A Red-Emitting, Microenvironment-Insensitive Fluorophore for Lysosome-Specific Imaging in Live Cells

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S1. Comprehensive synthetic procedures and characterization

4-(diethylamino)-2-(2-morpholinoethoxy)benzaldehyde (3)

To a solution of 4-(diethylamino)-2-hydroxybenzaldehyde (1) (1.00 g, 5.37 mmol) in anhydrous DMF (15 mL), 4-(2-chloroethyl)morpholine (2) (0.79 g, 5.76 mmol) and potassium carbonate (K_2CO_3, 1.48 g, 10.72 mmol) were added. The reaction mixture was stirred at room temperature (25 °C) for 24 hours. After completion (monitored by TLC, eluent: ethyl acetate/hexane, 3:7), the reaction mixture was diluted with water (50 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, ethyl acetate/hexane, 1:4) to afford 4-(diethylamino)-2-(2-morpholinoethoxy)benzaldehyde (3) as a yellow solid (1.40 g, 92%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.14 (s, 1H), 7.71 (d, J = 9.0 Hz, 1H), 6.30 (dd, J = 9.0, 2.3 Hz, 1H), 6.02 (d, J = 2.3 Hz, 1H), 4.20 (t, J = 5.6 Hz, 2H), 3.74 (t, J = 4.7 Hz, 4H), 3.42 (q, J = 7.1 Hz, 4H), 2.88 (t, J = 5.6 Hz, 2H), 2.67 – 2.59 (m, 4H), 1.22 (t, J = 7.1 Hz, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 186.99, 163.31, 153.83, 130.46, 114.30, 104.60, 93.32, 77.29, 77.03, 76.78, 66.91, 66.44, 57.51, 54.19, 44.80, 12.61.

Synthesis of 2-(2,6-bis((E)-4-(diethylamino)-2-(2-morpholinoethoxy)styryl)-4H-pyran-4-ylidene)malononitrile (DM)

To a solution of 4-(diethylamino)-2-(2-morpholinoethoxy)benzaldehyde (3) (1.40 g, 4.94 mmol) in methanol (20 mL), 2-(2,6-dimethyl-4H-pyran-4-ylidene)malononitrile (4) (0.94 g, 4.94 mmol) and piperidine (0.10 mL, 1.00 mmol) were added. The reaction mixture was refluxed at 65 °C for 16 hours under an inert atmosphere. After cooling to room temperature, the solvent was removed under reduced pressure, and the residue was triturated with cold methanol (10 mL). The resulting solid was collected by filtration, washed with methanol, and dried under vacuum to yield DM as a dark red solid (1.40 g, 60%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68 (d, J = 15.9 Hz, 2H), 7.35 (d, J = 8.8 Hz, 2H), 6.79 (d, J = 15.8 Hz, 2H), 6.49 (s, 2H), 6.32 (dd, J = 8.8, 2.4 Hz, 2H), 6.15 (d, J = 2.4 Hz, 2H), 4.20 (t, J = 6.1 Hz, 4H), 3.71 (t, J = 4.7 Hz, 8H), 3.42 (q, J = 7.1 Hz, 8H), 2.92 (t, J = 6.1 Hz, 4H), 2.63 – 2.54 (m, 8H), 1.22 (t, J = 7.1 Hz, 12H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 160.27, 159.62, 156.37, 150.65, 134.16, 131.42, 116.78, 113.92, 111.95, 104.98, 104.84, 94.95, 77.29, 77.03, 76.78, 67.03, 65.77, 57.57, 54.10, 44.71, 12.74, 0.01. **HR-MS** (ESI-ToF) m/z: Calculated for $C_{44}H_{57}N_6O_5$ $[M+H]^+$: 749.4385; Found: 749.4409; error: 0.0024 m/z.

Synthesis of (E)-2-(2-(4-(diethylamino)-2-(2-morpholinoethoxy)styryl)-6-methyl-4H-pyran-4-ylidene)malononitrile (MM)

To a solution of 4-(diethylamino)-2-(2-morpholinoethoxy)benzaldehyde (3) (200 mg, 0.652 mmol, 1.0 equiv) and 2-(2,6-dimethyl-4H-pyran-4-ylidene)malononitrile (134 mg, 0.783 mmol, 1.2 equiv) in methanol, a catalytic amount of piperidine (0.2 equiv) was added. The reaction mixture was stirred under reflux conditions for 12 hours, and progress was monitored periodically using thin-layer chromatography (TLC). Upon completion, the reaction mixture was allowed to cool to room temperature and poured into ice-cold water. The crude product was extracted using ethyl acetate and concentrated under reduced pressure. The resulting residue was purified by column chromatography using hexane/ethylacetate solvent system to yield the desired compound, (E)-2-(2-(4-(diethylamino)-2-(2-morpholinoethoxy)styryl)-6-methyl-4H-pyran-4-ylidene)malononitrile, as a red solid in 32% yield (96.2 mg). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.53 (dd, J = 15.9, 2.6 Hz, 1H), 7.25 (dd, J = 8.8, 2.8 Hz, 1H), 6.60 (dd, J = 15.9, 2.7 Hz, 1H), 6.43 – 6.33 (m, 2H), 6.23 (dd, J = 9.1, 2.5 Hz, 1H), 6.03 (d, J = 2.3 Hz, 1H), 4.11 (s, 2H), 3.66 (t, J = 4.8 Hz, 4H), 3.33 (t, J = 7.1 Hz, 4H), 2.82 (s, 2H), 2.54 (t, J = 4.6 Hz, 4H), 2.28 (d, J = 2.6 Hz, 3H), 1.14 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 160.63, 160.45, 158.65, 155.53, 149.95, 133.63, 129.90, 115.00, 114.90, 111.36, 110.42, 104.99, 103.88, 103.79, 103.77, 93.55, 76.37, 76.12, 75.86, 66.04, 64.93, 56.50, 53.08, 43.68, 18.90, 11.72. **HR-MS** (ESI-ToF) m/z: Calculated for $C_{27}H_{33}N_4O_3$ $[M+H]^+$: 461.2547; Found: 461.2569; error: 0.0022 m/z.

