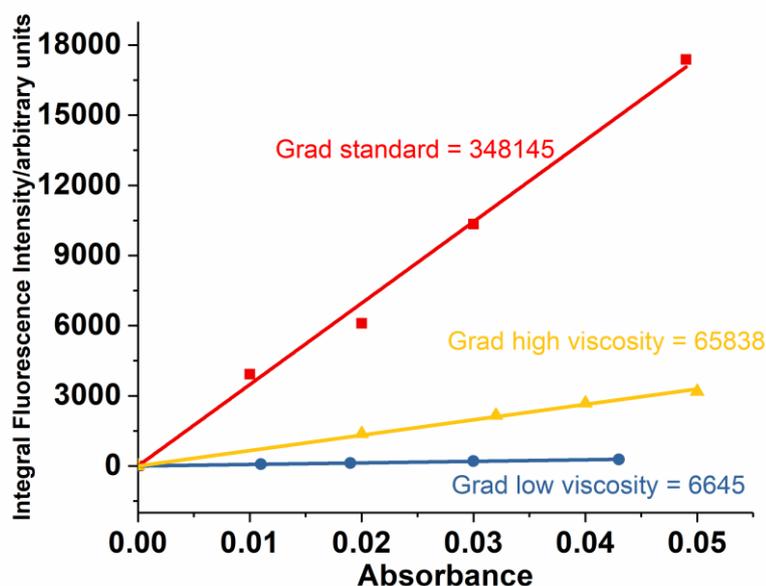


Figure S2. Plots of integrated fluorescence intensity vs absorbance for **GE-Y** in buffer with low and high viscosity and coumarin 153 respectively.

Table S2. Fluorescence quantum yields for **GE-Y**

Viscosity	Fluorescence quantum yield (Φ)
0.89 cP	0.01
438.4 cP	0.13

6. Stability of GE-Y towards interferences

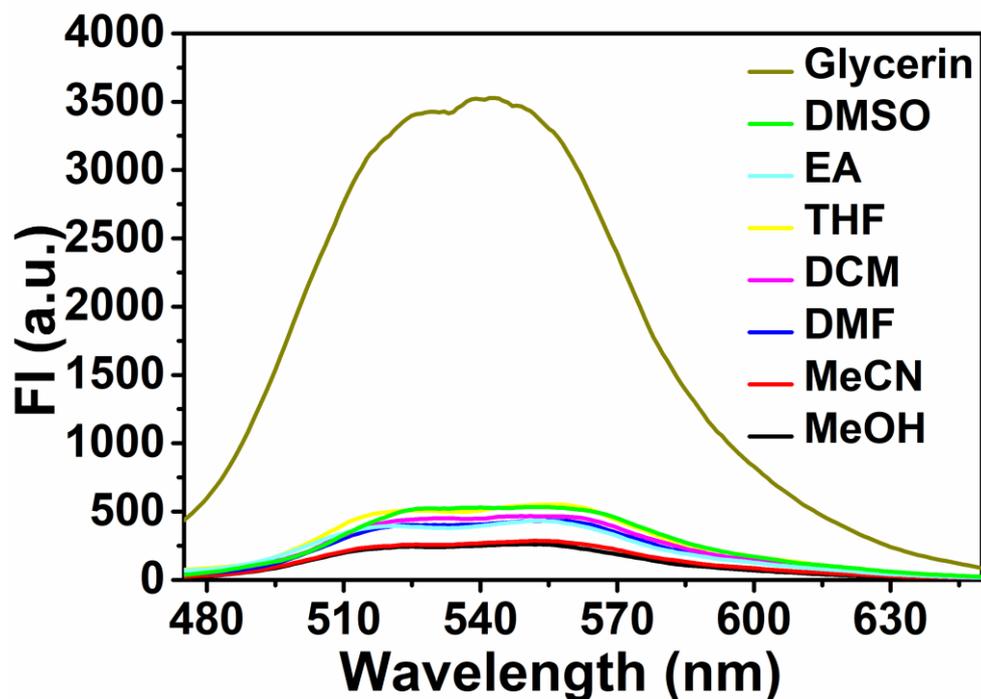


Figure S3. Fluorescence responses of GE-Y (10 μM) in glycerin, dimethyl sulfoxide (DMSO), ethyl acetate (EA), tetrahydrofuran (THF), dichloromethane (DCM), N,N-dimethylformamide (DMF), acetonitrile (MeCN) and methanol (MeOH) respectively. $\lambda_{\text{ex}} = 430$ nm, $\lambda_{\text{em}} = 550$ nm. Slit widths $\text{ex} = 10$ nm and $\text{em} = 10$ nm; temperature, 25°C.

