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Electronic Supporting Information (ESI)

A dual-labeling fluorescent probe to track lysosomal polarity and endoplasmic reticulum dynamics during ferroptosis

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1. Experimental section

1.1 Reagents and instruments

Reagents. 5-(Dimethylamino) naphthalene-1-sulfonyl chloride (DNSCl), 6-Carboxytetramethy -lrhodamine (6-TAMRA), N,N diisopropylethyl a mine (DIEA), O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyl uronium hexafluorophosphate (HATU), and BODIPY 493/503 were purchased from Bide Pharmatech Ltd. Cell Counting Kit (CCK-8), LysoTracker Green DND-26, and Mito-Tracker Green were bought from Yeasen Biotechnology (Shanghai) Co., Ltd. ER-Tracker Red, ER-Tracker Green, and Tubulin-Tracker Deep Red were purchased from Beyotime Biotech Inc, Shanghai, China. LysoTracker Deep Red was purchased from Maokang Biotech. pDsRed2-ER (P0141) was obtained from MiaoLingBio,China. The organic solvents were used without purification.

Instruments. High-resolution mass spectra (HR-MS) were obtained using an orbitrap Mass Spectrometer (Q Exactive Plus, Thermo Fisher Scientific). ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AV II NMR spectrometer. Photoluminescence spectra were performed on a fluorescence spectrophotometer (LS-55, Perkin Elmer). Absorbance values of 96-well plate were recorded from a microplate reader (iMark, ThermoFisher). Fluorescent images were performed on a confocal microscopy (OLYMPUS SpinSR10).

1.2 Preparation of multiple analytes

Hypochlorite ion (ClO^-) was prepared by diluting NaClO solution in deionized water. Hydrogen peroxide (H_2O_2) was made by dilution of H_2O_2 solution (30%) with deionized water. Nitric oxide (NO) was generated from the $Na_2[Fe(CN)_5NO]$ $2H_2O$ stock solution. Peroxynitrite ion ($ONOO^-$) was prepared by simultaneous addition of $NaNO_2$, HCl and H_2O_2 solution into NaOH solution at 0 °C. The concentration of $ONOO^-$ was estimated with Lambert-Beer law (the molar absorption coefficient is 1670 $M^{-1}cm^{-1}$) by measuring the absorbance value at 302 nm. Nitrite ion (NO_2^-) was generated from $NaNO_2$. Superoxide ($O_2^{\cdot-}$) was obtained from a saturated solution of KO_2 in DMSO. Hydroxy radical (·OH) was prepared by addition of $FeCl_2$ solution into H_2O_2 solution through Fenton reaction. The stock solutions of biothiol and metal ion were prepared from glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and glutamate (Glu), $CuSO_4$, $MgCl_2$, $ZnCl_2$, $FeCl_3$, and $CaCl_2$.

1.3 Spectroscopic measurements

Probe DRHB was dissolved in dimethyl sulfoxide (DMSO) for a stock solution (1 mM). The final test

solution of DRHB and DEA (10 μM) was prepared in various organic solvents (toluene, ethyl acetate, acetonitrile, dimethylformamide, isopropanol, acetone, dioxane), the dioxane-water mixtures with different polarity (0% - 30% H_2O, v/v), PBS buffer with different pH values (pH 4.0 - pH 8.0), and analyte-containing PBS buffer (ROS, RNS, RSS, and metal ions). The fluorescence spectra of these resulting solutions (containing 1% DMSO) were tested at room temperature.

1.4 Synthesis and characterization

The dansyl ethylenediamine (DEA) was prepared by following methods reported in the literature.[1] 6-TAMRA (215 mg, 0.5 mmol), DEA (147 mg, 0.5 mmol), DIEA (0.2 mL), and HATU (190 mg, 0.5 mmol) were dissolved in 200 mL CH_3CN, then the solution was stirred overnight at room temperature. The solvent was evaporated in vacuum, and the crude product was purified by silica gel column chromatography with the eluent as CH_2Cl_2/CH_3OH (10/1, v/v) to give the red product DRHB (141 mg) was obtained. Yield: 40 %. ^1H NMR (800 MHz, d6-DMSO, δ): 8.64 (t, J = 5.7 Hz, 1H), 8.40 (dd, J = 25.5, 6.2 Hz, 2H), 8.22 (t, J = 7.7 Hz, 2H), 8.09 – 8.02 (m, 4H), 7.57 – 7.53 (m, 2H), 7.49 – 7.46 (m, 1H), 7.17 (d, J = 7.5 Hz, 1H), 6.53 – 6.51 (m, 4H), 3.21 (dd, J = 12.6, 6.4 Hz, 2H), 2.94 (s, 12H), 2.92 (dd, J = 12.8, 6.4 Hz, 2H), 2.79 (s, 6H). ^{13}C NMR (201 MHz, d6-DMSO, δ): 168.73, 165.22, 152.63, 152.46, 151.78, 140.78, 139.78, 136.30, 134.91, 129.89, 129.51, 128.98, 128.66, 128.25, 127.77, 125.10, 124.00, 122.77, 119.40, 115.52, 109.55, 106.10, 98.43, 56.50, 55.38, 45.50, 41.94. HRMS (ESI): calcd for $C_{39}H_{39}N_5O_6S$ $[M + H]^+$ 706.2655, found 706.2687.

Scheme S1. The synthetic route of the probe DRHB.

1.5 Cell culture

U2OS cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37℃ in a humidified 5% CO_2 incubator.

