Supporting information

for

Nanoscale morphometric visualization of the endoplasmic reticulum under stress using a bright zinc complex

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Experimental Section

Materials

Chemicals for synthesis

All commercially available reagents and solvents were purchased and used without further purification, including zinc acetate dihydrate, m-anisidine, boron tribromide (BBr_3), 1,4-dibromobutane, Phosphorus(V) oxychloride ($POCl_3$), potassium hydroxide (KOH), dichloromethane, ethanol, dimethylformamide, and acetonitrile. Experiments were conducted under a dry nitrogen atmosphere. All solvents utilized were of spectral grade unless otherwise specified. Thin-layer chromatography (TLC) plates were developed and visualized under a short-wave UV lamp. The reagents were used as received without further purification. For reactions, clean, oven-dried glassware was employed. The progress of the reactions was monitored by TLC using Merck silica gel (60 F254) precoated plates (0.25 mm thickness), and compound spots were visualized with the naked eye under UV light (365 or 254 nm). The crude product obtained was purified by column chromatography using Merck silica gel (60-120 mesh). A mixture of hexane and ethyl acetate was used as the mobile phase for elution.

Chemicals for cellular staining and maintenance

All chemicals were used without further purification. ER-Tracker™ Blue-White DPX, Mito-Tracker™ Green were purchased from Invitrogen (Thermo Fisher Scientific, India). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin (10,000 units/mL) were all purchased from Gibco (Thermo Fisher Scientific, India). All fluorescent dyes were used following the product manuals.

Characterization

Mass spectrometry

Mass spectra were predicted with the support of ChemDraw 20.1.1, and high-resolution electrospray ionization (ESI) mass spectra were recorded on a Bruker impact-HD spectrometer with a quadrupole time-of-flight mass analyzer.

Nuclear magnetic resonance spectroscopy

^{1}H NMR spectra were recorded on a JEOL JNM-ECX500 spectrometer operating at 500 MHz, using tetramethylsilane (TMS) as an internal standard in $CDCl_3$ and DMSO-d_6.

UV-visible and steady-state fluorescence

The UV-visible spectrum of the Zn-complexes was recorded on a Shimadzu UV-Vis 2450 spectrophotometer using a 10 mm path-length quartz cuvette. The Agilent Cary Eclipse Fluorescence spectrometer was used to record the steady-state fluorescence of the samples.

Fluorescence lifetime spectroscopy

The fluorescence lifetime decays were assessed using the Horiba Scientific DeltaFlex TCSPC system with pulsed LED sources. A 560 nm pulsed Nano LED was utilized for excitation. For spectral value deconvolution, Ludox was employed to measure the instrument response function (IRF). Data acquisition was performed using Data Station software, and the data were analyzed using DAS 6 analysis software from Horiba.

Cleaning of the glass coverslip

For single-molecule fluorescence studies, glass coverslips require ultra-high purity and therefore cannot be cleaned using conventional methods. A freshly prepared piranha solution (100 mL, 3:1 v/v

H_2SO_4:H_2O_2) was used for cleaning. Approximately 25-30 glass coverslips were individually immersed in the piranha solution with gentle shaking and incubated for 0.5-1 h. After treatment, the piranha solution was carefully discarded, and the coverslips were rinsed with deionized water 3-4 times. The coverslips were then sonicated for 15 min, followed by another 3-4 rinses with deionized water. This cleaning cycle was repeated four times in total. The thoroughly cleaned coverslips were subsequently used for single-molecule fluorescence experiments.

Single-molecule fluorescence spectroscopy

The Zn complex was spin-coated onto pre-cleaned glass coverslips for the acquisition of single-molecule fluorescence time traces. Excitation was performed using a 532 nm diode laser with a maximum output power of 50 mW. Emission from the Zn complex was collected using a 630/69 nm bandpass filter. An oil-immersion Nikon TIRF objective (100× magnification, 1.49 NA) was mounted on a custom-built inverted optical microscope. A 550 nm high-pass dichroic mirror (AHF Analysentechnik) was employed to separate excitation and emission light. Single-molecule emission videos were recorded using an Andor iXon Ultra EMCCD camera with an exposure time of 50 ms. The EMCCD was operated at an EM gain of 300 with a pixel readout rate of 17 MHz, and both frame-transfer and photon-counting modes were enabled. Fluorescence from individual molecules appeared as diffraction-limited spots during observation. Data acquisition was performed using Andor Solis software. Incident photons were converted into electrons by the EMCCD and recorded as digital counts. These counts were subsequently back-calculated to photon numbers by accounting for instrumental parameters, including EM gain, pre-amplifier gain, and count values, using the photon-counting mode of the detector. MATLAB scripts were employed to identify single molecules and extract quantitative parameters such as total photon counts, photons per cycle, and temporal fluorescence traces. Movies recorded in the kinetic mode provided time- and frame-resolved intensity trajectories at selected pixels over the specified exposure time, which were stored for further analysis.

Relative quantum yield

The relative quantum yield of the Zn-complex was calculated using an optically matching solution of Rhodamine B in 95% ethanol (φ = 0.68) as a reference. The optical density of Zn-complex and Rhodamine B was less than 0.1. The values of fluorescence quantum yield, φ (sample), were calculated by using the following equation:

$$\varphi_S = \varphi_R \left(\frac{Grad_S}{Grad_R}\right)\left(\frac{\eta_S^2}{\eta_R^2}\right)$$

Where the subscripts 'S' and 'R' denote the sample and reference, respectively, φ is the quantum yield, 'Grad' is the gradient from the plot of integrated fluorescence intensity vs absorbance, and η is the refractive index of pure solvents.

Cell viability

For the assessment of cell viability, HeLa cells were seeded in 96-well plates at a density of 5,000 cells per well. After incubating overnight, the cells were washed with 1X PBS, and the media was replaced with fresh DMEM and a Zn complex. Following incubation periods of 6, 12, and 24 hours, 40 µL of the XTT labeling mixture, composed of 5 mL of XTT labeling reagent and 0.1 mL of an electron-coupling reagent from the Cell Proliferation Kit II-XTT (Roche), was added to each well. The plates were then incubated for an additional 6 hours. During this time, the tetrazolium salt XTT was cleaved in the presence of the electron-coupling reagent, producing soluble formazan salts. The absorbance was measured at 450 nm with a reference read at 660 nm using a Tecan Infinite M200 PRO plate reader.

Cell culture and staining

HeLa, HepG2, and HEK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in T-25 flasks, maintained at 37 °C with 5% CO_2 and 95% humidity for optimal growth. When the cells reached greater than 80% confluency, they were trypsinized and reseeded onto poly-L-lysine-coated glass coverslip in 35 mm confocal dishes at a density of 3×10^5 cells per well. Each well was filled with 2 mL of cell suspension in growth medium, and the cells were incubated overnight to ensure proper adherence and growth. The attachment and morphology of the cells were assessed using an optical microscope. Once the cells were adequately adhered and confluent, they were stained with a 1 μM concentration of Zn-complex for 30 minutes under live conditions. After that, the cells were washed with the PBS solution and proceeded to live cell imaging.