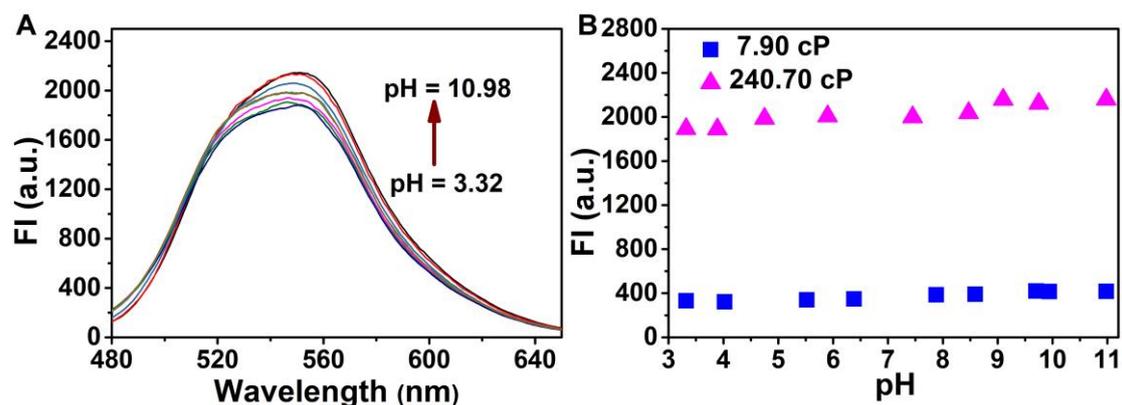


Figure S4. (A) Fluorescence spectra of GE-Y (10 μM) in PBS/glycerin binary solutions (240.70 cp) at different pH values (varied from 3.32 to 10.98, PBS is 10 mM). (B) Effect of pH on the fluorescence of GE-Y (10 μM) at low (7.90 cP) and high (240.70 cP) viscosity. ($\lambda_{\text{ex}} / \lambda_{\text{em}} = 430 / 550$ nm. Slit width $\text{ex}=10$ nm and $\text{em}=10$ nm; temperature 25°C).

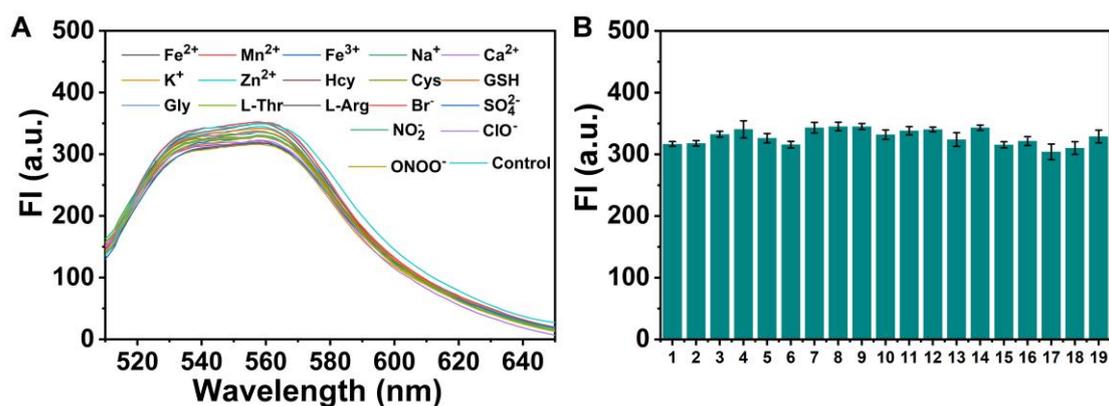


Figure S5. (A) Fluorescence responses of **GE-Y** (10 μM) towards different competing species in PBS buffer (10 mM, pH 7.4); (B) Histogram of panel (A) at 550 nm. 1) Fe^{2+} , 2) Mn^{2+} , 3) Fe^{3+} , 4) Na^+ , 5) Ca^{2+} , 6) K^+ , 7) Zn^{2+} , 8) homocysteine (Hcy), 9) cysteine (Cys), 10) glutathione (GSH), 11) glycine (Gly), 12) L-threonine (L-Thr), 13) L-arginine (L-Arg), 14) Br^- , 15) SO_4^{2-} , 16) NO_2^- , 17) ClO^- , 18) ONOO^- , 19) control (10 μM **GE-Y** without the addition of anything). 1 mM for ClO^- , ONOO^- and amino acid, 100 mM for other cations and anions. Slit width ex = 10 nm and em = 10 nm; temperature 25°C.

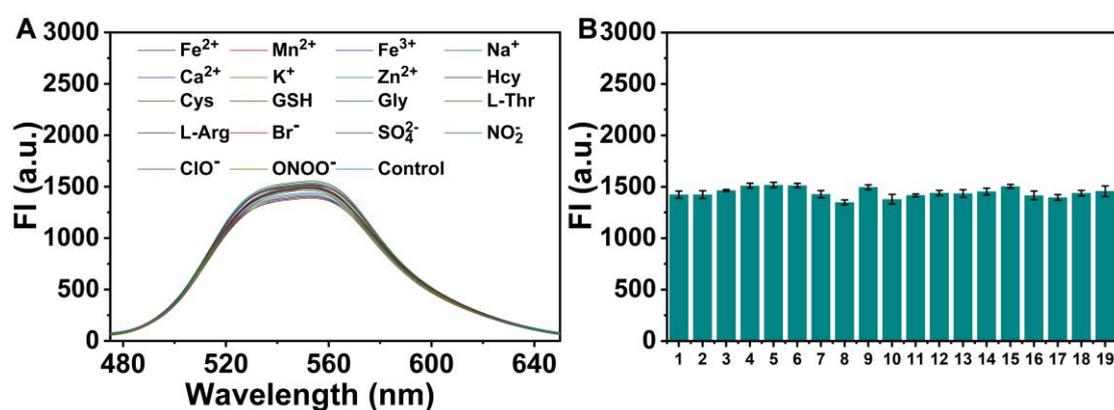


Figure S6. (A) Fluorescence responses of **GE-Y** (10 μM) to different competing species in PBS/glycerin binary solutions (126.80 cP); (B) Histogram of (A) at 550 nm. 1) Fe^{2+} , 2) Mn^{2+} , 3) Fe^{3+} , 4) Na^+ , 5) Ca^{2+} , 6) K^+ , 7) Zn^{2+} , 8) homocysteine (Hcy), 9) cysteine (Cys), 10) glutathione (GSH), 11) glycine (Gly), 12) L-threonine (L-Thr), 13) L-arginine (L-Arg), 14) Br^- , 15) SO_4^{2-} , 16) NO_2^- , 17) ClO^- , 18) ONOO^- , 19) control (10 μM **GE-Y** without the addition of anything). 1 mM for ClO^- , ONOO^- and amino acids, 100 mM for other cations and anions. Slit width ex = 10 nm and em = 10 nm; temperature 25°C.