1.6 Cell imaging and colocalization experiments

U2OS Cells (1×10^4 cells/well) were seeded in glass-bottom 24-well plates and incubated in a CO_2 incubator. The DRHB working solution (10 μM) was prepared by diluting DRHB stock solution (5 mM, DMSO) in DMEM medium containing 10% FBS. After 24 h cellular growth, the cells were incubated with DRHB working solution for 30 min and washed with PBS buffer. Then, LysoTracker Green DND-26 (LYG, 500 nM), ER-Tracker green (ERG, 1 μM), Mito-Tracker Green (MTG, 100 nM), BODIPY 493/503 (LDs, 1 μM), or Tubulin-Tracker Deep Red (Tub-DR, 1 μM) were added and incubated for another 30 min. After that, the washed cells were treated with DMEM medium containing erastin/oleic acid for different time and imaged.

Fluorescence images were performed on an ultrahigh-resolution spinning-disk confocal microscopy (OLYMPUS SpinSR 10) equipped with a sCMOS camera (ORCA-Fusion, 2304×2304) using an oil-immersion objective lens (100×OHR, NA.1.5). Especially, the Olympus super-resolution imaging mode (at least 120 nm resolution) was used in the photostability experiments and time-lapse imaging experiments for dynamic tracking of lysosomes, the ER, and LDs in living cells. Fluorescence images were acquired and processed by CellSense software. The average fluorescence intensity and pearson correlation coefficient (PCC) were analyzed by the ImageJ (FiJi) software.

1.7 Lysosomal polarity imaging experiments

U2OS Cells (1×10^4 cells/well) were seeded in glass-bottom 24-well plates and incubated in a CO_2 incubator. The cuproptosis inducer elesclomol-Cu (1 mM, DMSO) was prepared by mixing the elesclomol and $CuCl_2$ stock solutions with equivalent molar amount for 5 minutes. After 24 h cellular growth, the cells were incubated with DRHB working solution for 30 min and washed. Then, the DRHB-stained cells were treated with tunicamycin, erastin, elesclomol-Cu, or EBSS (Earle's Balanced Salt Solution) for 4 h and imaged. The ratiometric images (F_{red}/F_{green}) were processed by CellSense software. The lysosomal area and mean ratio in lysosomes were analyzed by the ImageJ (FiJi) software.

1.8 Cytotoxicity Assays

The toxicity of the probe DRHB toward living U2OS cells was tested using a Cell Counting Kit (CCK-8) assay. Live cells (1×10^4 cells/well) were seeded in 96-well plates and incubated for 24 h. The cell culture medium containing gradient concentrations (0-500 μM) of DRHB replaced the inoculation medium for 24 h incubation. Then, the cells were washed and incubated with CCK8-containg medium for 2 h. Finally, the absorbance of each well was read at 450 nm by a microplate reader.

2. Existing ER/lysosome fluorescent probes

2.1 Reported lysosomal polarity probes

Table S1. Comparison of reported polarity-sensitive probes for lysosomal polarity.

Probe structure	$\lambda_{ex}/\lambda_{em}$ (nm)	Ratio response	Ferroptosis	Applications	References
Lyso-OC	375/461	No	No	Real-time monitoring of autophagy in MCF-7 cells	*Chem. Commun.*, 2017, **53**, 3645–3648.
NOH	410/474 479/552	Yes	No	Lysosomal polarity in MCF-7 cells is equivalent to DCM	*Biomaterials*, 2018, **164**, 98–105.
MND-Lys	411/537	No	No	Lysosomal polarity changes in zebrafish, inflammatory and obese mice	*Chem. Commun.*, 2019, **55**, 11063–11066.
CPM	453/550	No	No	Lysosomal polarity for cancer diagnosis in organ, animal, and clinical tissue	*Chem. Commun.*, 2019, **55**, 4703–4706.
Lyso-OSC	372/515	No	No	Lysosomal polarity changes during autophagy in MCF-7 cells.	*Chin. Chem. Lett.*, 2020, **31**, 447–450.
Lyso-TPFP	365/532	No	No	Lysosomal polarity variations in oxidative stress and autophagy in LM-3 cells	*Anal. Chim. Acta*, 2020, **1136**, 34–41.
KSLP1	594/686	No	No	Lysosomal polarity alterations in senescent MRC5 cells and *C. elegans*.	*Sens. Actuators B Chem.*, 2020, **319**, 128302.
DCM-ML	--/655 --/705	Yes	No	Lysosomal polarity difference in normal/cancer cells and changes during zebrafish development.	*Sens. Actuators B Chem.*, 2021, **345**, 130397.
Lyso-NRB	412/544 550/580	No	No	Lysosome polarity fluctuation during autophagy in HepG2 cells and zebrafish	*J. Mater. Chem. B*, 2022, **10**, 4285–4292.
NCIC-Pola	480/692	No	No	Lysosome polarity changes during autophagy in BV-2 cells and mouse brain	*Biosens. Bioelectron.*, 2023, **237**, 115453.
DRHB	340/505 544/585	Yes	Yes	Increased Lysosomal polarity during ferroptosis in U2OS cells	This work

-- Not mentioned.

2.2 Commercial ER-Trackers

CLogP: 3.5

CLogP: 6.3

CLogP: 8.5

ER-Tracker Blue-White DPX ER-Tracker Green ER-Tracker Red

Scheme S2. Molecular structure of commercial endoplasmic reticulum probes.

2.3 Reported ER probes for ferroptosis model

Table S2. Comparison of reported endoplasmic reticulum probes for ferroptosis model.