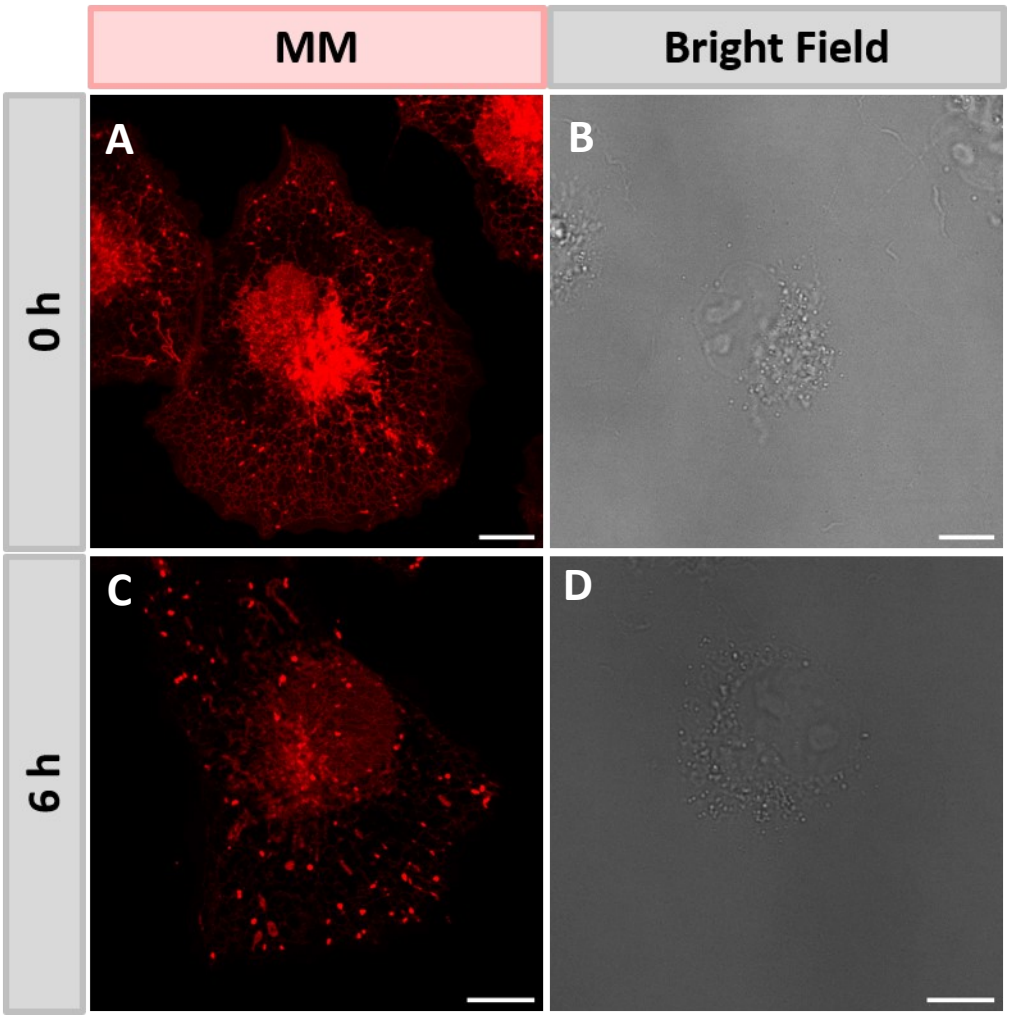

Figure S1. Confocal fluorescence images showing the localization of MM in COS-7 cells at (A, B) 0 hours and (C, D) 6 hours of incubation. At 0 hours, MM predominantly localizes in a mesh-like network resembling an organelle structure, likely the endoplasmic reticulum (ER). After 6 hours of incubation, the fluorescence fades from the network-like structure and shifts to punctate structures, indicating a change in subcellular localization. (Scale bar: 10 μm)

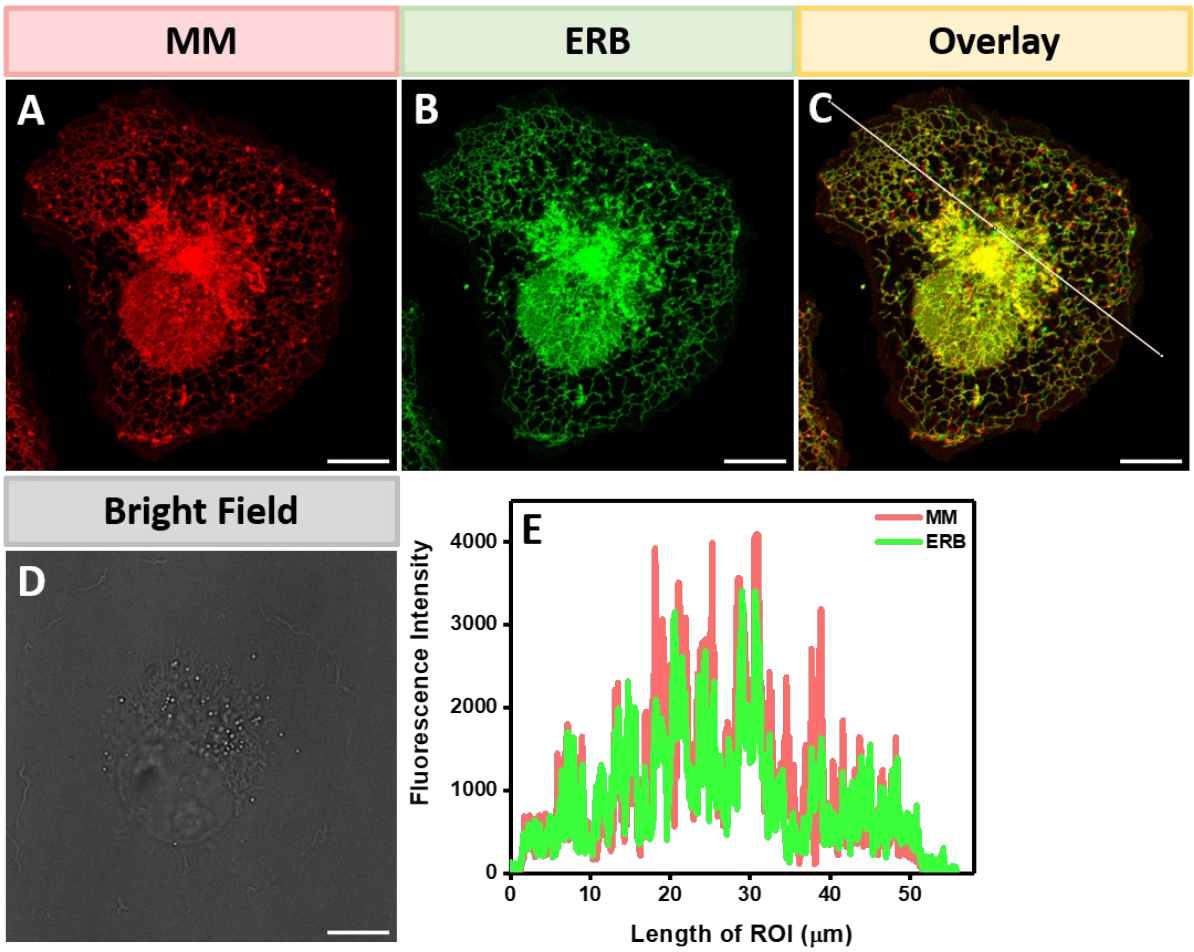

Figure S2. Subcellular colocalization of MM and ER-Tracker™ Blue-White DPX (ERB) in live COS-7 cells at 10 min incubation. (A) MM fluorescence (Ex: 561 nm, Em: 575–650 nm) showing a mesh-like network pattern. (B) ERB fluorescence (Ex: 405 nm, Em: 500–550 nm) highlighting the endoplasmic reticulum. (C) Overlay of MM and ERB signals demonstrating significant colocalization within the ER. (D) Bright-field image of the COS-7 cells. (E) Line profile analysis of fluorescence intensities (marked in panel C) for MM and ERB channels, confirming colocalization through overlapping intensity peaks. (Scale bar: 10 μm)

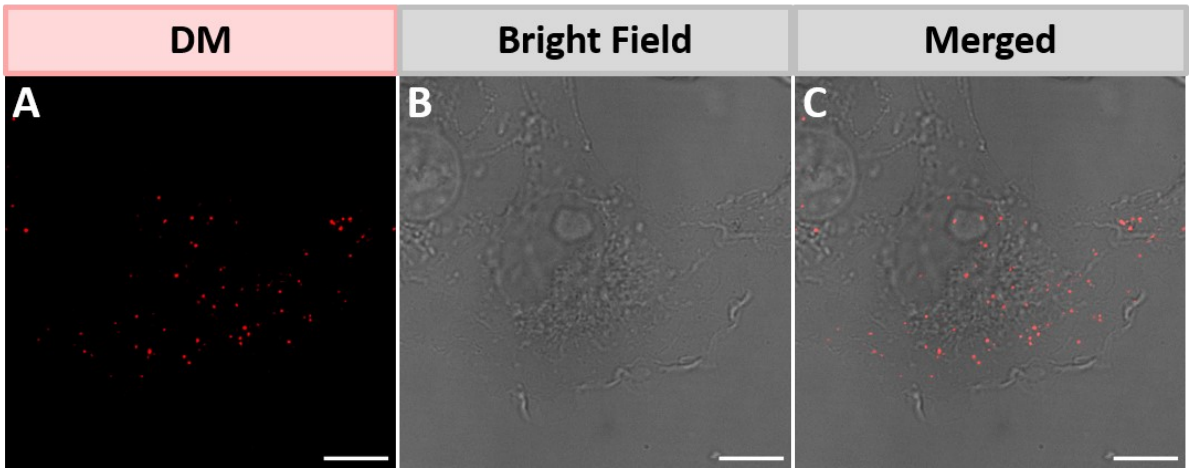

Figure S3. Confocal microscopy images of DM (1 μM) in a living COS-7 single cell. (A) Fluorescence image of DM; (B) corresponding bright-field image; and (C) merged image of fluorescence and bright field. Imaging parameters: DM, λ_{ex} = 561 nm, λ_{em} = 575–650 nm. Scale bar = 10 μm.