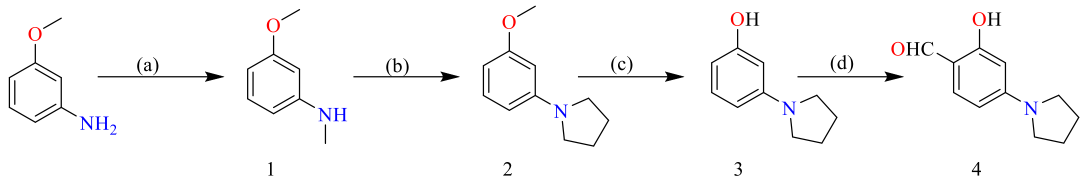
Colocalization study

For the colocalization study, once the seeded cells adhered to the surface of the confocal dishes, they were initially stained with ERB (0.5 μM) or MTG (0.3 μM) for 20-30 minutes. After washing the cells twice, they were then stained with a Zn-complex (1 μM) for an additional 30 minutes. The cells were thoroughly washed twice before proceeding to imaging.

Confocal SRRF

For confocal SRRF imaging, time-series image stacks were acquired using a Nikon Eclipse Ti inverted confocal microscope. The resulting image sequences were processed using the NanoJ SRRF plugin in ImageJ (Henriques Lab) to generate super-resolved images. These images were subsequently analyzed to determine the size and morphology of endoplasmic reticulum (ER) tubules. Samples were excited with a 561 nm laser, and emission was collected using a 595/50 nm bandpass filter. Images were acquired with a Nikon Plan Apo 60× oil-immersion objective (NA 1.42). Each SRRF reconstruction was generated from 200 consecutive frames, analyzed in Fiji using the NanoJ SRRF plugin with a radiality magnification of 5 and a ring radius of 1.

Synthetic route for the Zn-complex



Scheme S1: (a) 1,4-dibromobutane, KOH, CH_3CN. (b) BBr_3, CH_2Cl_2, 0 °C. (c) $POCl_3$, DMF. (d) diaminomaleonitrile, $Zn(OAc)_2 \cdot 2H_2O$, EtOH.

Synthesis of 3-methoxy-*N*-methylaniline (1): To a solution of m-anisidine 1 (5.0 g, 40.6 mmol) and sodium methoxide (13.0 g, 241 mmol) in methanol, paraformaldehyde (1.81 g) was slowly added and the mixture was stirred at room temperature for 16 h. Then NaBH4 (1.5 g, 39.6 mmol) was added to the mixture. After refluxing for 2 h, the mixture was evaporated, treated with 1 M KOH, and extracted with ethyl acetate. The combined organic layers were dried over $NaSO_4$ and evaporated to give the crude product. This was purified by silica gel column chromatography to obtain the pure product as oil. ^1H NMR (500 MHz, $CDCl_3$-D): δ 7.11 (t, J = 8.2 Hz, 1H), 6.30 (dd, J = 8.0, 2.5 Hz, 1H), 6.25 (dd, J = 8.0, 2.4 Hz, 1H), 6.18 (t, J = 2.4 Hz, 1H), 3.79 (s, 3H), 2.83 (s, 1H). ESI-MS calcd for $C_8H_{11}NO$ $[M+H]^+$: 137.0841, Found: 137.0841. Yield: 4.25 g.

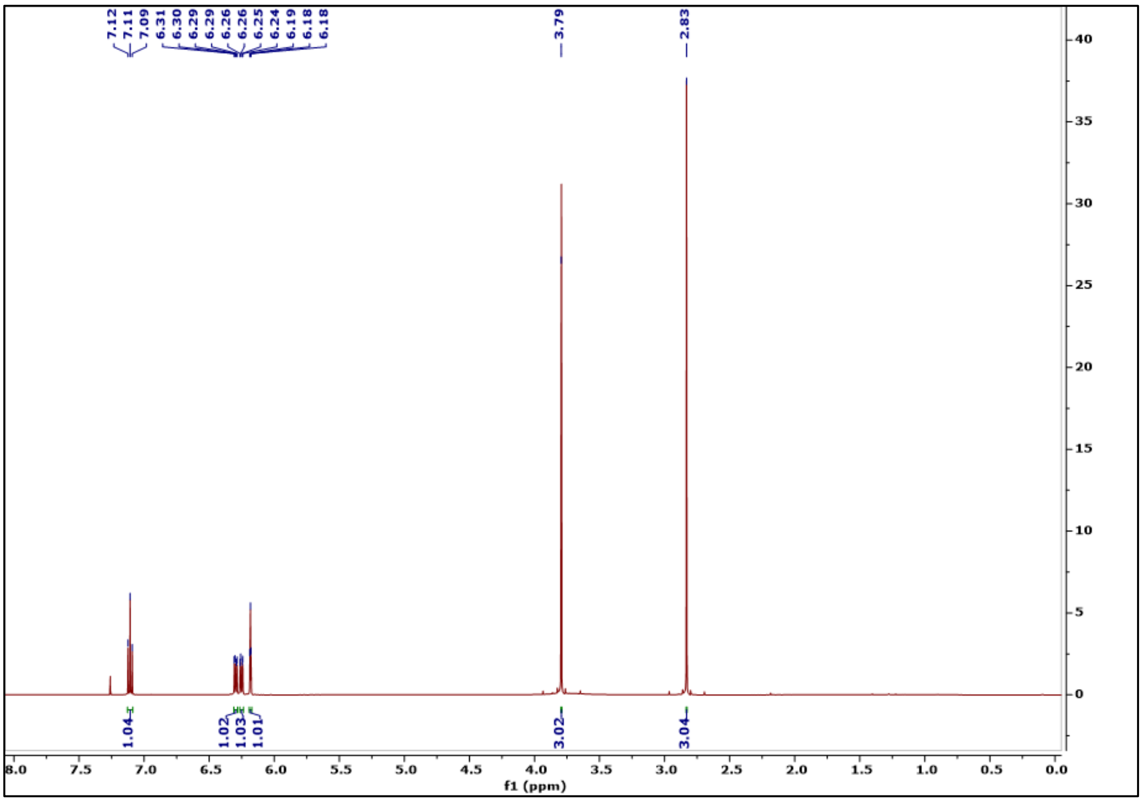


Fig. S1 ^1H NMR spectrum of compound 1.