Probe structure	ClogP	$\lambda_{ex}/\lambda_{em}$ (nm)	Response	Micro-structure tracking	Translocation to newborn LDs	Applications	References
Ir-ER	6.2	405/530	Viscosity	No	--	ER viscosity increases	*Chem. Commun.*, 2021, **57**, 5040-5042
DSPI-3	11.9	360/528 560/620	pH and viscosity	No	--	ER viscosity increases	*Anal. Chem.*, 2022, **94**, 6557–6565
PV1	5.0	481/616	Viscosity	No	--	ER viscosity increases	*Anal. Chim. Acta*, 2022, **1232**, 340454
JER	6.8	540/645	Viscosity	No	--	ER viscosity increases from 159 cP to 213 cP	*Chem. Commun.*, 2023,**59**, 1769-1772
BNS	6.2	447/590	Polarity ("ON" in low-polarity)	No	--	ER polarity increases	*Anal. Chim. Acta*, 2023, **1275**,341571.
SBD-CH	4.2	431/543	Polarity ("ON" in low-polarity)	No	--	ER polarity Increases	*Chem. – Eur. J.*, 2024, e202401285.
BODIQPy-TPA	7.1	488/640	Polarity ("ON" in low-polarity)	No	--	Induction of lipid peroxidation and ferroptosis.	*J. Med. Chem.*, 2024, **67**, 1900–1913.
DRHB	1.7	544/585	Polarity ("OFF" in low-polarity)	Yes	No	Dynamic fragmentation, rearrangement, and ER-phagy of ER micro-structure	This work

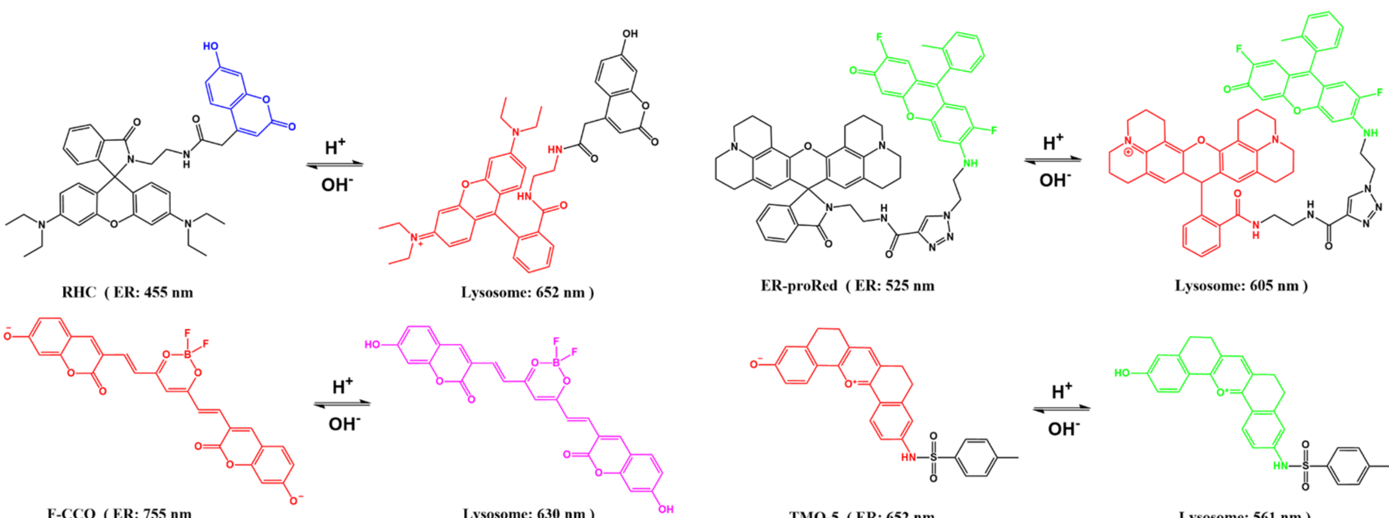
-- Not mentioned.

2.4 Reported ER/lysosome dual-labeling probes

Table S3. Comparison of reported fluorescent probes for ER/lysosome dual-labeling.

Probe	ClogP	Polarity detection	Translocation to newborn LDs	Ferroptosis / oleic acid	Applications	References
RHC	7.1	No	--	No/No	Monitoring of ER-phagy and regeneration in thapsigargin model	*Anal. Chem.*, **2022**, 94, 5173–5180.
ER-proRed	10.4	No	--	No/No	Imaging of starvation-induced ER-phagy	*Autophagy*, **2023**, 19, 2015–2025.
TMO-5	7.2	No	--	No/No	No	*Anal. Chem.*, **2024**, 96, 876–886.
F-CCO	4.2	No	--	No/No	pH changes in dexamethasone-induced apoptosis	*Dyes and Pigments*, **2024**, 224, 111997.
DRHB	1.7	Yes	No	Yes/Yes	Monitoring of lysosomal polarity changes and ER dynamics in ferroptosis	This work

-- Not mentioned.



Scheme S3. The structure and sensing mode of reported ER/lysosome dual-labeling fluorescent probes.