7. MTT assay for GE-Y

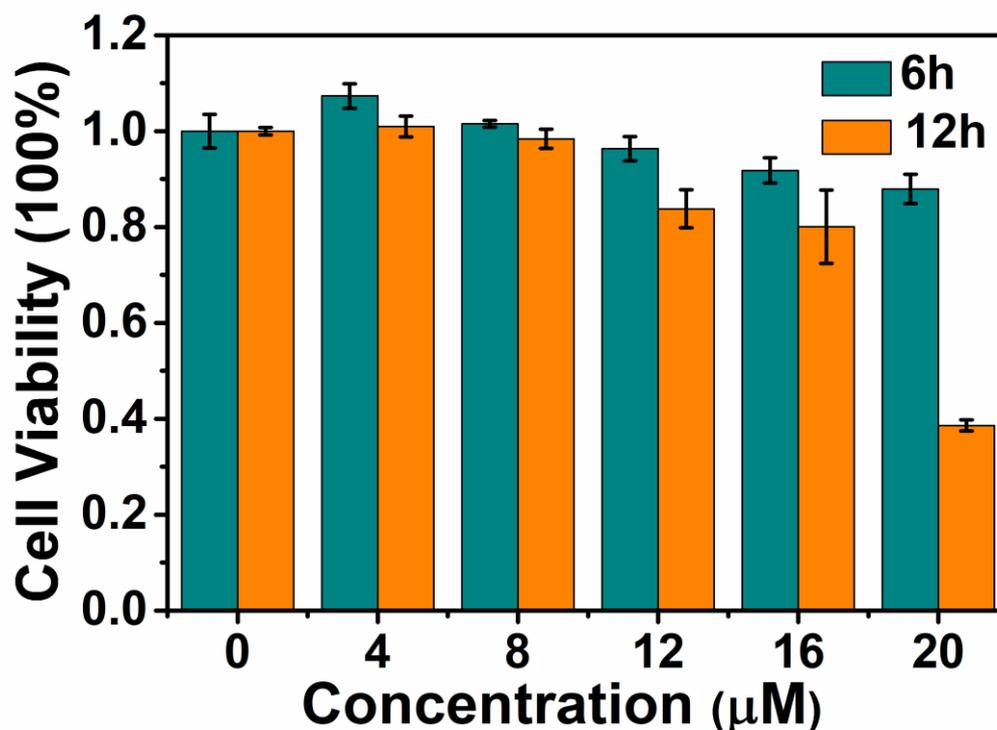


Figure S7. Cell viability of MCF-7 cells stained with different concentrations of GE-Y.

8. Live cell imaging with GE-Y

Co-localization experiment: Cells were co-stained with GE-Y (10 µM) and commercially available ER-Tracker Red (1 µM) for 20 min. Then the cells were washed three times with PBS, and 1 ml DMEM was added to the dish plate. Cells were imaged by Leica TCS SP5 II Confocal Laser Scanning Microscope. Green channel: GE-Y ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520\text{-}560 \text{ nm}$, voltage of PMT: 550 V). Red channel: ER-Tracker Red ($\lambda_{\text{ex}} = 561 \text{ nm}$, $\lambda_{\text{em}} = 600\text{-}640 \text{ nm}$, voltage of PMT: 540 V).

Cell imaging of tunicamycin-induced ER-stress: MCF-7 cells were initially treated with 40 µg/mL tunicamycin for 0, 10, 40 or 70 min, respectively. Followed by the addition of GE-Y into the cells at a final concentration of 10 µM and continued to incubate for another 20 min. Then the cells were washed three times with DMEM medium, and 1 ml DMEM was added to the dish plate. For the control group, the cells were not treated with GE-Y, other procedure is the same. The fluorescence images were

obtained using Leica TCS SP5 II Confocal Laser Scanning Microscope. Green channel:
 $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520\text{-}560 \text{ nm}$, voltage of PMT: 450 V.

Imaging of autophagy: MCF-7 cells were incubated with Hanks' Balanced Salt Solution (autophagy cells) or DMEM for 40 minutes, and then **GE-Y** (10 μM) were added for staining for 20 minutes. Then the cells were washed three times with DMEM medium, and 1 ml DMEM was added to the dish plate. The fluorescence images were obtained using Leica TCS SP5 II Confocal Laser Scanning Microscope. Green channel:
 $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520\text{-}560 \text{ nm}$, voltage of PMT: 450 V.