Synthesis of 1-(3-methoxyphenyl)pyrrolidine (2): 1.21 g (8.8 mmol) of 3-methoxy-N-methylaniline and 1.06 g (18.9 mmol) of KOH were dissolved in 30 mL of CH_3CN, and then 10.21 g (47.3 mmol) of 1,4-dibromobutane was added to the solution slowly. The solution was refluxed under nitrogen for 12 h, and the solvent was removed under reduced pressure. The brown residue was extracted with CH_2Cl_2 and then purified by column chromatography to give a yellow oil (Yield: 1.0 g). ^1H NMR (500 MHz, $CDCl_3$): δ 7.14 (t, J = 8.2 Hz, 1H), 6.25 (dd, J = 8.0, 2.4 Hz, 1H), 6.21 (dd, J = 8.2, 2.3 Hz, 1H), 6.13 (t, J = 2.5 Hz, 1H), 3.81 (s, 3H), 3.26 (m, 4H), 1.97 (m, 4H). ESI-MS calcd. for $C_{11}H_{16}NO$ $[M+H]^+$: 178.1154; found 178.1154.

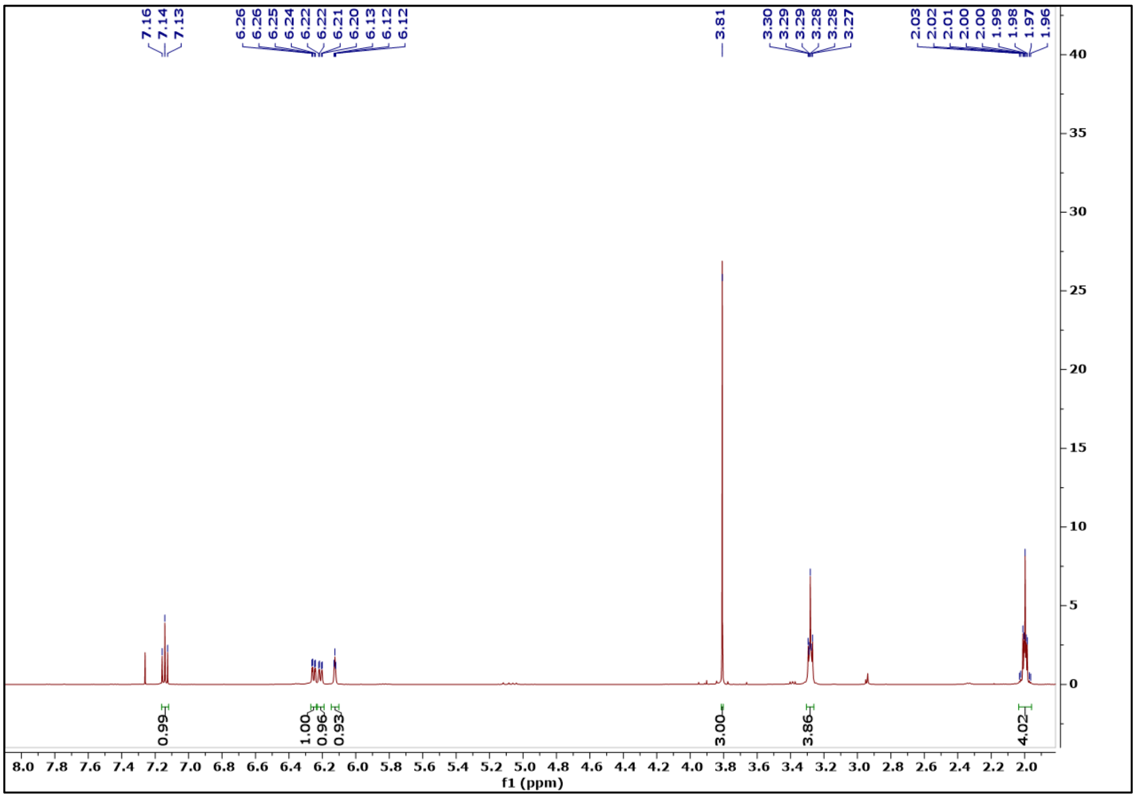


Fig. S2 ^{1}H NMR spectrum of compound 2.

Synthesis of 3-(pyrrolidin-1-yl)phenol (3): 0.86 g (4.8 mmol) of 1-(3-methoxyphenyl)pyrrolidine was dissolved in 10 mL of dichloromethane. Added 1.0 mL (10.6 mmol) of BBr_3 to the solution at 0°C, and the solution was slowly warmed to room temperature and stirred overnight. Added iced methanol to the

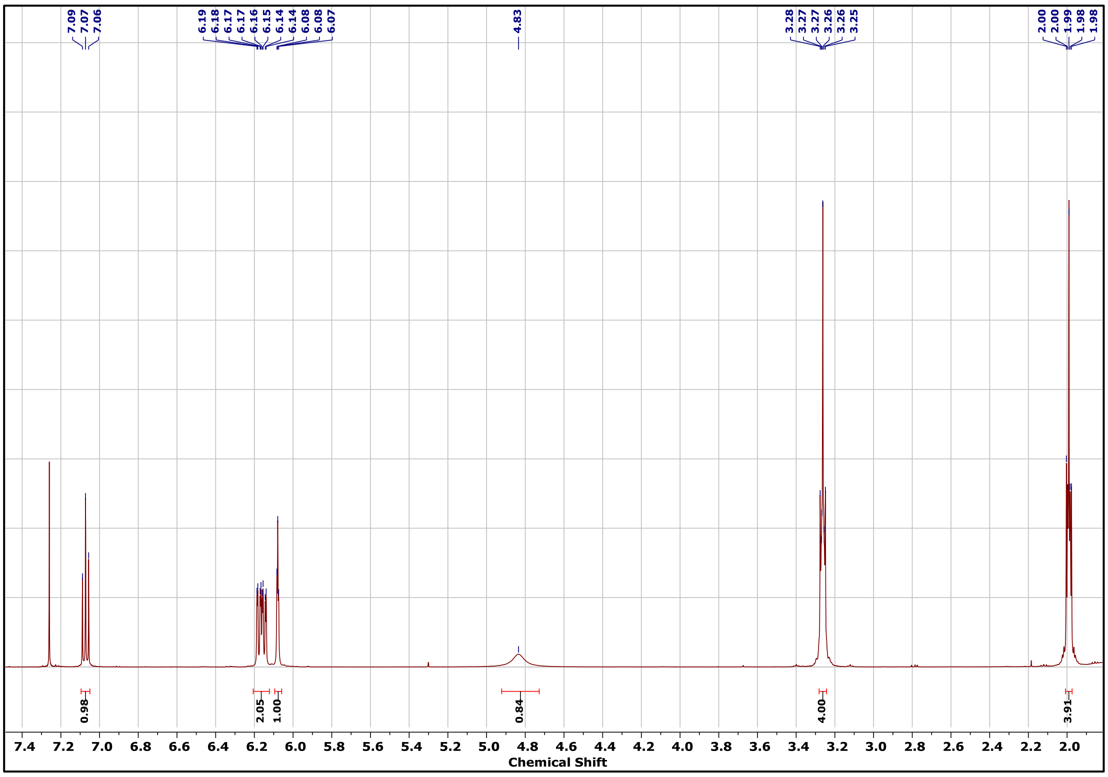


Fig. S3 ^{1}H NMR spectrum of compound 3.