3. Supplemental Figures

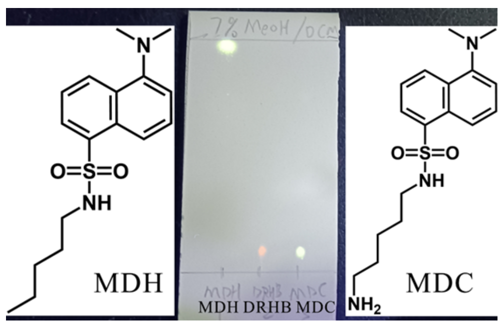


Fig. S1 The comparison of molecular polarity of probe DRHB, monodansylpentane (MDH), and monodansylcadaverine (MDC) via thin layer chromatography under 365 nm light, MeOH: DCM = 7: 100. MDC, a commercial autophagosome probe, non-specifically targets lysosomes due to its amino group;[2] MDH, a commercial LDs probe due to its lipophilic alkyl chain.[3]

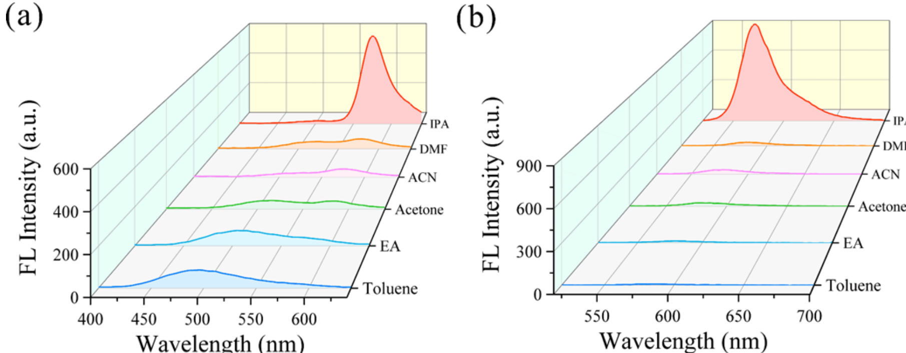


Fig. S2 Emission spectra of probe DRHB in various solvents upon 380 nm excitation (a) and 510 nm excitation (b), respectively.

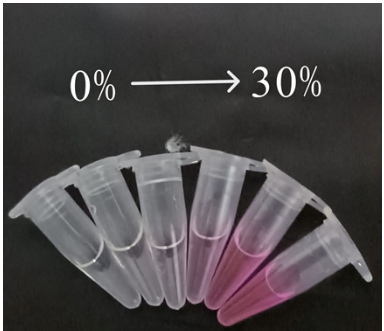


Fig. S3 Photograph of probe DRHB in the mixture of dioxane and water under visible light.

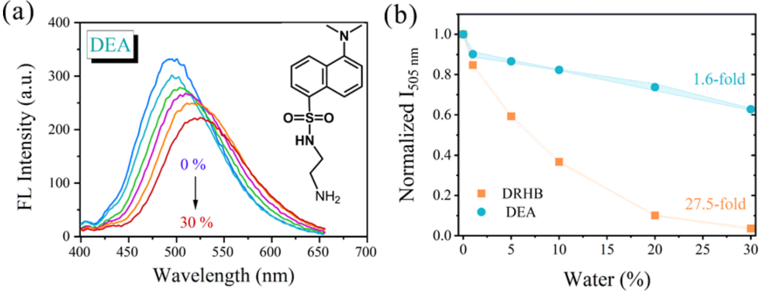


Fig. S4 (a) Fluorescence emission spectra and molecular structure of control probe (DEA, dansyl ethylenediamine) in the mixture of dioxane and water (λex: 380 nm). (b) Comparison of the sensitivity of DEA and probe DRHB toward water contents. Error bars: SD, n = 3.

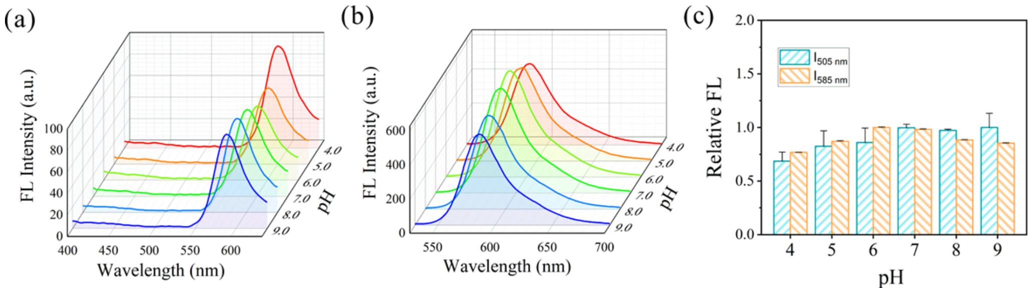


Fig. S5 Emission spectra of probe DRHB (10 μM) in 10 mM PBS buffer with different pH value upon 380 nm excitation (a) and 510 nm excitation (b), respectively. (c) Fluorescence intensity at 505 nm and 585 nm from (a) and (b). Error bars: SE, n = 3.

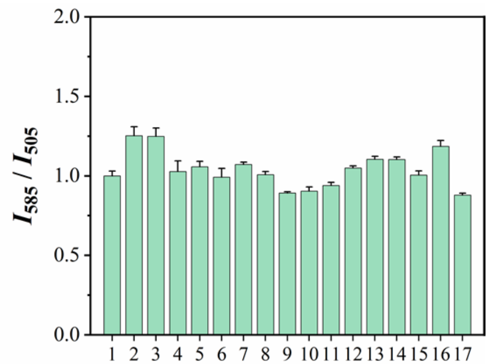


Fig. S6 Fluorescence intensity ratios (I_{585}/I_{505}) of probe DRHB in the presence of the following species in 10 mM PBS buffer. [1]: blank, 500 μM reactive oxygen ([2]: H_2O_2, [3]: ClO^-, [4]: $^{\bullet}OH$, and [5]: $O_2^{\bullet-}$); 100 μM nitrogen species ([6]: $ONOO^-$, [7]: NO_2^-, and [8]: NO); biothiols ([9]: 1 mM GSH, 500 μM [10]: Hcy, [11]: Cys, and [12]: Glu); 200 μM metal ions ([13]: Fe^{3+}, [14]: Mg^{2+}, [15]: Zn^{2+}, [16]: Cu^{2+}, and [17]: Ca^{2+}). Error bars: SE, n = 3.