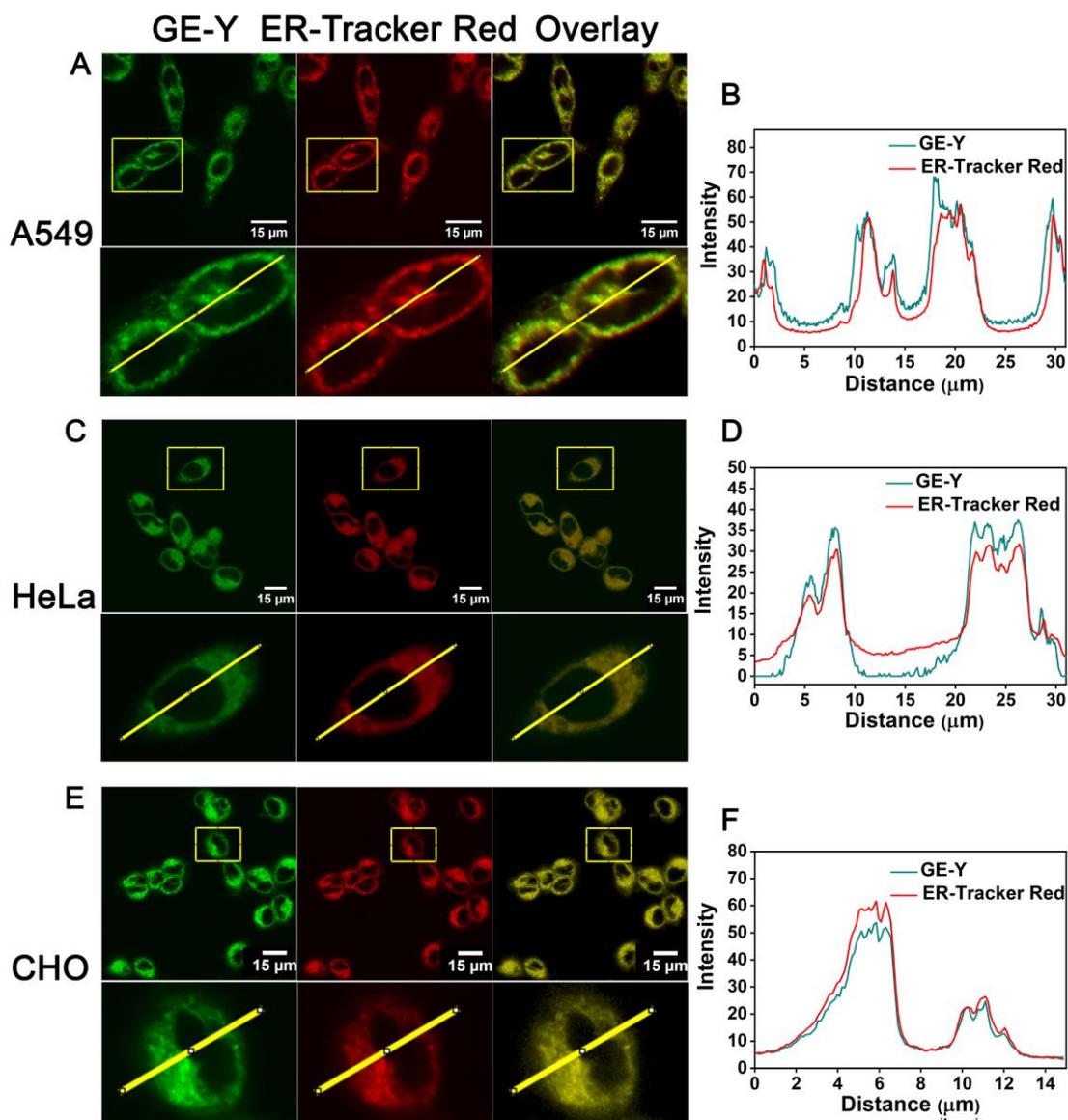


Figure S8. Colocalization cell images of **GE-Y** in (A) A549 cells, (C) HeLa cells, (E) CHO cells. Cells co-stained with 10 μM **GE-Y** and 1 μM ER-Tracker Red for 20 min. Green channel ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520\text{-}560$ nm): **GE-Y**. Red channel ($\lambda_{\text{ex}} = 561$ nm, $\lambda_{\text{em}} = 600\text{-}640$ nm): ER-tracker Red. Up lane: the whole images; down lane: enlarged regions of interest (ROI) of up lane. Scale bar is 15 μm . (B)(D)(F) Image J software was used for fluorescence intensity profile of down lane's ROI analysis (panel B is for panel A, panel D is for panel C, panel F is for panel E). The cyan line was from **GE-Y**, and the red line was from ER-Tracker Red.

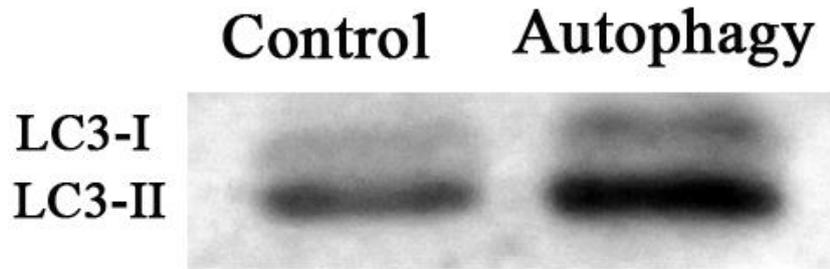


Figure S9. LC3-I and LC3-II protein in live MCF-7 cells analyzed using Western blot analysis. The cells were incubated with Hanks' Balanced Salt Solution (Autophagy panel) or DMEM (Control panel) for 40 minutes, and then **GE-Y** (10 μ M) were added for staining for 20 minutes.

Table S3. Gray value of LC3-I and LC3-II protein in live MCF-7 cells in **Figure S9**. The gray values were obtained by using the ImageJ software.