solution in an ice bath, and removed the solvent under reduced pressure. Then the product was purified by column chromatography to give a colorless oil (Yield: 0.52 g).^{1}H NMR (500MHz, $CDCl_3$): δ 2.00 (m, 4H), 3.26 (m, 4H), 4.54 (s, 1H), 6.05 (t, 1H, J = 2.4 Hz), 6.15 (m, 2H), 7.07(t, 1H, J = 7.8 Hz). ESI-MS calcd. for $C_{10}H_{13}NO$ $[M+H]^+$: 164.1075; found 164.1069

Synthesis of 2-hydroxy-4-(pyrrolidin-1-yl)benzaldehyde (4): $POCl_3$ (0.2 mL) was added slowly in dry DMF at 0 °C and stirred for 30 min. Then 162.8 mg (1.0mmol) 3-(pyrrolidin-1-yl)phenol dissolved in dry DMF was added to the reaction mixture at 0 °C slowly. The solution was slowly warmed to room temperature and stirred for 14 hours. Then the reaction mixture was poured into ice and stirred for several minutes. The product was extracted with DCM and purified by column chromatography to afford a colourless oil (Yield: 101 mg). 1H NMR (500 MHz, CHLOROFORM-*D*) δ 11.61 (s, 1H), 9.43 (s, 1H), 7.21 (d, *J* = 8.9 Hz, 1H), 6.11 (dd, *J* = 8.6, 2.3 Hz, 1H), 5.91 (d, *J* = 2.2 Hz, 1H), 3.33 – 3.30 (m, 4H), 1.99-1.96 (m, 4H). ESI-MS calcd. for $C_{11}H_{14}NO_2$ $[M+H]^+$: 192.1025; found 192.0946

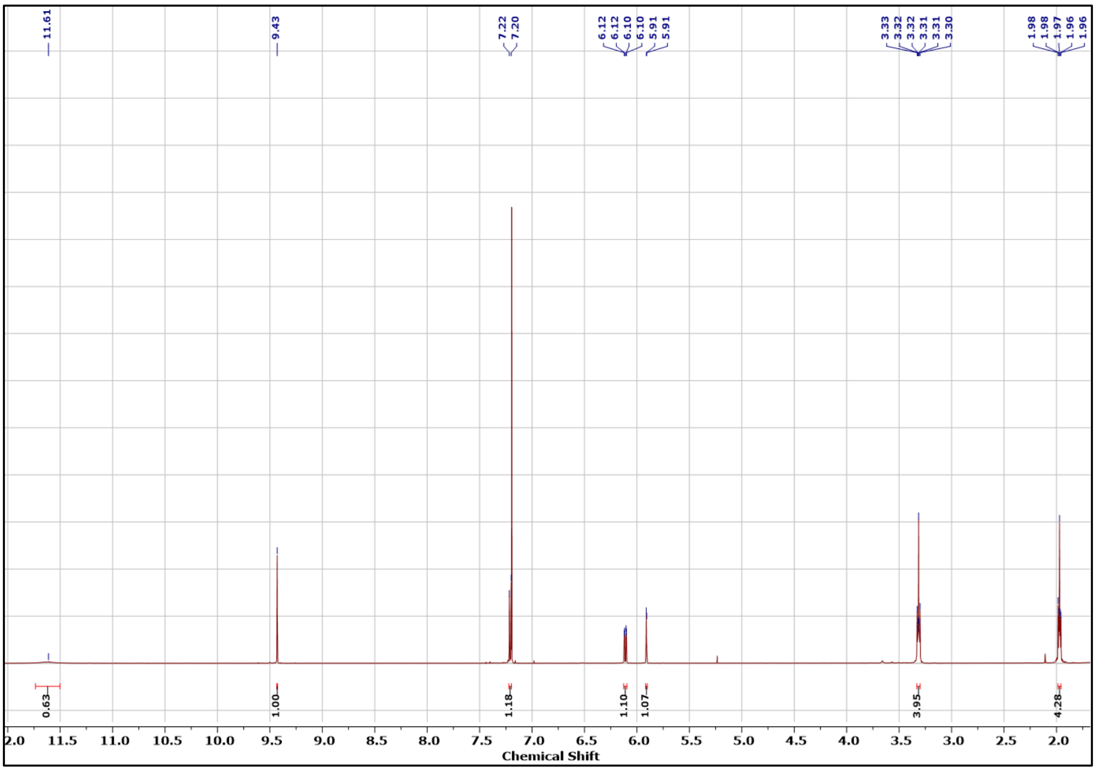


Fig. S4 ^{1}H NMR spectrum of compound 4.

Synthesis of Zn-complex: A mixture of 60.5 mg (0.32 mmol) of 2-hydroxy-4-(pyrrolidin-1-yl)benzaldehyde, 17.5 mg (0.15 mmol) of diaminomaleonitrile, and 65.8 mg (0.15 mmol) of $Zn(OAc)_2 \cdot 2H_2O$ was dissolved in 50 mL of ethanol and refluxed at 60 °C for 8-10 hours. The solvent was removed by rotary evaporators, and the crude product was washed repeatedly with iced ethanol to give 32.8 mg of Zn-complex. ^{1}H NMR (500 MHz, DMSO-*D*6) δ 8.13 (s, 2H), 7.16 (d, *J* = 9.1 Hz, 2H), 6.14 (dd, *J* = 9.0, 2.3 Hz, 2H), 5.71 (d, *J* = 2.2 Hz, 2H), 3.36 (t, *J* = 5.8 Hz, 8H), 1.98 –1.94 (m, 8H). ESI (m/z): $[M+H]^+$ calcd. for 517.5315. ^{13}C NMR (125 MHz, DMSO-*D*6) δ 175.17, 154.26, 118.89, 112.99, 106.28, 101.67, 47.91, 25.39