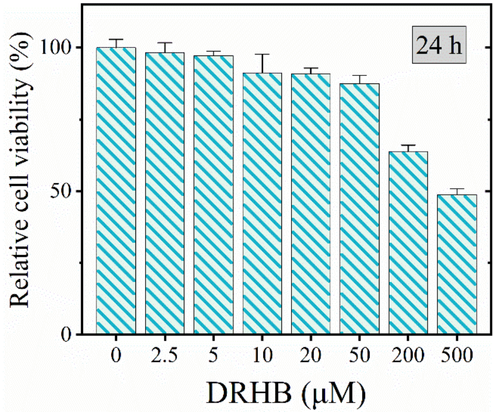


Fig. S7 Cytotoxicity of probe DRHB on U2OS cells. Error bars: SD, n = 4.

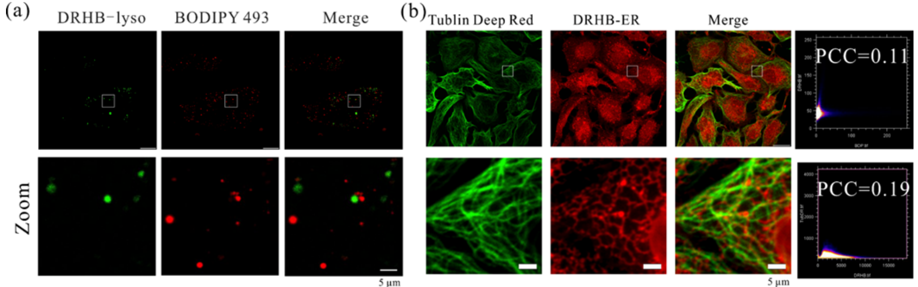


Fig. S8 Colocalization of probe DRHB with BODIPY 493/503 (a) and Tubulin-Tracker Deep Red (b) in U2OS living cells, respectively. DRHB-lyso: λ_{ex} = 405 nm, λ_{em} = 417-477 nm; BODIPY: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; DRHB-ER: λ_{ex} = 561 nm, λ_{em} = 580-654 nm; Tub-DR: λ_{ex} = 640 nm, λ_{em} = 665-705 nm.

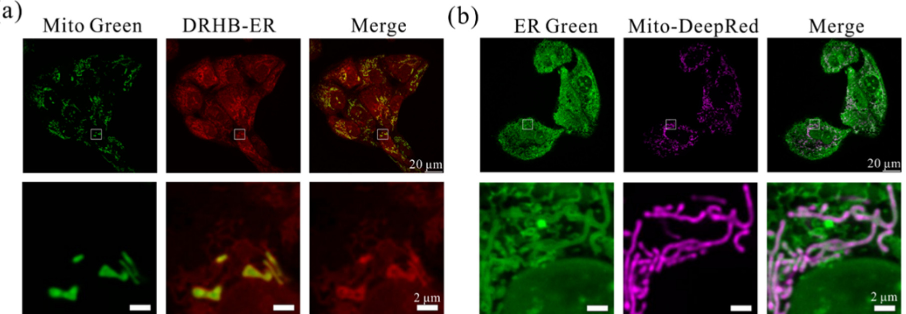


Fig. S9 (a) Colocalization of DRHB with MitoTracker Green. (b) Colocalization of ER Green with MitoTracker Deep Red in U2OS living cells. MTG/ERG: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; DRHB-ER: λ_{ex} = 561 nm, λ_{em} = 580-654 nm; MTDR: λ_{ex} = 640 nm, λ_{em} = 665-705 nm.

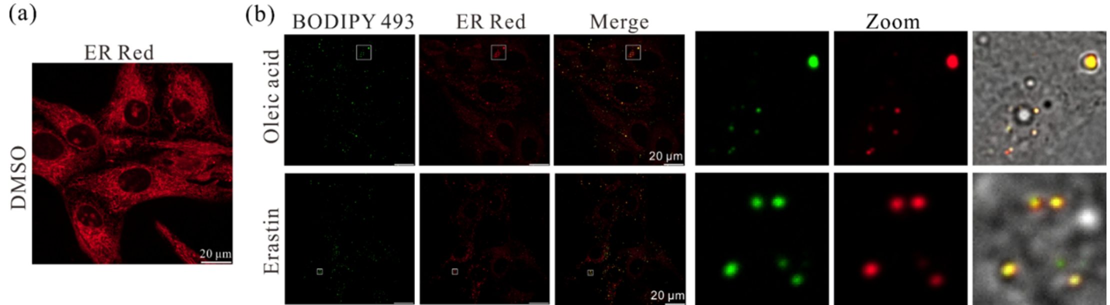


Fig. S10 Colocalization of ER Tracker Red with BODIPY, U2OS cells were prestained and then treated with DMSO (a), oleic acid (500 μM), or Erastin (50 μM, ferroptosis inducer) for 1 h to generate LDs (b). BODIPY: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; ER Red: λ_{ex} = 561 nm, λ_{em} = 580-654 nm.