Group	Control	Autophagy
Gray value of LC3-I	2234	2937
Gray value of LC3-II	4422	9541
LC3-II/ LC3-I	1.98	3.25

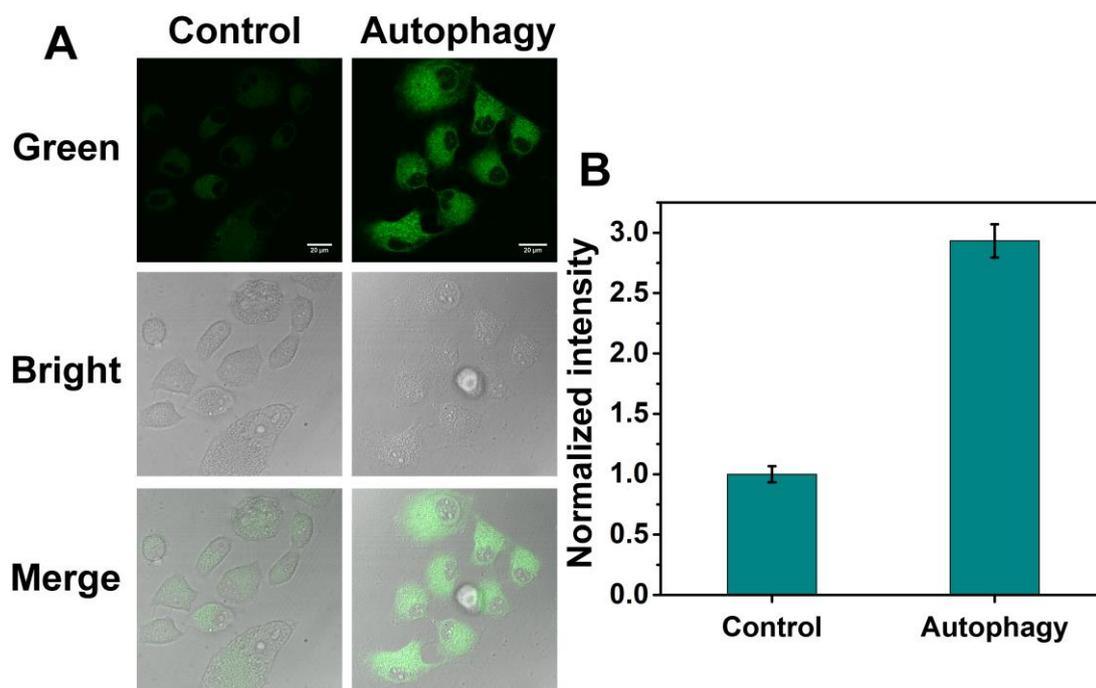


Figure S10. (A) Fluorescence imaging of autophagy in live MCF-7 cells with **GE-Y**. The cells were incubated with Hanks' Balanced Salt Solution (autophagy panel) or DMEM (control panel) for 40 minutes, and then **GE-Y** (10 μ M) were added for staining for 20 minutes. Herein, Hanks' Balanced Salt Solution was used for the stimulus of autophagy. λ_{ex} =488 nm, λ_{em} =520-560 nm, voltage of PMT: 450 V. Scale bar, 20 μ m. (B) Semiquantitative analysis of averaged fluorescence intensity of (A). error bar represents s.d..

9. ^1H and ^{13}C spectra

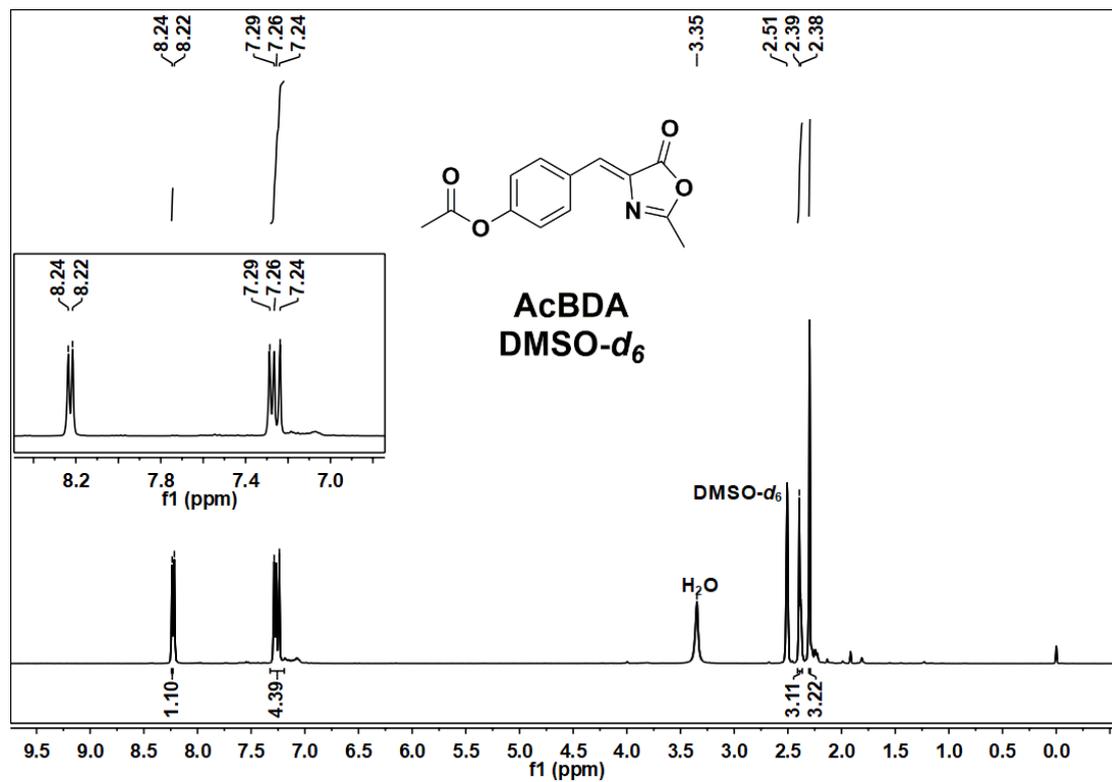


Figure S11. ^1H NMR spectra of AcBDA.