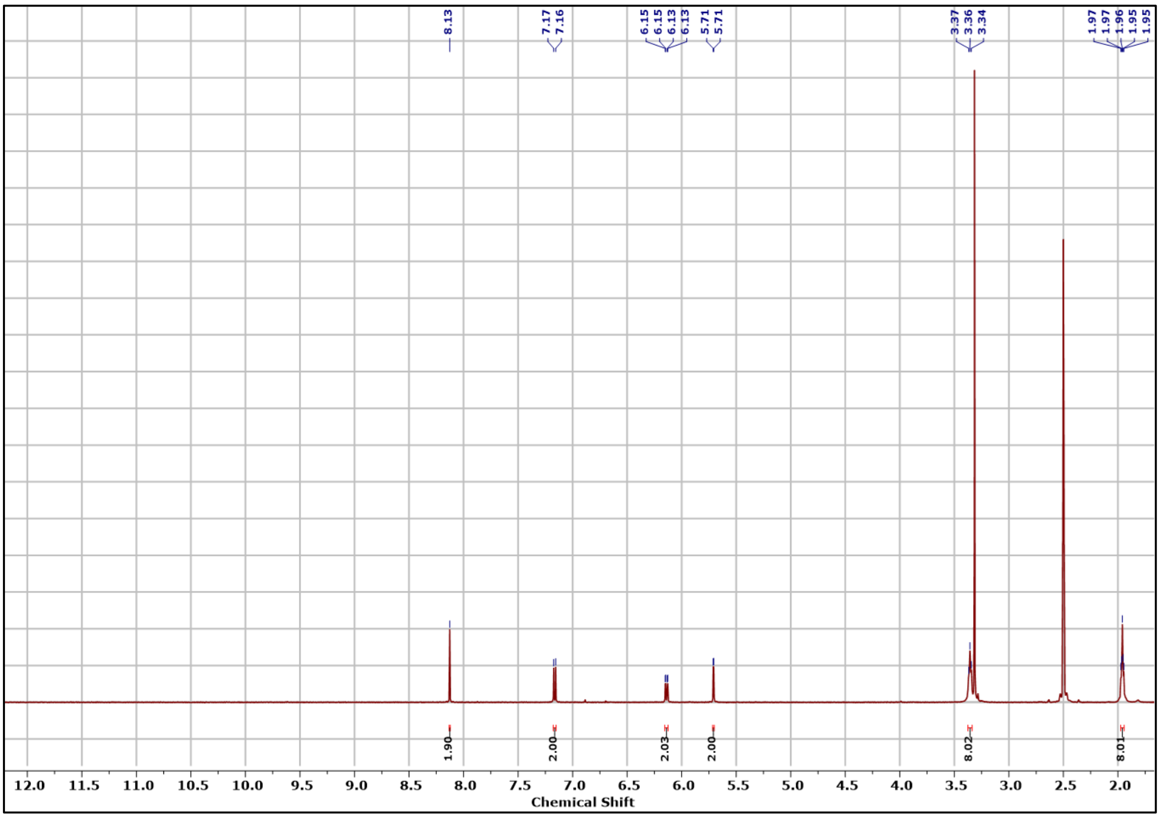


Fig. S5 ^{1}H NMR spectrum of Zn-complex.

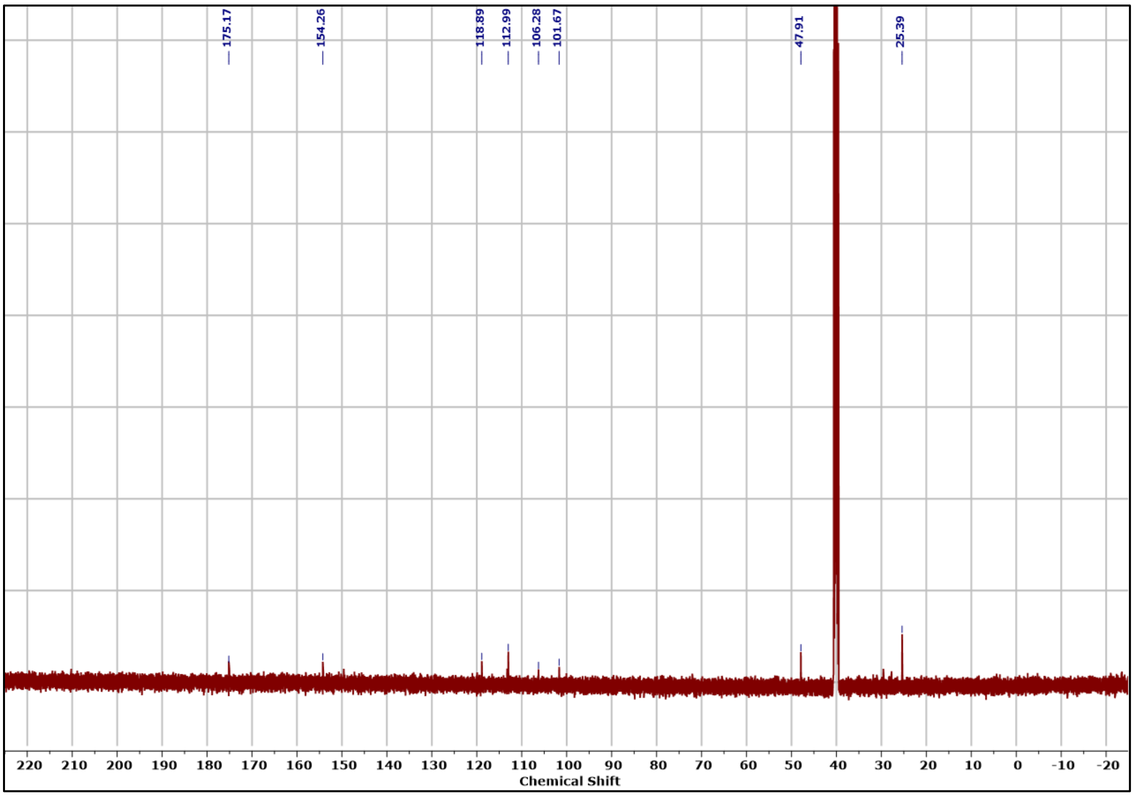


Fig. S6 ^{13}C-NMR spectrum of Zn-complex.