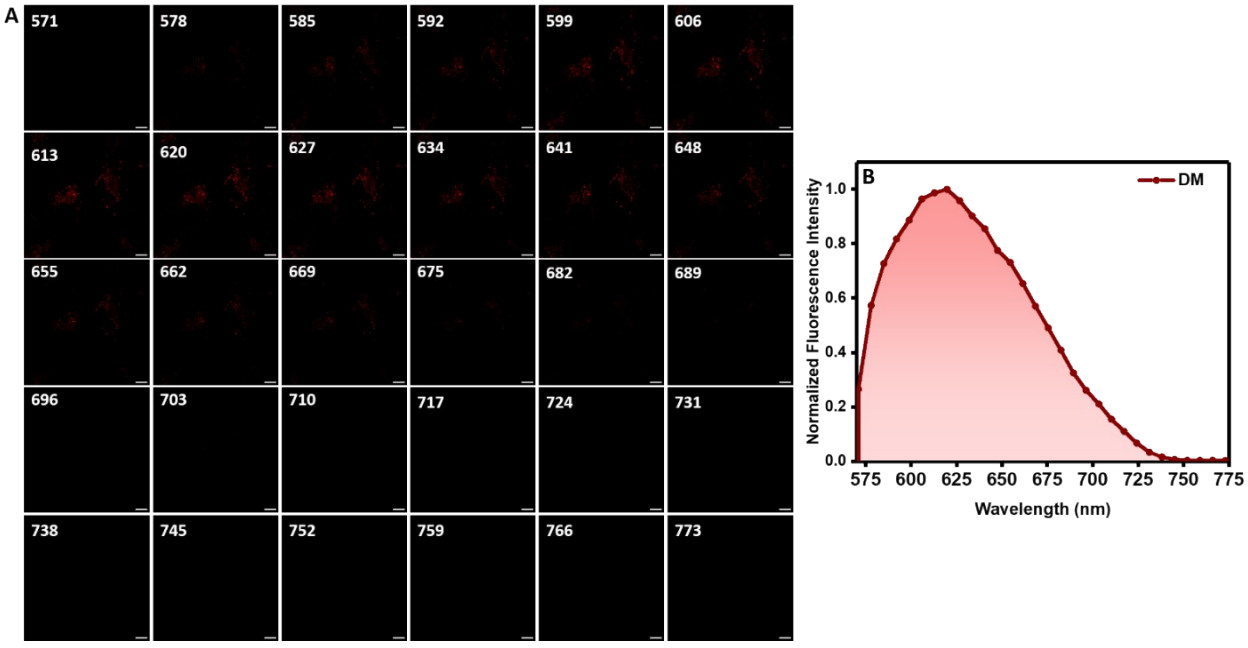

Figure S4. Emission Spectra of DM in COS-7 Cells (A) Confocal images of COS-7 cells stained with DM (1 μM), captured at emission wavelengths ranging from 571 nm to 775 nm with a 10 nm interval. The excitation wavelength (λ_{ex}) was set to 561 nm. Scale bars represent 10 μm. (B) Normalized emission spectra of DM from COS-7 cells, highlighting the peak emission at approximately 620 nm.

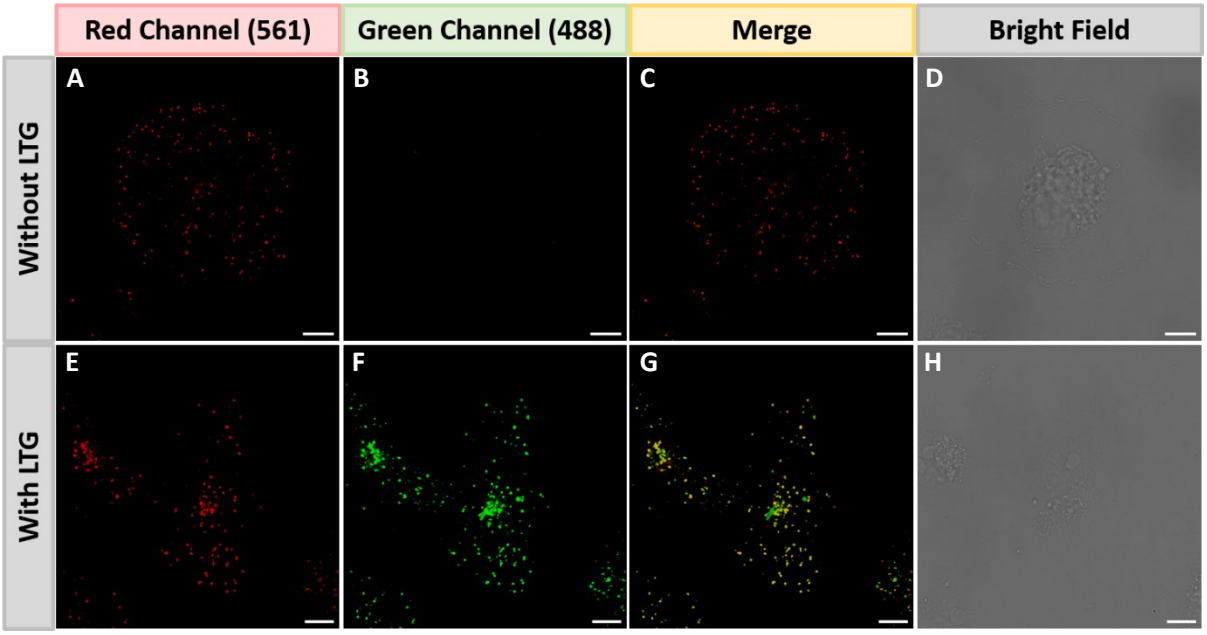

Figure S5. Confocal microscopy images demonstrating the fluorescence of DM and its compatibility with Lysotracker Green (LTG) in living cells. (Top row) Imaging without LTG: (A) fluorescence of DM (λ_{ex} = 561 nm, λ_{em} = 575–675 nm), (B) absence of fluorescence in the 488 nm channel, (C) combined red and green channels, and (D) corresponding cellular morphology in bright field. (Bottom row) Imaging with LTG: (A) fluorescence of DM, (B) fluorescence of LTG (λ_{ex} = 488 nm, λ_{em} = 500–550 nm), (C) overlap of red and green signals indicating colocalization, and (D) corresponding cellular morphology in bright field. Scale bar = 10 µm.