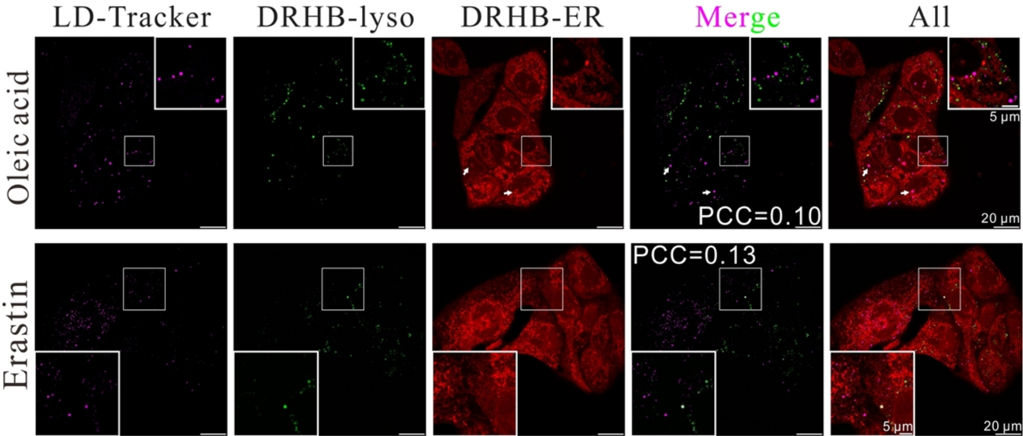


Fig. S11 Colocalization of probe DRHB with BODIPY 493/503, U2OS cells were prestained and then treated with oleic acid (500 μM) or erastin (50 μM) for 4 h. BODIPY: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; ER Red: λ_{ex} = 561 nm, λ_{em} = 580-654 nm.

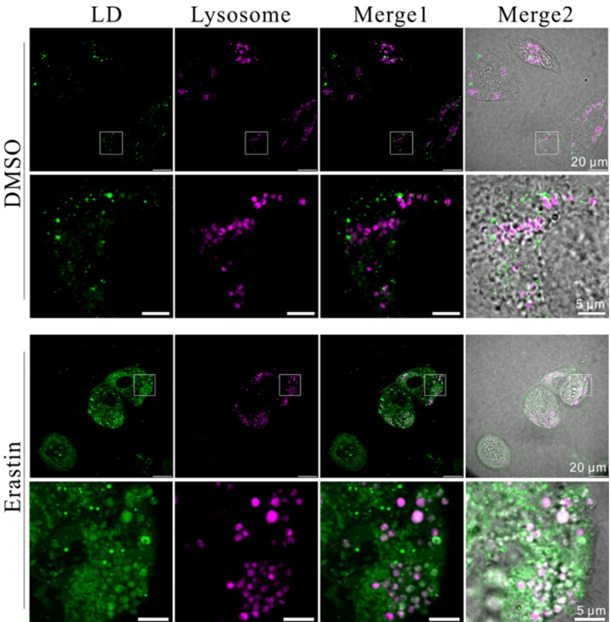


Fig. S12 Validation of the activated lipophagy during ferroptosis via the colocalization of BODIPY493 with Lyso Deep Red. U2OS cells were prestained and then treated with DMSO or erastin (50 μM) for 4 h. BODIPY: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; LYDR: λ_{ex} = 640 nm, λ_{em} = 665-705 nm.

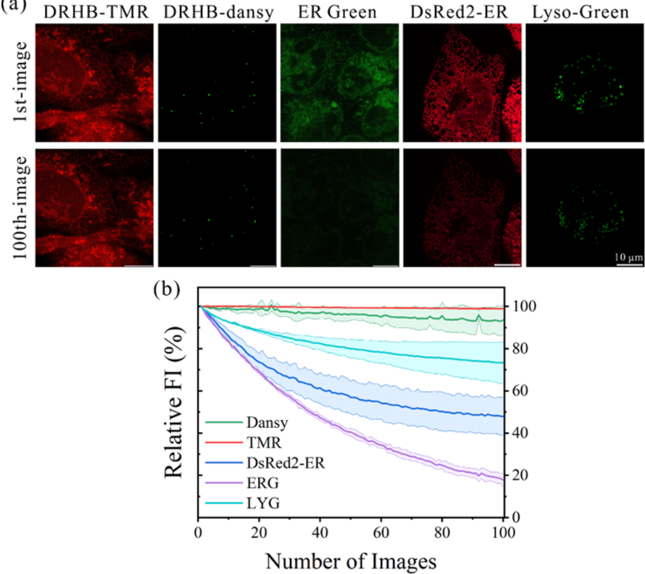


Fig. S13 (a) Photostability of DRHB, ER Green, DsRed2-ER, and Lyso Green in living U2OS cells after continuous super-resolution imaging. (b) Mean fluorescence intensity of (a). DsRed2-ER (a commercially genetic ER probe). Error bars: SD, n = 3. DRHB-TMR/DsRed2-ER: λ_{ex} = 561 nm, λ_{em}=580-654 nm; DRHB-Dansy: λ_{ex} = 405 nm, λ_{em} = 500-550 nm; ERG/ LYG: λ_{ex} = 488 nm, λ_{em} = 500-550 nm.