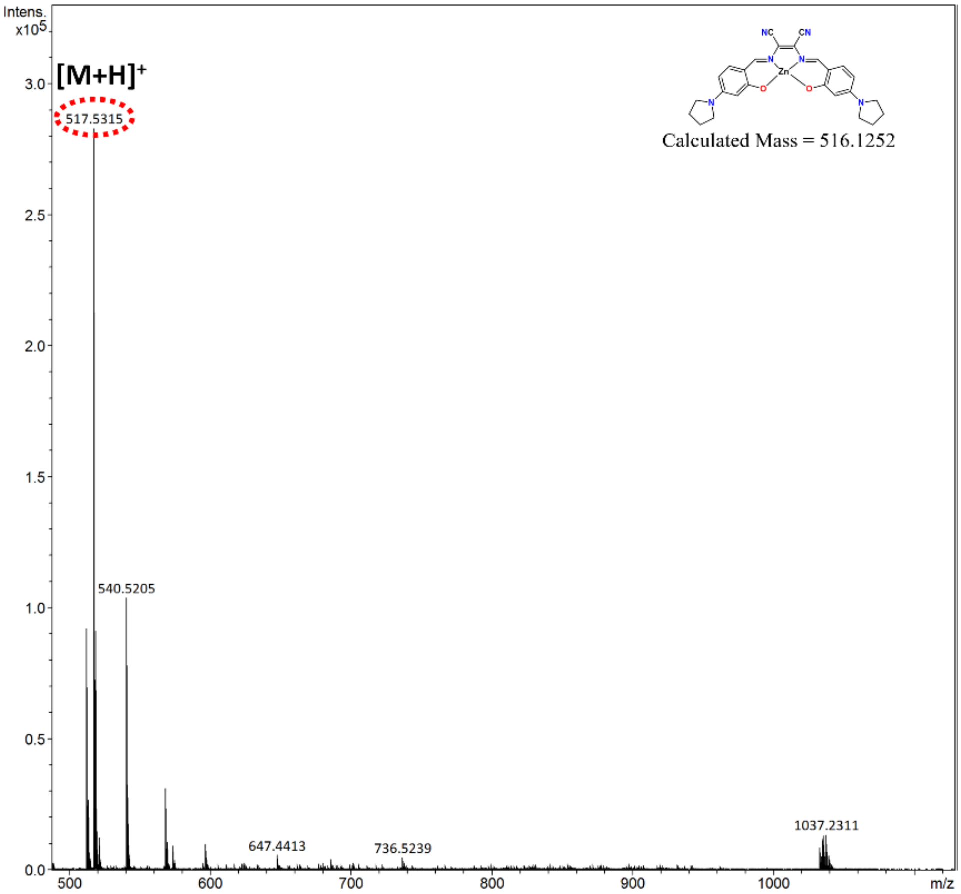
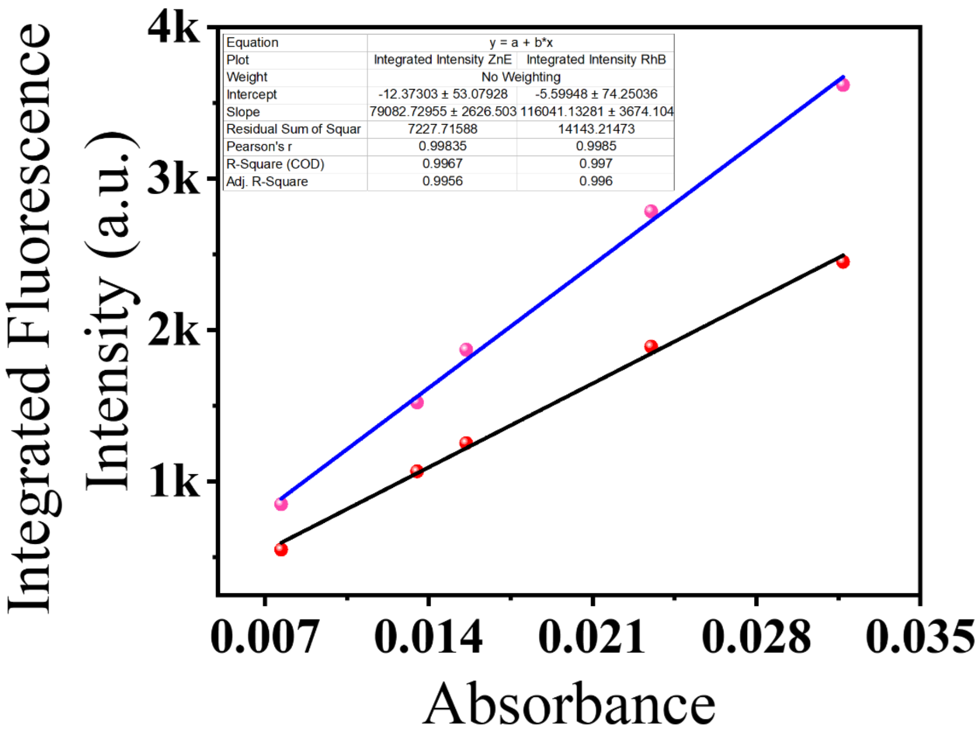


Fig. S7 ESI-MS data of Zn-complex.

$$\varphi_S = \varphi_R \left(\frac{Grad_S}{Grad_R}\right)\left(\frac{\eta_S^2}{\eta_R^2}\right)$$

$Grad_S = 79082.72955$, $Grad_R = 116041.13281$

$\eta_S^{DMSO} = 1.47$, $\eta_R^{EtOH(95\%)} = 1.36$



Equation	y = a + b*x	
Plot	Integrated Intensity ZnE	Integrated Intensity RhB
Weight	No Weighting	
Intercept	-12.37303 ± 53.07928	-5.59948 ± 74.25036
Slope	79082.72955 ± 2626.503	116041.13281 ± 3674.104
Residual Sum of Squar	7227.71588	14143.21473
Pearson's r	0.99835	0.9985
R-Square (COD)	0.9967	0.997
Adj. R-Square	0.9956	0.996

Fig. S8 The QY of the Zn-complex was found to be around 0.55 in reference to the Rhodamine B dye.

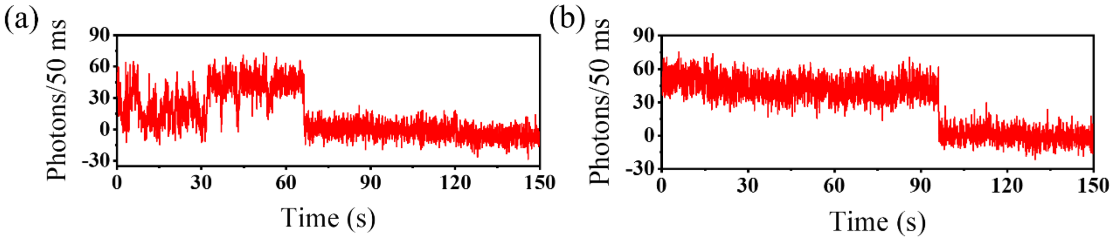


Fig. S9 Real-time traces showing both (a) blinking and (b) Single-step bleaching of the individual Zn-complex.

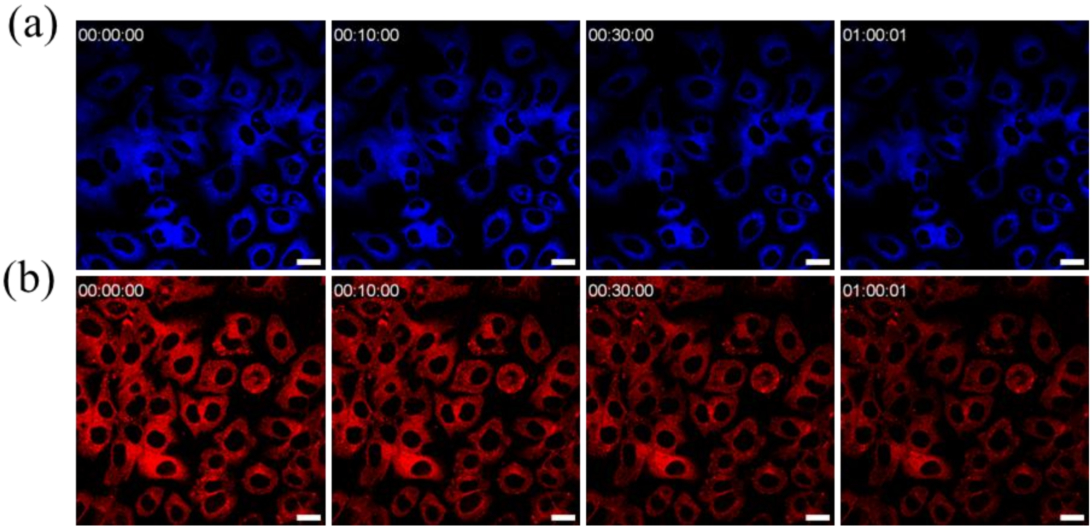


Fig. S10 Time-lapse CLSM imaging for the comparison of photostability of the Zn-complex with ER-Tracker Blue at the 3.65±0.03 µW laser power at the cellular level. Scale bar: 10 µm.