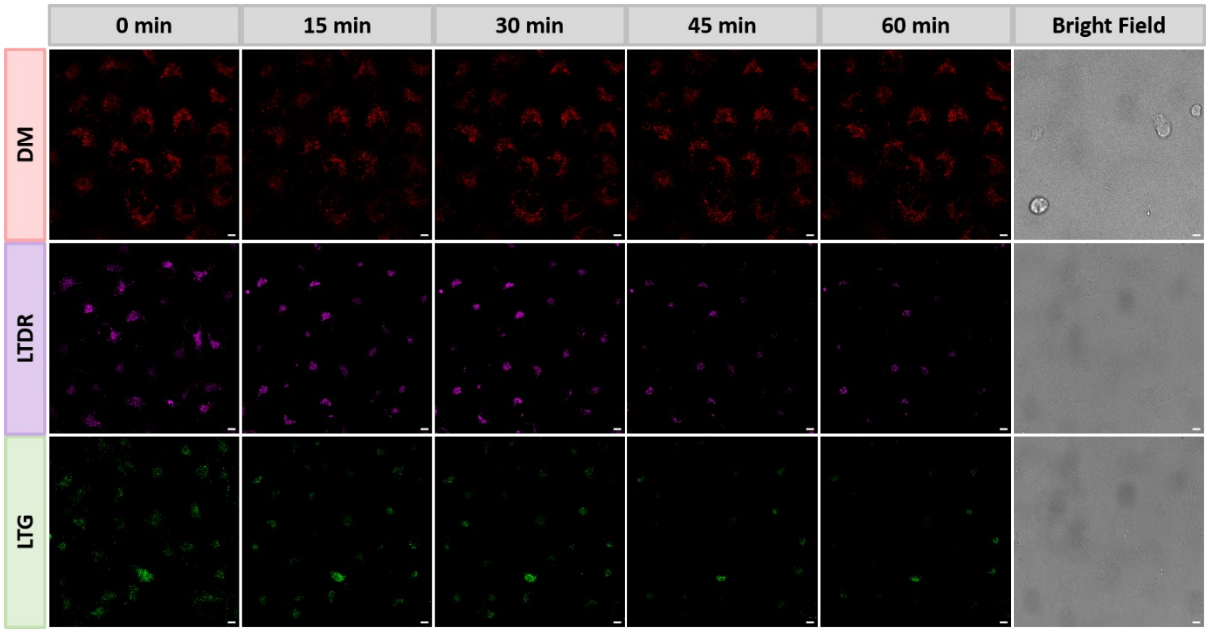

Figure S6. Confocal images of COS-7 cells stained with DM, LTDR, and LTG under continuous irradiation. Images were captured per 1.29 s; representative images of consecutive frames are displayed (more frames are shown in Videos S1−S3). Imaging wavelength DM: λex/λem = 561/575-650 nm; LTDR: λex/λem = 633/650-720 nm; LTG: λex/λem = 488/500-570 nm

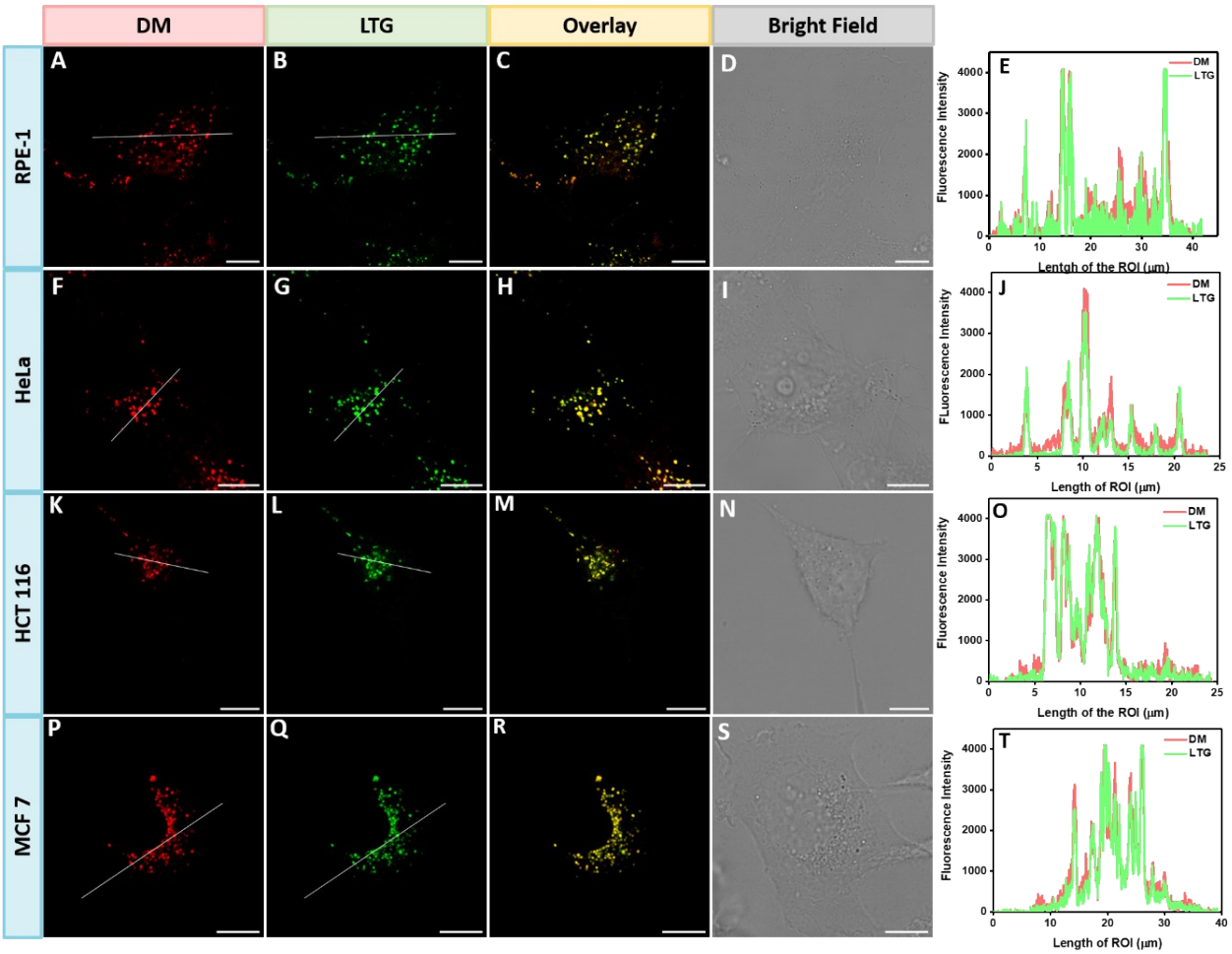

Figure S7. Confocal microscopy images validating the lysosomal targeting ability of DM in various cell lines: RPE-1, HeLa, HCT-116, and MCF-7. (A, F, K, P) Fluorescence images of DM (λ_ex = 561 nm, λ_em = 575–675 nm). (B, G, L, Q) Fluorescence images of Lysotracker Green (LTG; λ_ex = 488 nm, λ_em = 500–550 nm). (C, H, M, R) Merged images showing colocalization of DM and LTG in lysosomes. (D, I, N, S) Corresponding bright-field images for cellular morphology. (E, J, O, T) Fluorescence intensity profiles along the indicated region of interest (ROI) demonstrating colocalization of DM and LTG fluorescence. Scale bars = 10 μm.

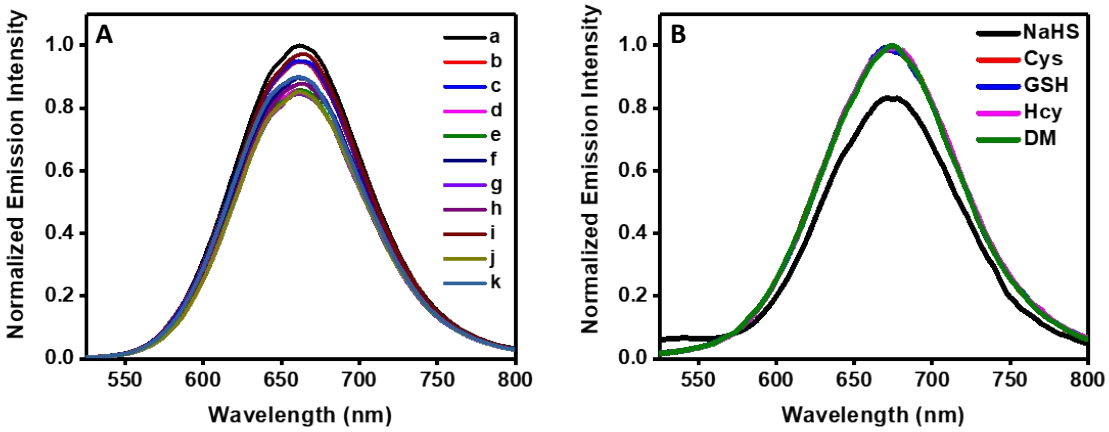

Figure S8. Selectivity studies of DM toward various ions and thiols. (A) Normalized emission spectra of **DM** in the presence of different ions: (a) S^{2-}, (b) NO_2^-, (c) F^-, (d) SO_4^{2-}, (e) NO_3^-, (f) I^-, (g) Cl^-, (h) Br^-, (i) CH_3COO^-, (j) Ca^{2+}, and (k) DM alone. (B) Normalized emission spectra of DM in the presence of NaHS, Cys, GSH, and Hcy, compared to DM alone. DM exhibited no significant fluorescence response to any of the tested nucleophiles or ions, confirming its insensitivity and high selectivity under the tested conditions.