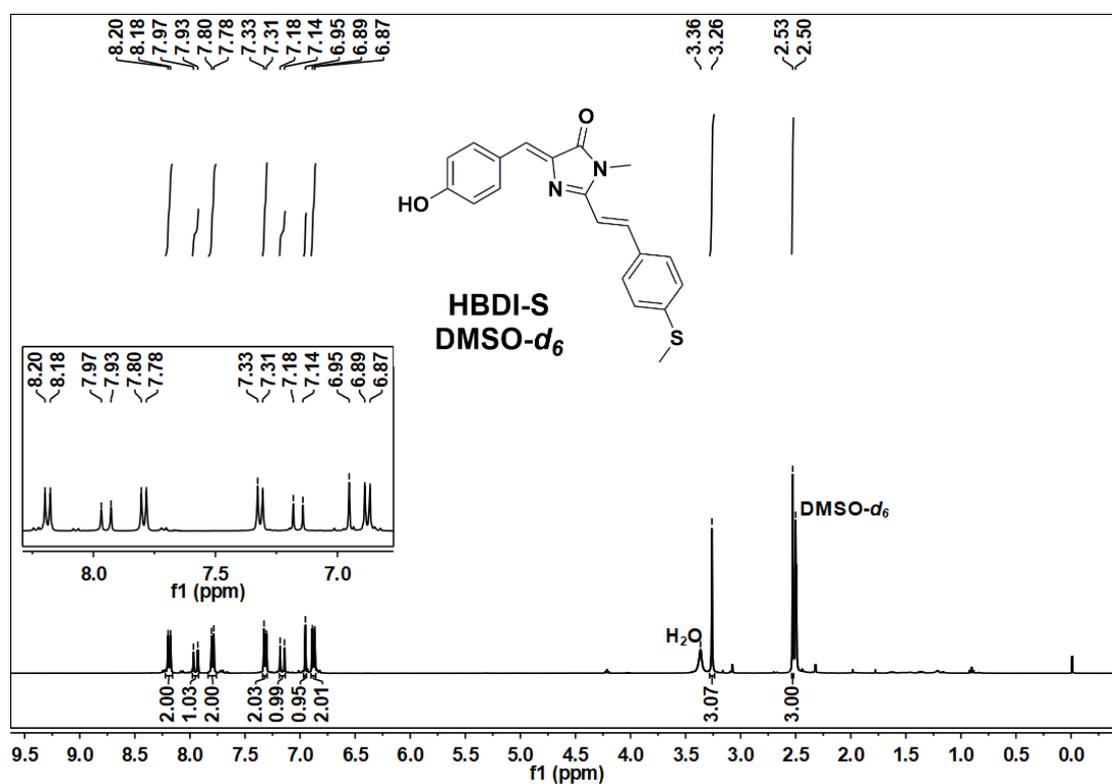


Figure S12. ^1H NMR spectra of HBDI-S.

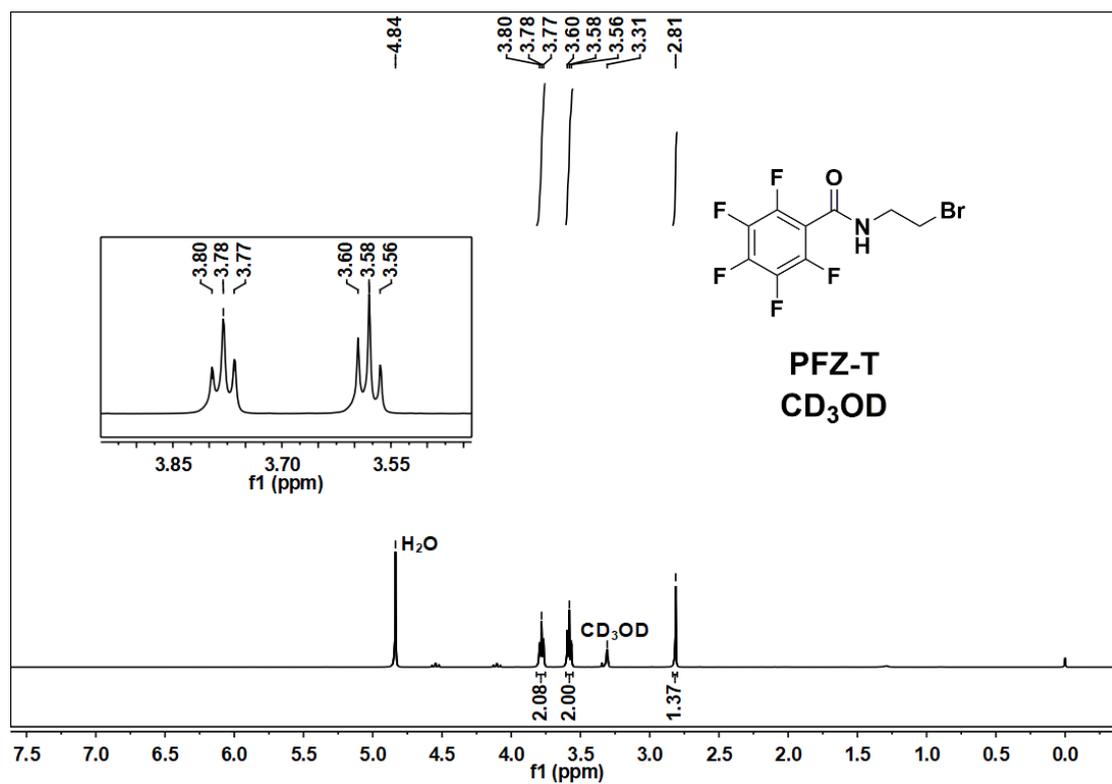


Figure S13. ¹³C NMR spectra of HBDI-S.