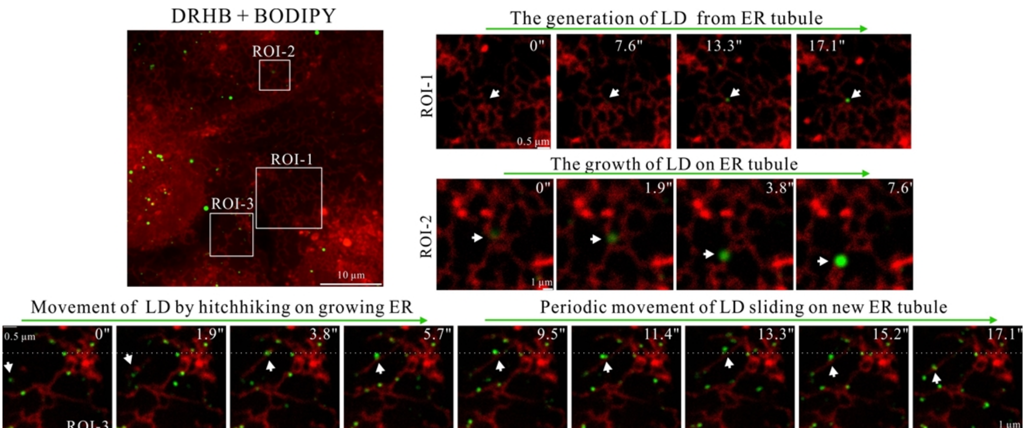


Fig. S14 Time-lapse super-resolution imaging of dynamic ER-LD interactions during ferroptosis. U2OS cells were co-stained with probe DRHB and BODIPY 493/503, then treated with erastin (100 μM) for 1h. BODIPY: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; DRHB-ER: λ_{ex} = 561 nm, λ_{em} = 580-654 nm.

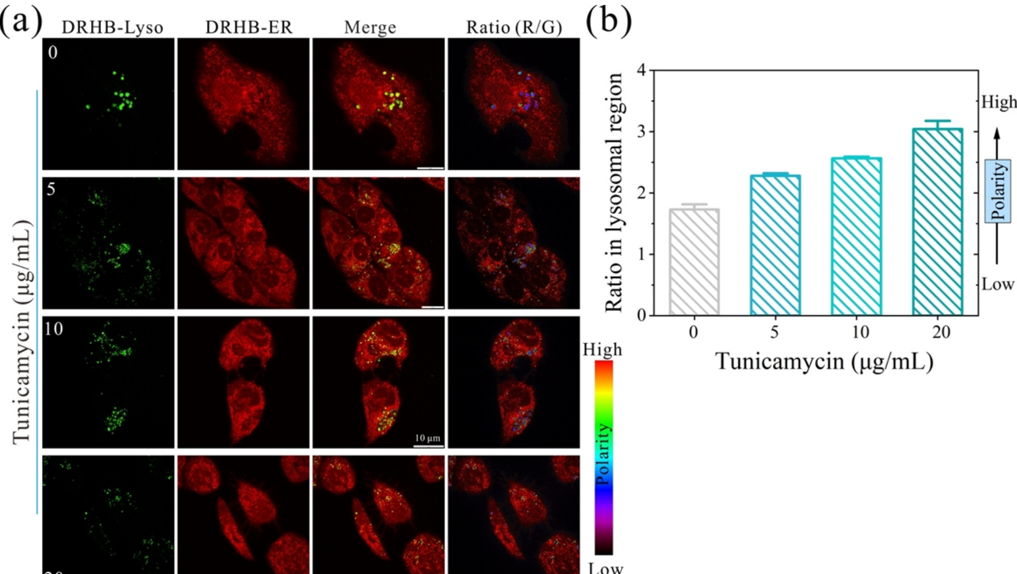


Fig. S15 (a) Fluorescence imaging of polarity in living U2OS cells in the presence of tunicamycin. The cells incubated with DRHB, then treated with tunicamycin (0, 5, 10 or 20 μg/mL, 4 h). (b) The mean ratio (F_{red}/F_{green}) in lysosomes of (a). Error bars: SE, n = 6 independent experiments. DRHB-lyso: λ_{ex} = 405 nm, λem = 500-550 nm; DRHB-ER: λ_{ex} = 561 nm, λ_{em} = 580-654 nm.