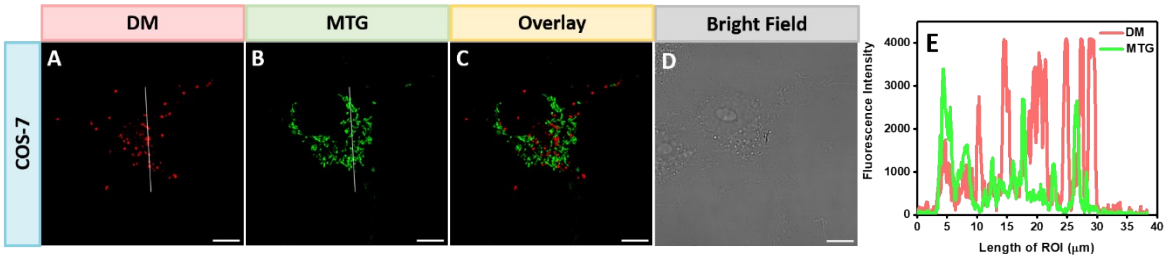

Figure S9. Colocalization analysis of **DM** with MitoTracker Green (**MTG**) in COS-7 cells. (A) Fluorescence image of DM (λ_{ex} = 561 nm, λ_{em} = 575–675 nm). (B) Fluorescence image of **MTG** (λ_{ex} = 488 nm, λ_{em} = 500–550 nm). (C) Overlay of **DM** and **MTG** fluorescence signals. (D) Corresponding bright-field image of the cell. (E) Intensity profile along the line drawn in (A–B) showing minimal overlap between the fluorescence signals of **DM** and **MTG**, indicating that **DM** does not colocalize with mitochondria. Scale bars = 10 μm.

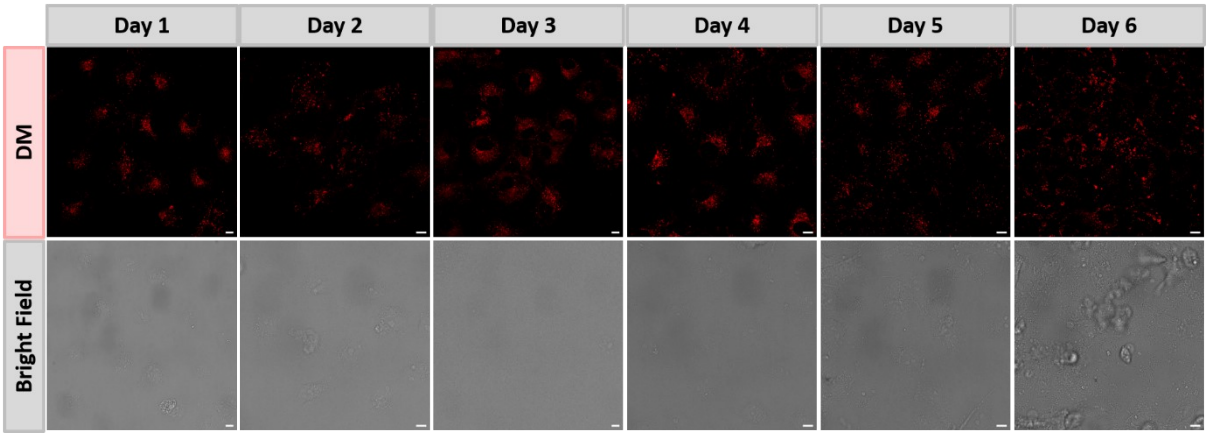

Figure S10. Confocal images of **DM**-stained multiple cells for several days. COS-7 cells were stained with **DM** (1 μM) (0 day: images recorded right after this time). Cells were imaged every day for another 6 days continuously

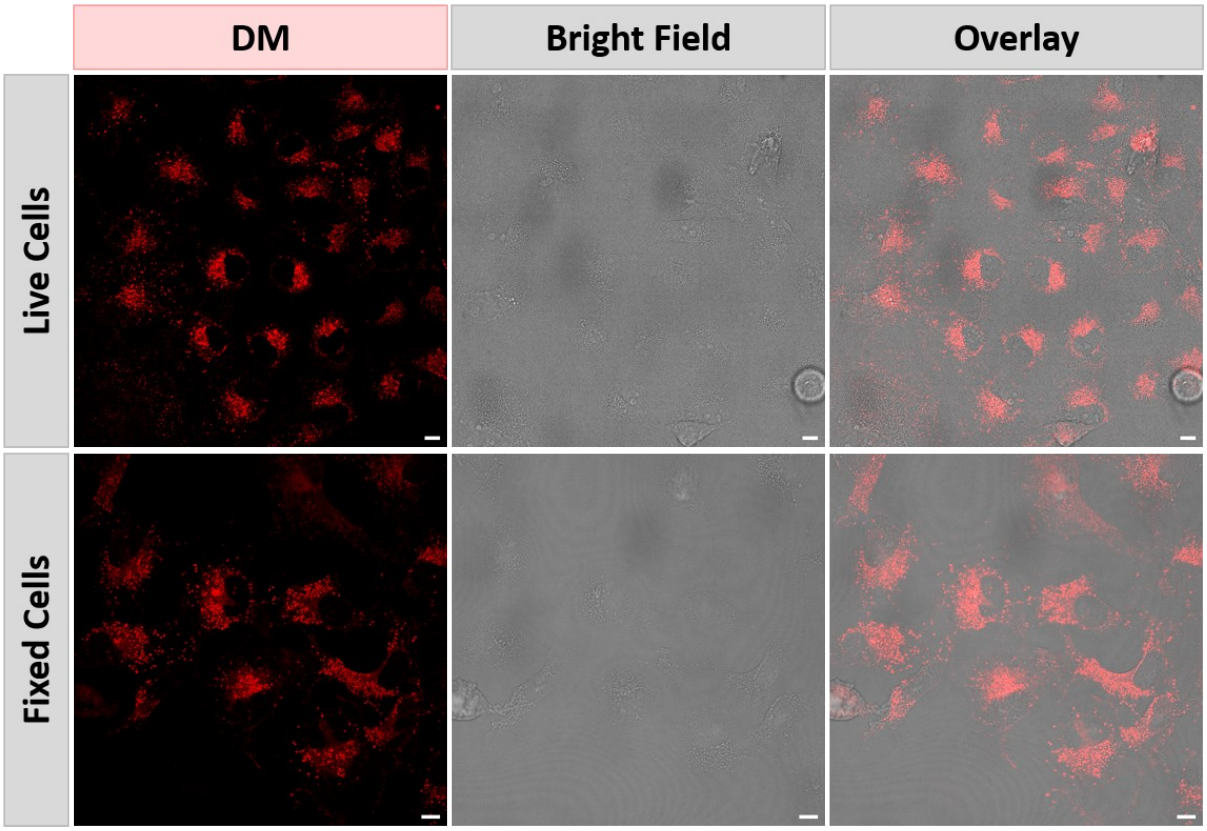

Figure S11. DM Localization in Live and Fixed COS-7 Cells. Confocal images of COS-7 cells stained with DM dye, showing fluorescence distribution in live (top row) and fixed (bottom row) cells. The columns represent (from left to right) DM, bright-field, and the overlay of both. Scale bars represent 10 μm.