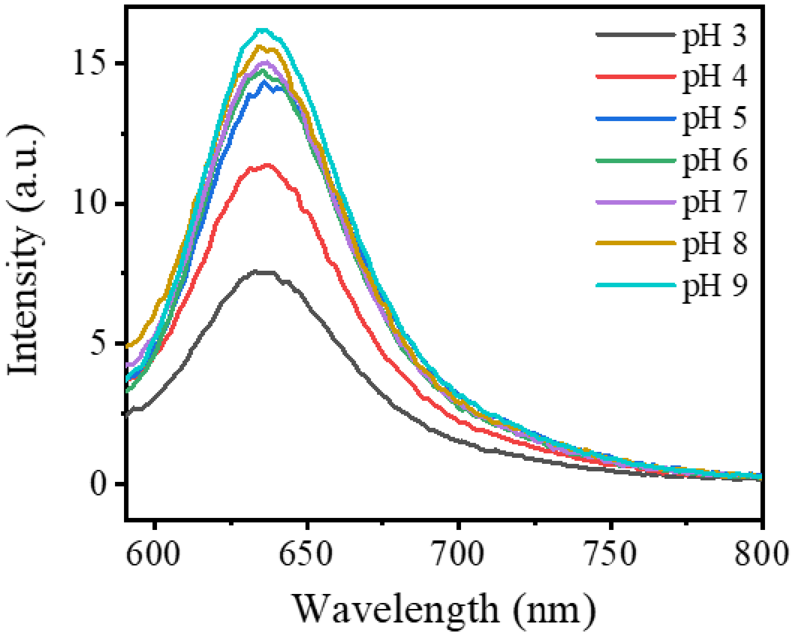


Fig. S11 Fluorescence response of the Zn–complex at various pH conditions, demonstrating the suitability of Zn-complex in the physiological pH range.

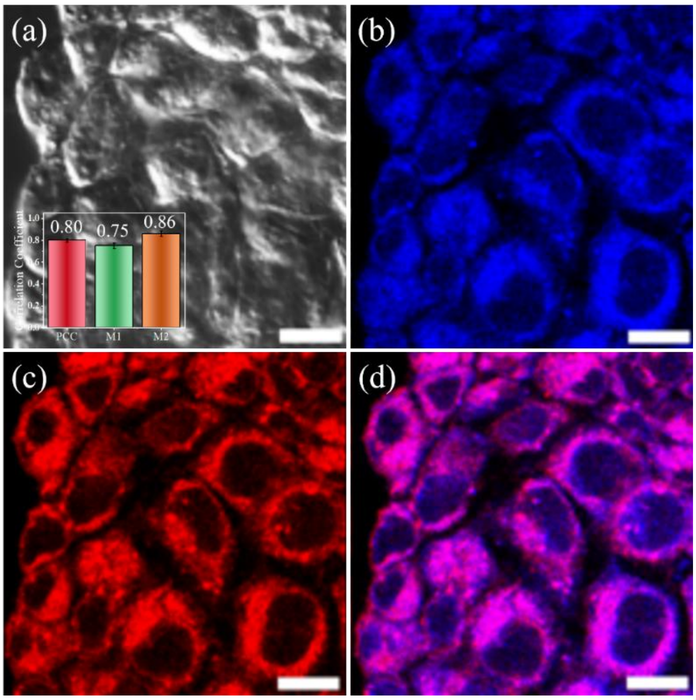


Fig. S12 Subcellular colocalization of the Zn-complex and MTG. (a) TD image; the inset shows colocalization analysis between MTG and the Zn-complex, quantified using PCC and MOC's (M1 and M2). (b) Mitochondria labelled with MTG (0.3 μM), showing emission in the 500-550 nm range under 488 nm excitation. (c) ER labelled with the Zn-complex (1 μM), showing emission in the 570–620 nm range under 561 nm excitation. (d) Merged image of panels (b) and (c). Scale bar: 10 μm

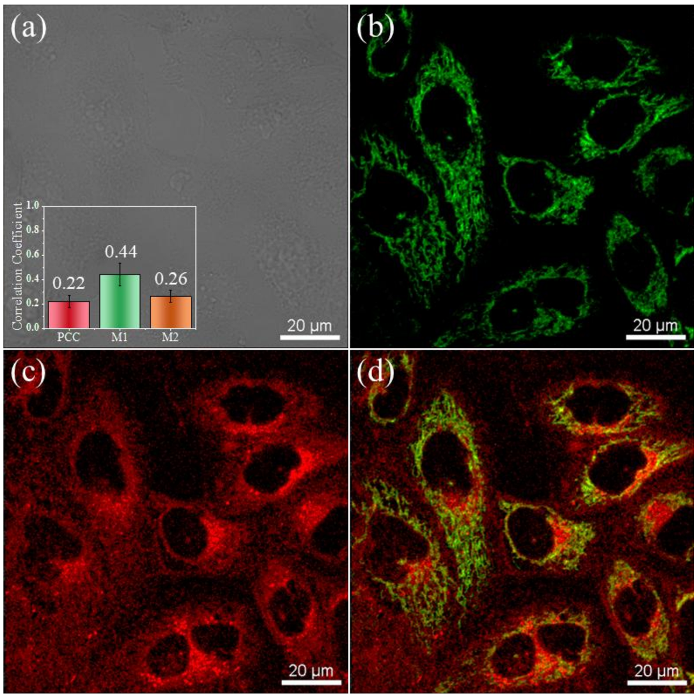


Fig. S13 Subcellular colocalization of the Zn-complex and MTG. (a) TD image; the inset shows colocalization analysis between MTG and the Zn-complex, quantified using PCC and MOC's (M1 and M2). (b) Mitochondria labelled with MTG (0.3 μM), showing emission in the 500-550 nm range under 488 nm excitation. (c) ER labelled with the Zn-complex (1 μM), showing emission in the 570–620 nm range under 561 nm excitation. (d) Merged image of panels (b) and (c).