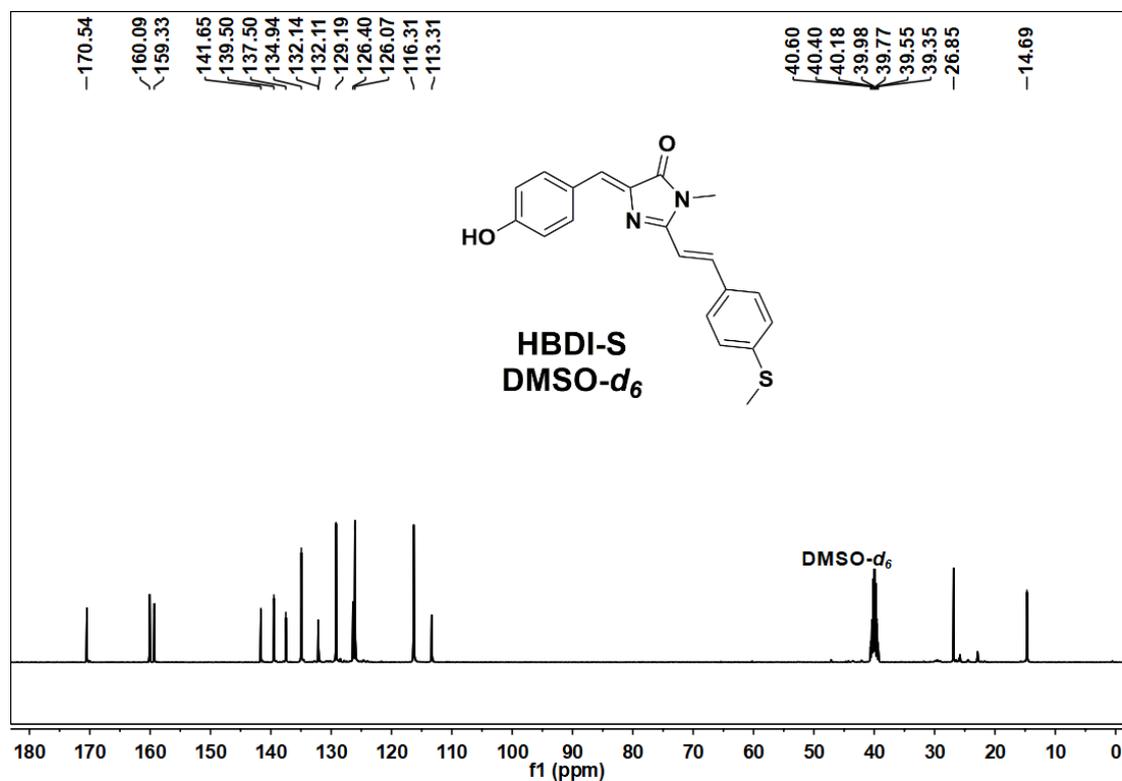


Figure S14. ¹H NMR spectra of PZF-T.