S. Characterization: 1H NMR, 13C NMR and HRMS Data for All Compounds

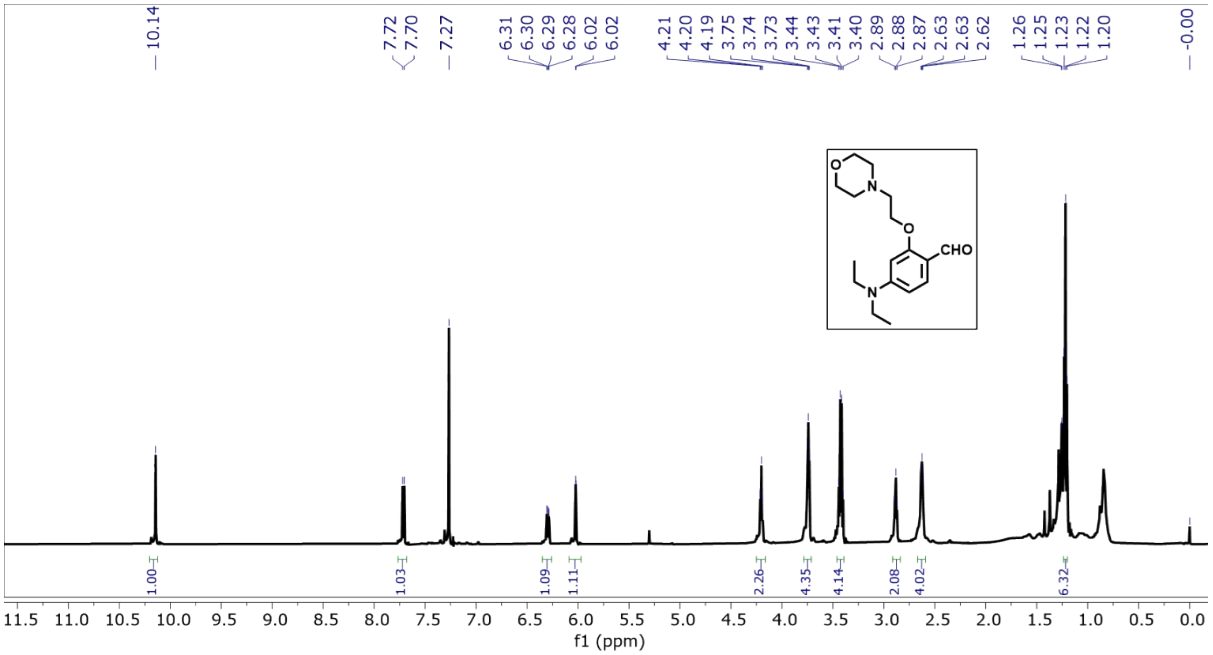

Figure S12. ^{1}H NMR spectrum of compound 3 in $CDCl_3$.

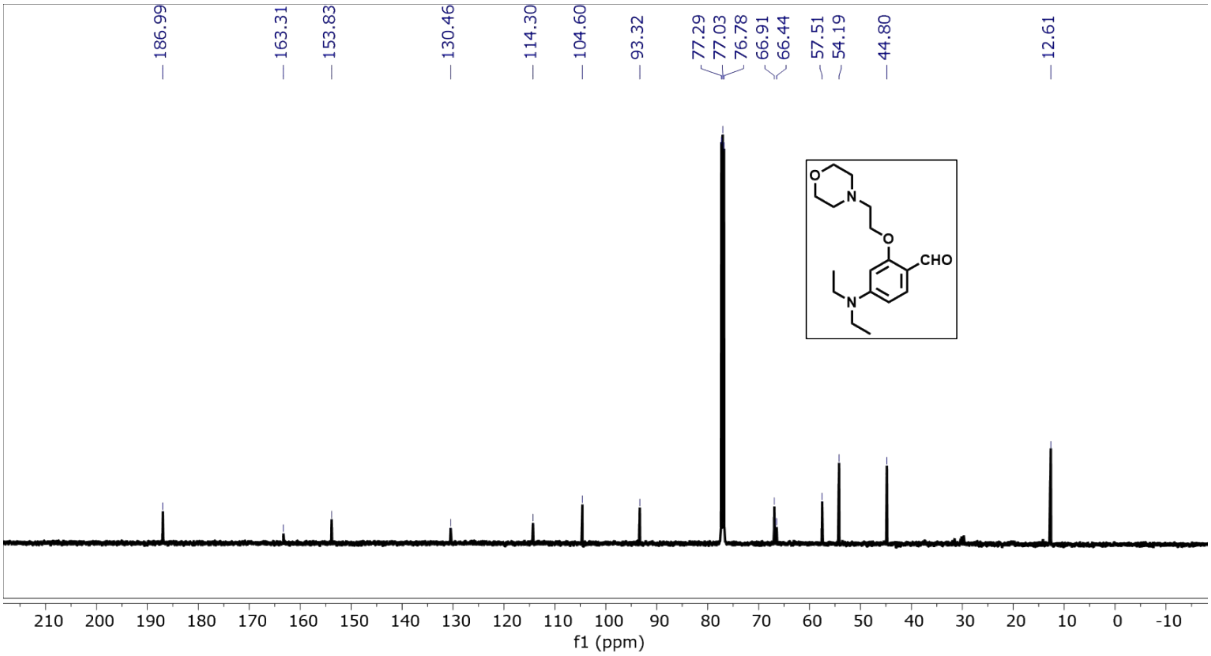

Figure S13. ^{13}C NMR spectrum of compound 3 in $CDCl_3$.

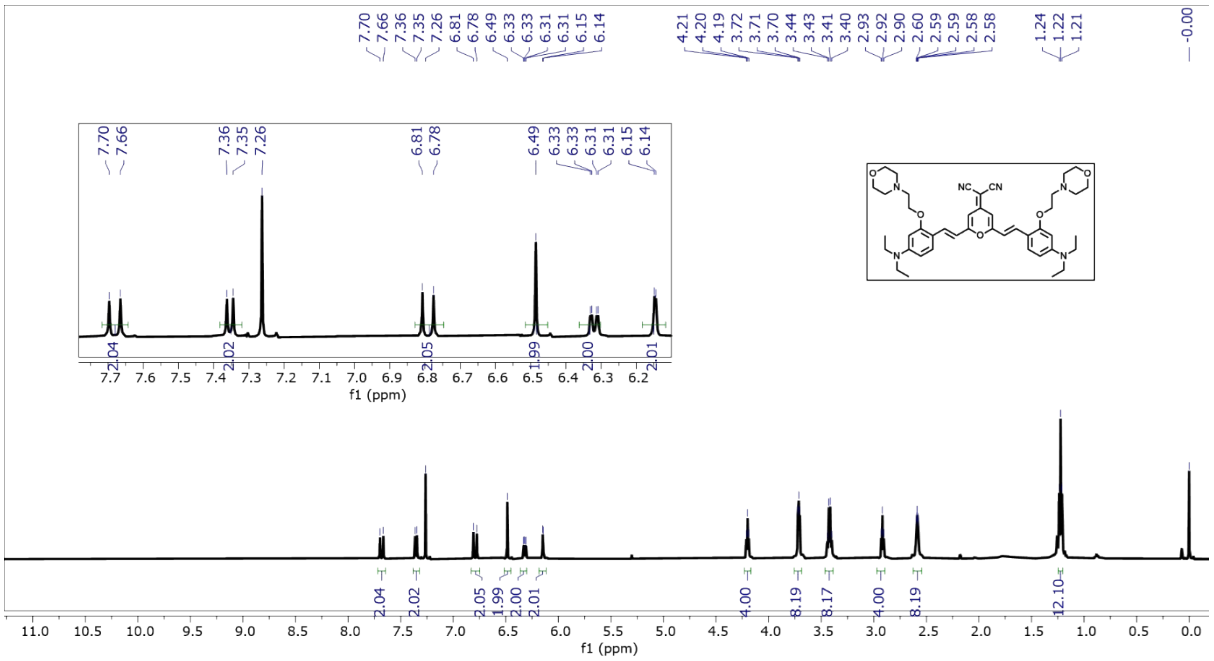

Figure S14. ^{1}H NMR spectrum of compound DM in $CDCl_3$.