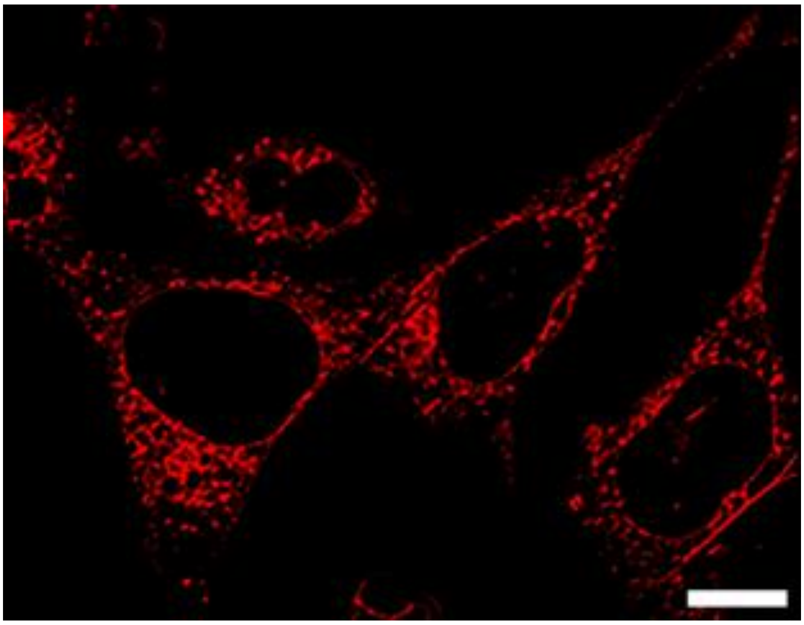


Fig. S14 Shows super-resolution imaging of the ER stained with a zinc complex in HEK cells, suggesting a tubular net-like structure. Scale bar: 5 µm

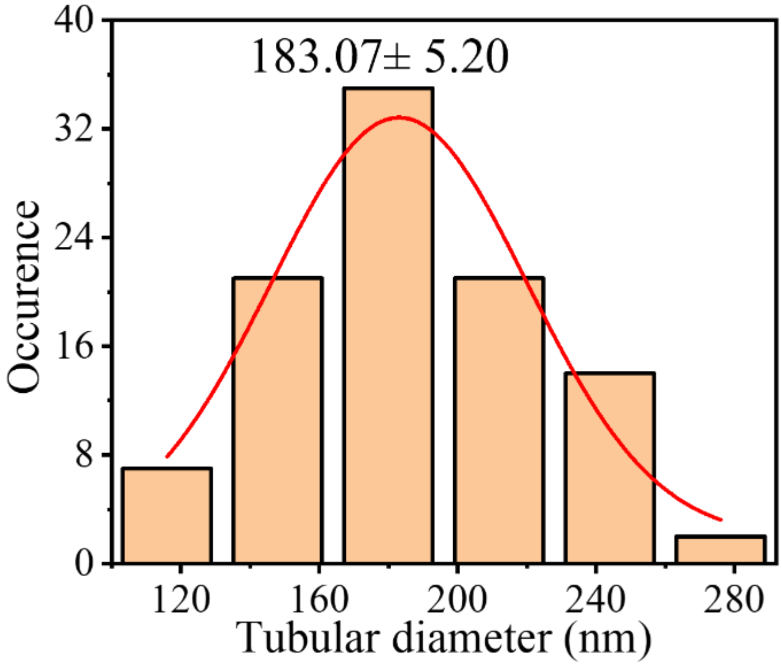


Fig. S15 Shows the average ER tubule diameter of approximately 183 nm, obtained from the analysis of 105 ER tubules.

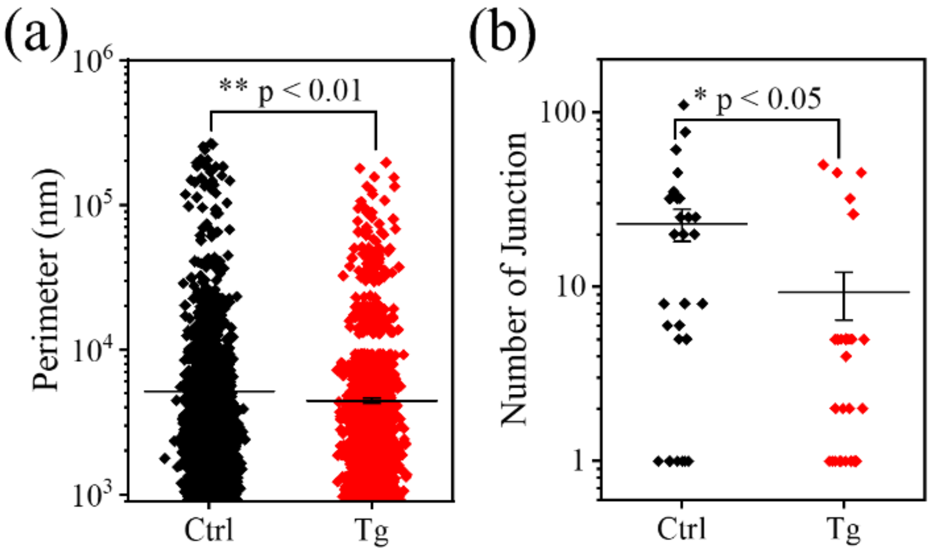
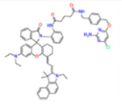
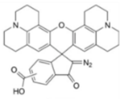
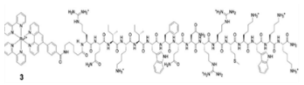
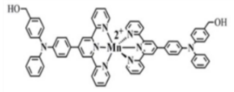
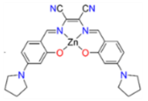


Fig. S16 Statistical analysis of ER (a) perimeter and (b) number of junctions after thapsigargin treatment compared with the control, determined by a paired two-tailed Student's t-test. $p < 0.05$; $p < 0.01$.

Table R1: Shows the comparison of the reported molecular probes and metal complexes used for SRM imaging.

Sr. No.	**Organic Dyes/ Metal complexes**	**Compound Structure**	**$\lambda_{ex/em}$ (nm)**	**QY (%)**	**Cell viability**	**Probe [μM]**	**Resolution of ER**	**Reference**
1.	SMI-NIR1-CLP		739/770	61.4	68 %	2.0	119 nm	*Anal. Chem.* 2024, 96, 10860-10869
2.	NH_2-BODIPY		---	---	100 %	0.25	71.5 nm	*Org. Biomol. Chem.*, 2019, 17, 3732-3736
3.	ER-1		561/610	---	---	---	---	*Helv. Chim. Acta* 2018, 101, e1800165
4.	Ru-ER		454/610	3.0	72 %	200	151.7 nm	*Chem. Sci.*, 2016, 7, 6551–6562
5.	MnFM1		366/504	28	85 %	50	90 nm	*Inor. Chem. Front.*, 2019,6, 2914-2920
6	Zn-complex		592/630	56	92 %	2.0	110 nm	This work