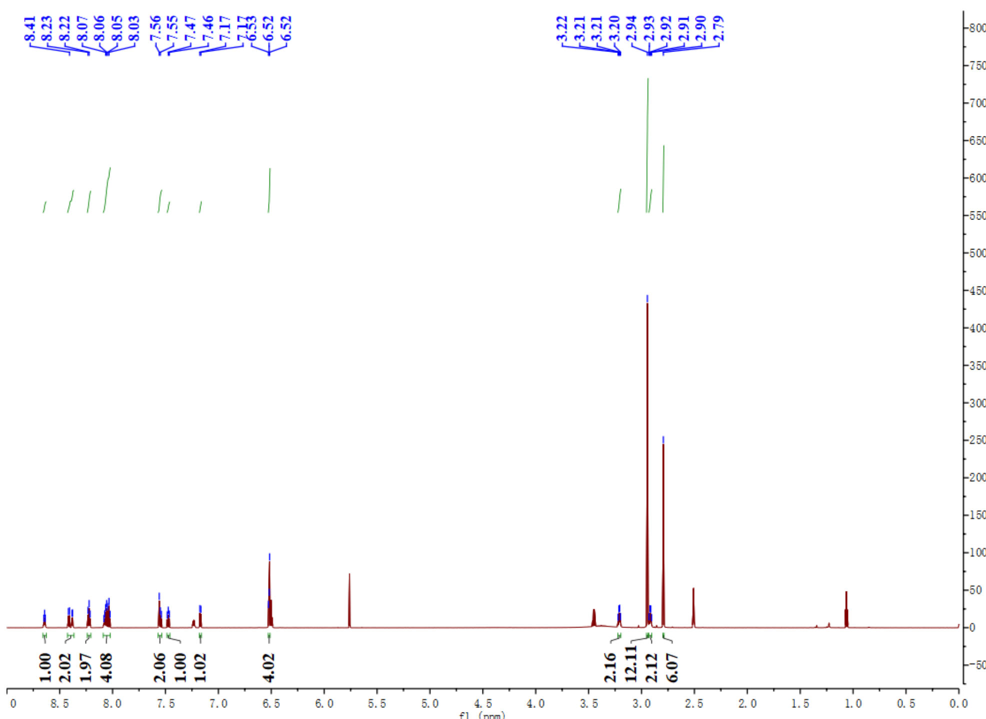


Fig. S16 ^{1}H-NMR spectrum of probe DRHB in d6-DMSO.

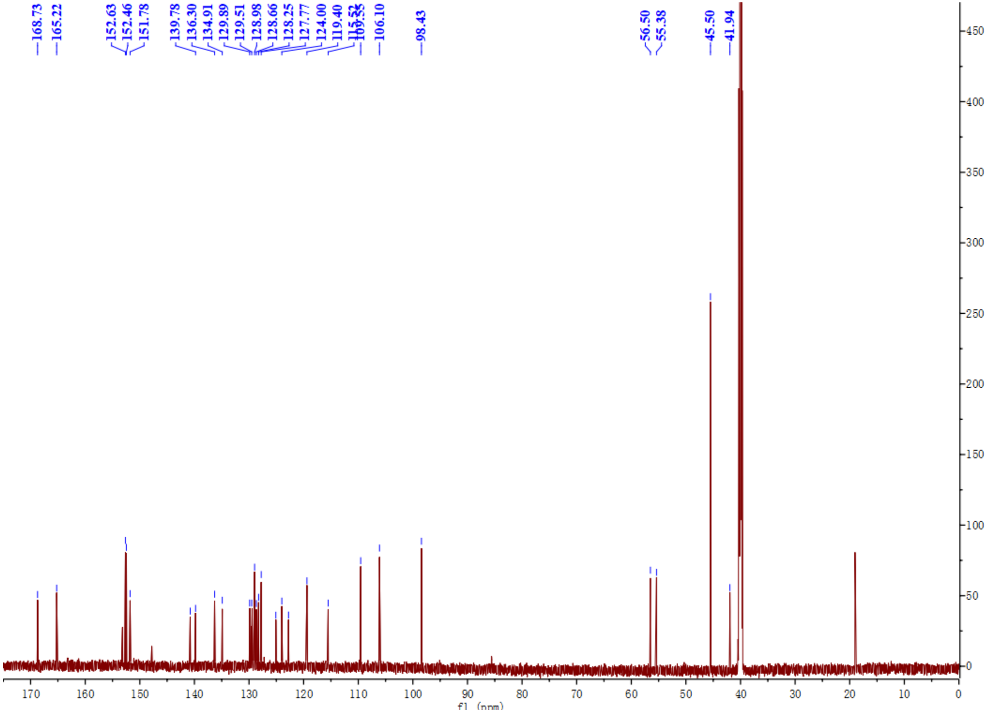


Fig. S17 ^{13}C-NMR spectrum of probe DRHB in d6-DMSO.

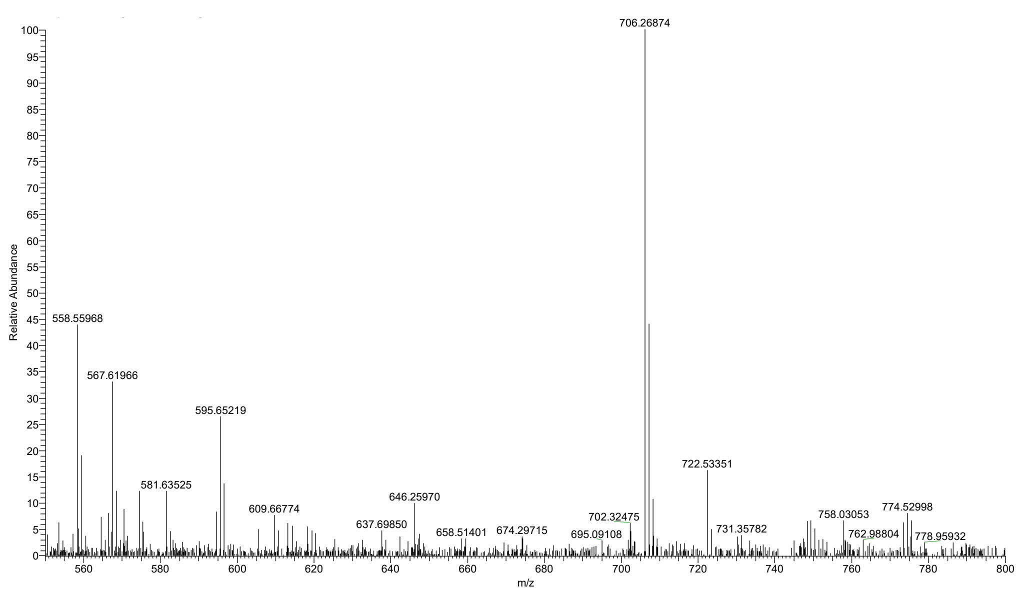


Fig. S18 HR-MS spectrum of probe DRHB.

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