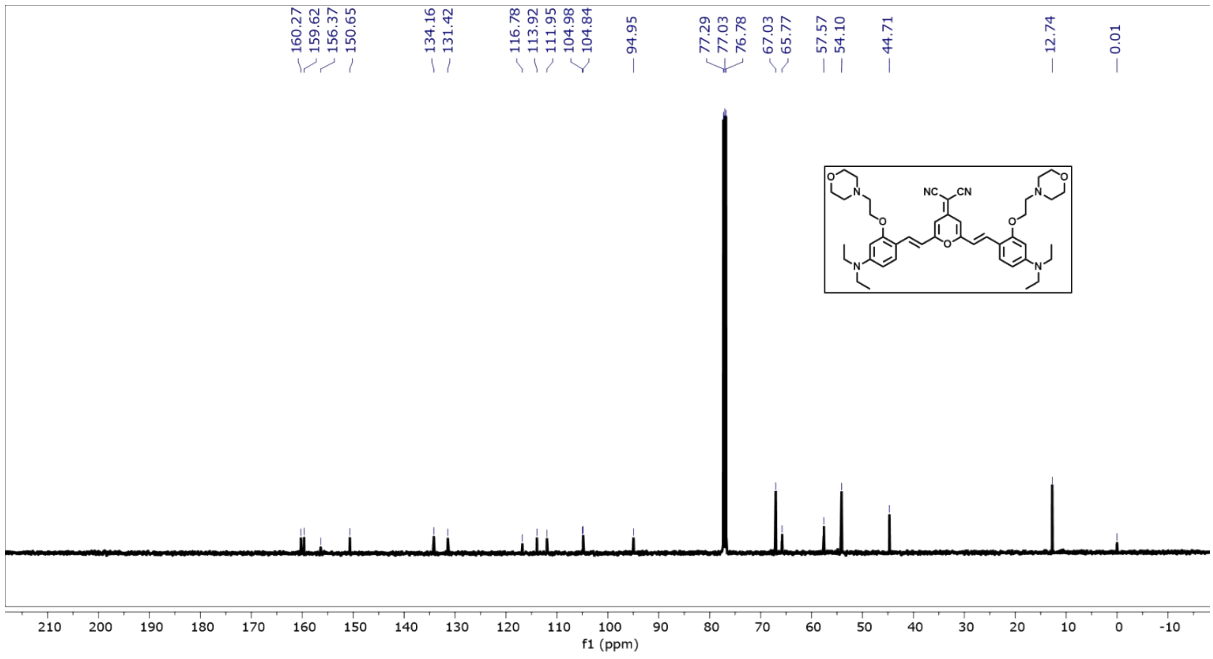

Figure S15. ^{13}C NMR spectrum of compound DM in $CDCl_3$.