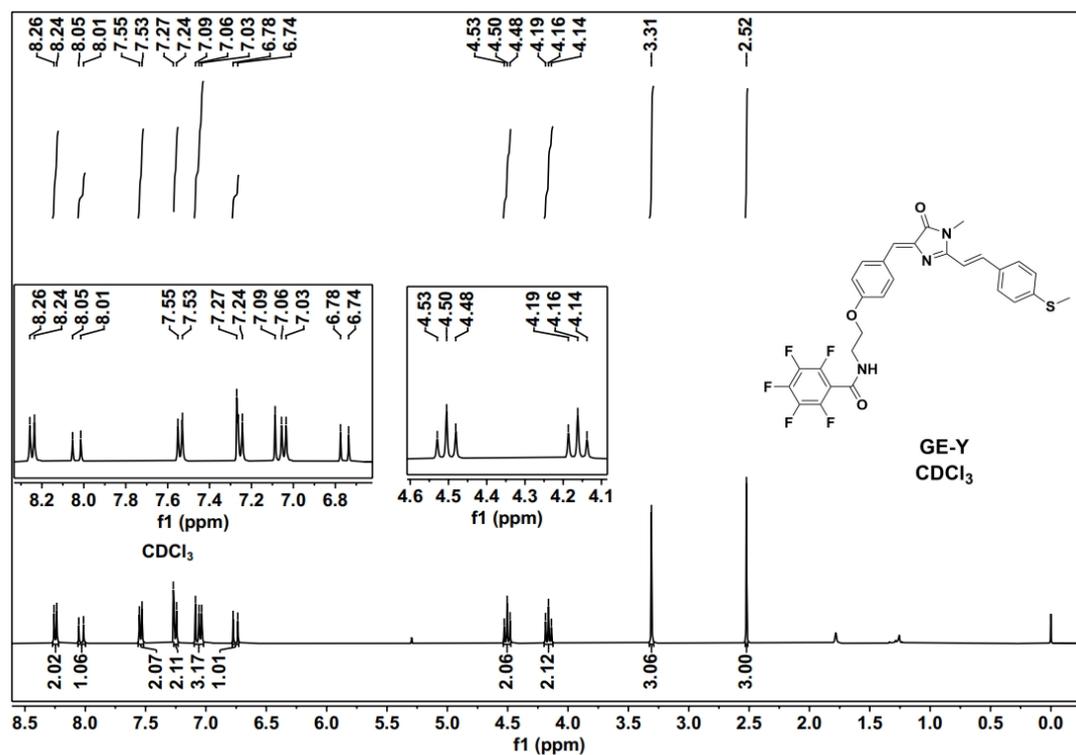


Figure S15. ¹H NMR spectra of GE-Y.

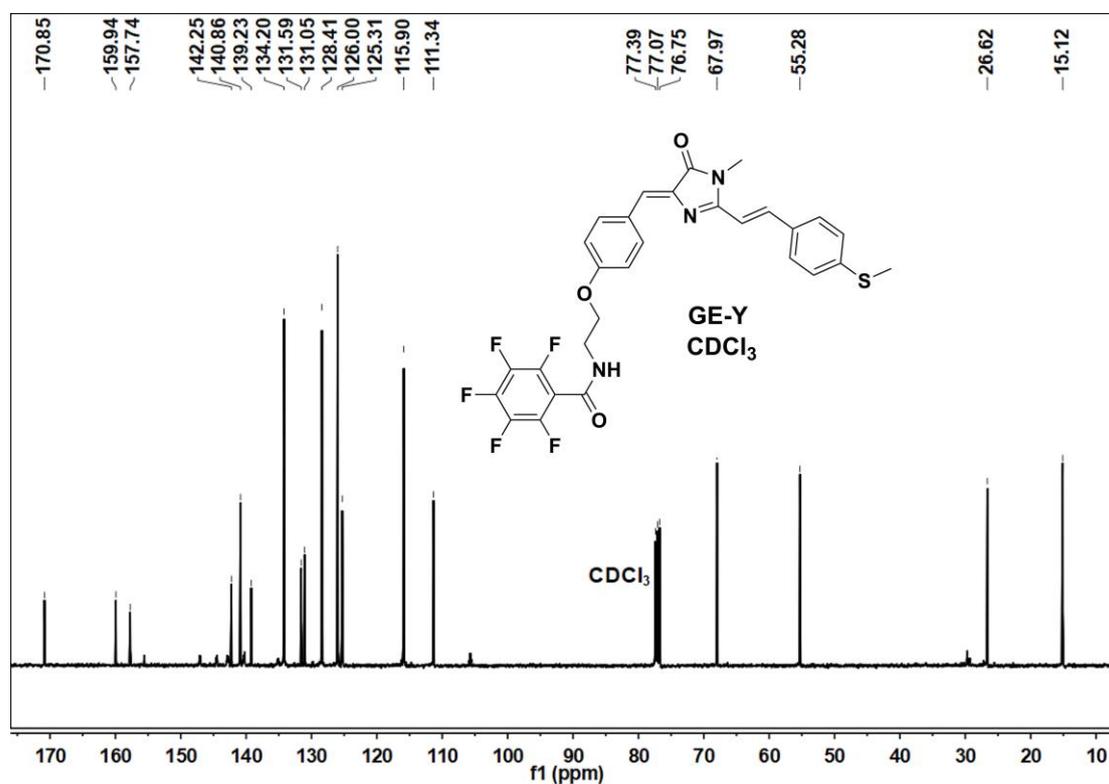


Figure S16 ¹³C NMR spectra of GE-Y.

10. HRMS of GE-Y

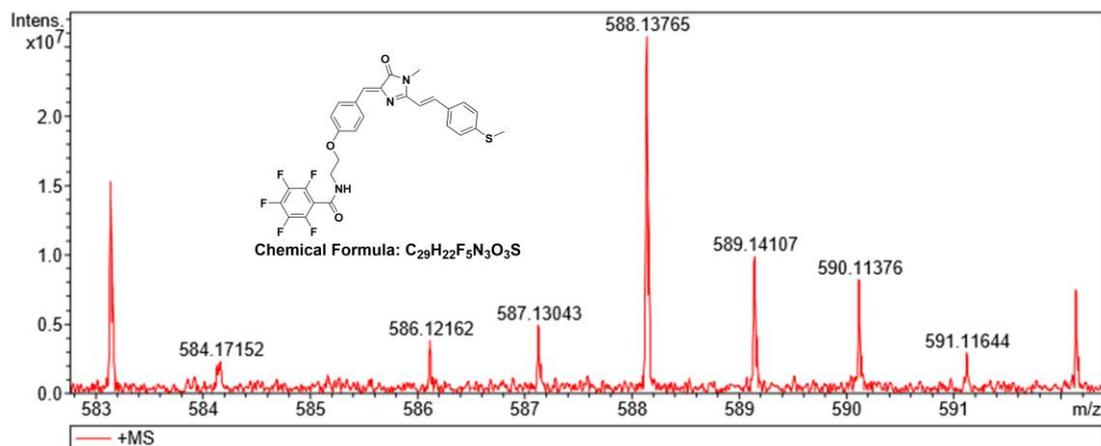


Figure S17. HRMS of GE-Y.

11. References

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