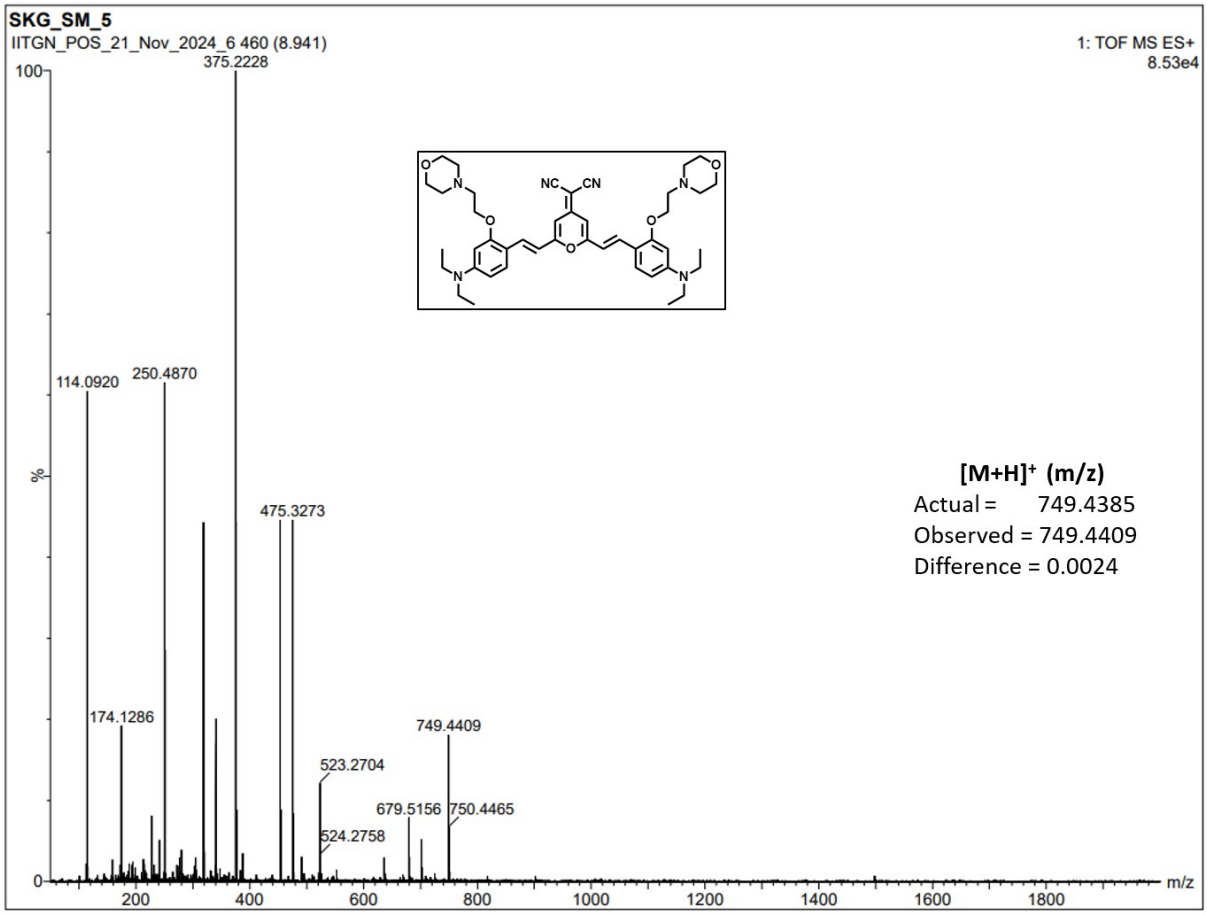

Figure S16. HR-MS spectrum of compound DM

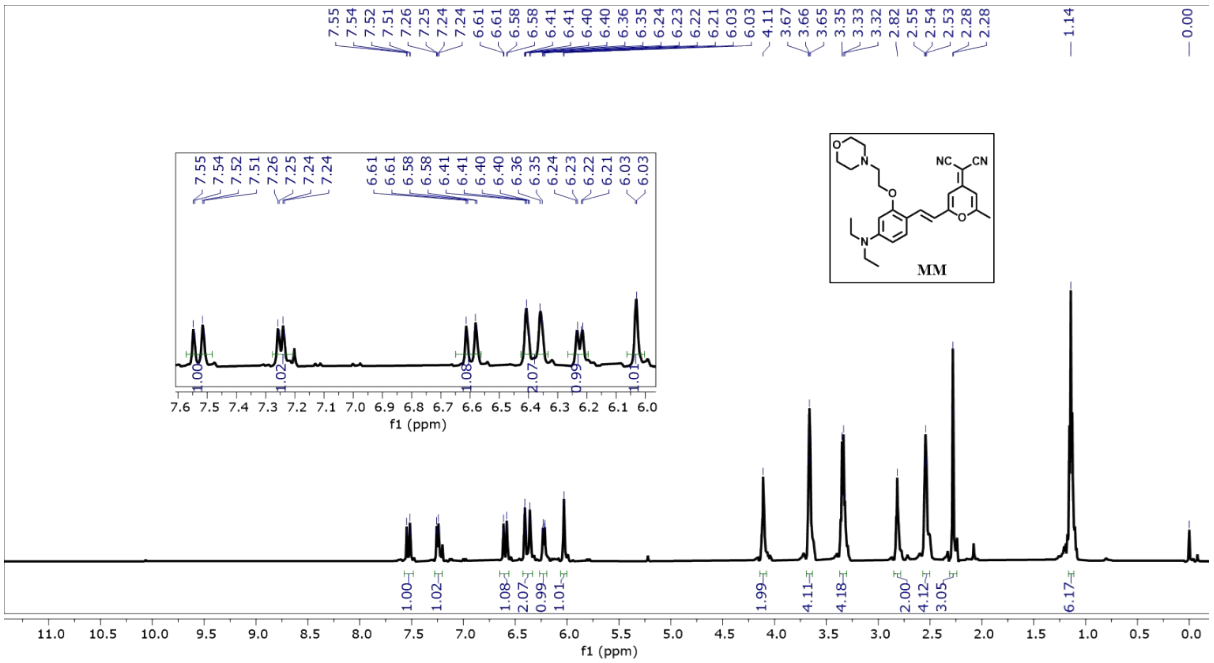

Figure S17. ^{1}H NMR spectrum of compound MM in $CDCl_3$.

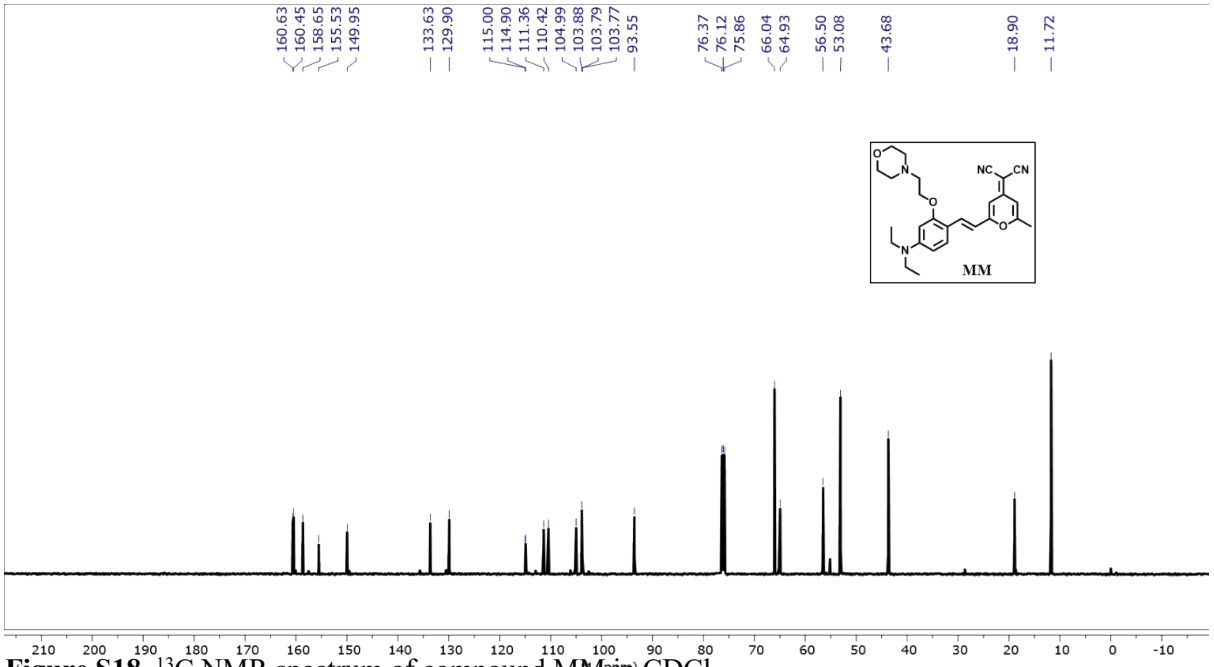

Figure S18. ^{13}C NMR spectrum of compound MM in $CDCl_3$.

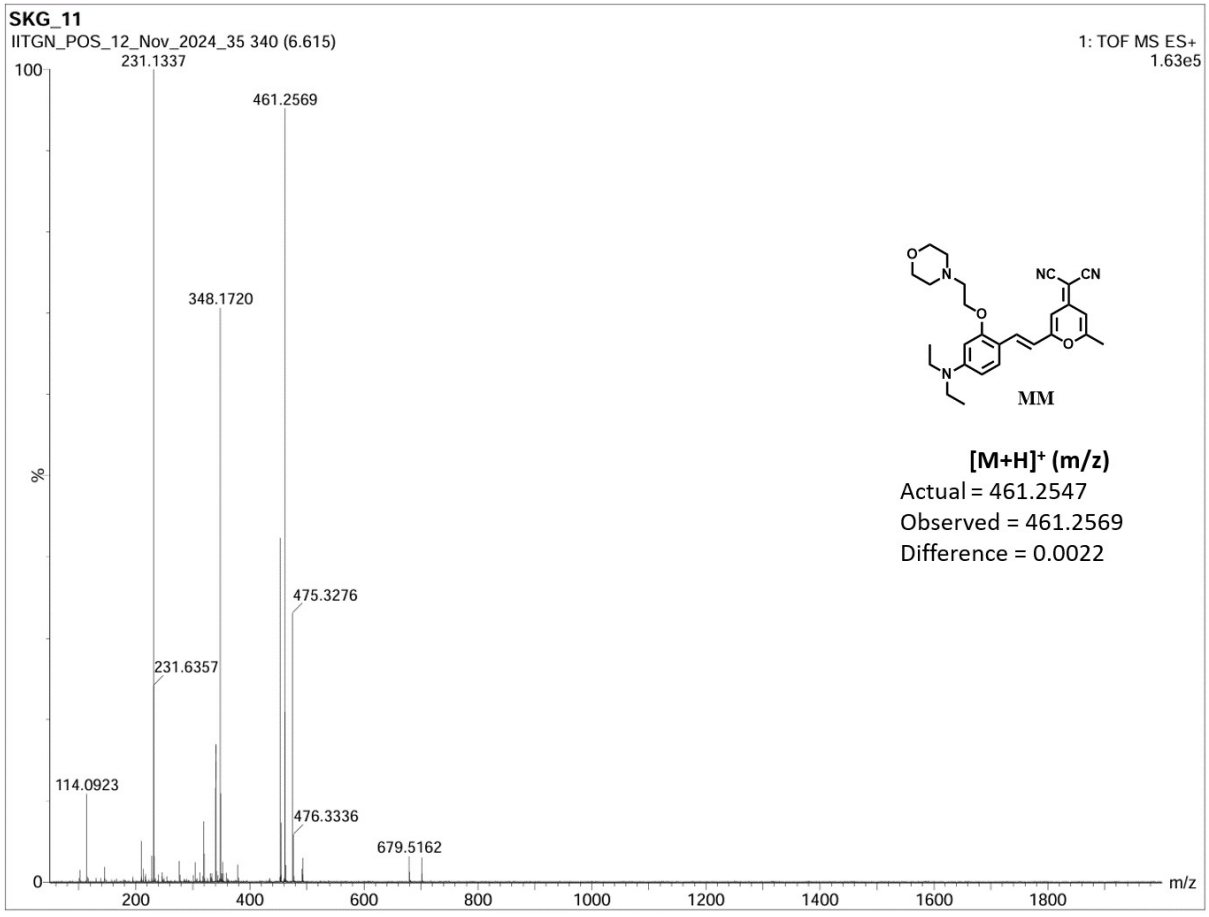

Figure S19. HR-MS spectrum of compound MM