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Supporting Information

Photocleavable Fluorescent Membrane Tension Probes: Fast Release with Spatiotemporal Control in Inner Leaflets of Plasma Membrane, Nuclear Envelope, and Secretory Pathway

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1. Materials and Methods

As in ref. S1 and S2. Briefly, reagents for synthesis were purchased from Fluka, Sigma-Aldrich, TCI and Across. PEG linker 14 was purchased from Broadpharm.com. Salts of the best grade available from Fluka or Sigma-Aldrich were used as received. Egg sphingomyelin (SM), 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC), and Mini-extruder were purchased from Avanti Polar Lipids, cholesterol (CL) was purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, pH = 7.4), DMEM (GlutaMAX, 4.5 g/L D-glucose, pyruvate, with phenol red) medium, FluoroBrite DMEM (high D-Glucose) medium, Leibovitz’s L-15 medium, Opti-MEM reduced serum medium with GlutaMAX supplement, Penicillin-Streptomycin, Fetal Bovine Serum, TrypLE Express Enzyme, Lipofectamine 2000, T4 DNA ligase, puromycin and ER-Tracker™ Blue-White were obtained from Thermo Fisher Scientific. Polyethyleneimine (linear, MW~25000) was obtained from Polysciences. 35 mm glass-bottom dishes were obtained from MatTek (P35G-0.170 14-C); 96-well μ-plates and μ-Slide 8-Well Glass Bottom was obtained from Ibidi. Column chromatography was carried out on silica gel 60 (SilicaFlash P60, 40-63 μm). Analytical (TLC) was performed on silica gel 60 (Merck, 0.2 mm).

IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer (ATR, Golden Gate) and are reported as wavenumbers ν in cm⁻¹ with band intensities indicated as s (strong), m (medium), w (weak), br (broad). All ¹H and ¹³C NMR spectra were recorded (as indicated) on a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer at room temperature (25 °C) and are reported as chemical shifts (δ) in ppm relative to TMS (δ = 0). Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t) and quartet (q) with coupling constants (J) given in Hz, or multiplet (m). Broad peaks are marked as br. ¹H and ¹³C resonances were assigned with the aid of additional information from 1D and 2D NMR spectra (H,H-NOESY, H,H-COSY, DEPT 135, HSQC and HMBC). ESI-MS for the characterization of new
compounds was performed on an ESI API 150EX. ESI-HRMS was measured on Xevo G2-S Tof (Waters). All mass data are reported as mass-per-charge ratio m/z (intensity in %, [assignment]). Analytical HPLC were recorded using a JASCO LC-2000 Plus system equipped with quaternary pump (JASCO PU-2089) and UV/Vis detector (JASCO UV-2077 Plus). Fluorescence measurements were performed using a FluoroMax-4 spectrofluorometer (Horiba Scientific) equipped with a stirrer and a temperature controller. All fluorescence spectra were background subtracted and corrected using correction factors supplied by the manufacturer. Fluorescence cellular imaging was performed using Leica SP8 DIVE confocal equipped with a white light laser as light source, HyDTM detectors and 63x oil immersion objective lens, or an IXM-C automated microscope from ImageXpress equipped with a Lumencor Aura III with solid-state light sources, bandpass filters and a 40x objective. Sample preparation and washing on 96-well plates was performed using a Plate washer Biotek EL406®. Fluorescence lifetime imaging microscopy (FLIM) was performed either on the Leica SP8 DIVE, at 20 MHz, with \( \lambda_{\text{exc}} = 488 \text{ nm} \) (white light laser), or on a Nikon Eclipse Ti A1R microscope upgraded with a FLIM kit from PicoQuant, equipped with a laser at \( \lambda_{\text{exc}} = 485 \text{ nm} \) (PicoQuant, LDH-D-C-485) at 20 MHz and a 100x oil immersion objective lens, and collecting the fluorescence between 550 and 650 nm. Flow cytometry was performed using a SONY SH800S flow cytometer. Bulk light irradiation was performed using a Thorlabs M365LP1 lamp (\( \lambda = 365 \text{ nm} \)) at 100% power.

**Abbreviations.** BSA: Bovine serum albumin; CAPA: Chloroalkane penetration assay; CL: Cholesterol; CLSM: Confocal laser scanning microscopy; DIPEA: Diisopropylethylamine; DMEM: Dulbecco’s Modified Eagle Medium; DMF: Dimethylformamide; DMSO: Dimethyl sulfoxide; DOPC: Dioleyl-sn-glycero-3-phosphocholine; ER: Endoplasmic reticulum; ESI: Electrospray ionization; FBS: Fetal bovine serum; FLIM: Fluorescence lifetime imaging; GA: Golgi apparatus; GSH: Glutathione; HK: HeLa Kyoto; HPLC: High pressure liquid chromatography; HRMS: High resolution mass
spectroscopy; LP: Laser power; LUVs: Large unilamellar vesicles; NLS: Nuclear localization sequence; PBS: Phosphate buffer saline; PCC: Pearson correlation coefficient; ROI: Region of interest; rt: Room temperature; SM: Egg sphingomyelin; TBTA: Tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine; TFA: Trifluoroacetic acid; Tris: tris(hydroxymethyl)aminomethane; Trx: Neutralized 1 mM Trolox.
2. **Synthesis**

**Scheme S1.** (a) Et₃N, CH₂Cl₂, rt, 12 h, 92%; (b) Et₃N, CH₂Cl₂, rt, 2 h, 85%; (c) TFA, CH₂Cl₂, rt, 1 h, quant.; (d) 12, Et₃N, CH₂Cl₂, rt, 2 h, 66%; (e) 18, Cu₂SO₄·5H₂O, TBTA, sodium ascorbate, rt, 1 h, 89%; (f) 14, HATU, DIPEA, DMF, rt, 1 h, 82%; (g) TFA, CH₂Cl₂, rt, 1 h, quant.; (h) 12, Et₃N, CH₂Cl₂, rt, 3 h, 50%; (i) 18, Cu₂SO₄·5H₂O, TBTA, sodium ascorbate, rt, 2 h, 98%.

**Compound 1** was synthesized according to procedures described in ref. S3.

**Compound 2** was synthesized according to procedures described in ref. S1.

**Compound 10** was synthesized according to procedures described in ref. S4.
Compound 18 was synthesized according to procedures described in ref. S3.

Compound 19 was synthesized according to procedures described in ref. S5.

Compound 23 was synthesized according to procedures described in ref. S6.

Compound 12. To a solution of 11 (125 mg, 0.420 mmol) in dry CH₂Cl₂ (4.0 mL), triethylamine (174 µL, 1.25 mmol) and then 10 (372 mg, 1.88 mmol) were added. The reaction mixture was stirred for 12 h at rt. Then, the crude product was directly purified by flash column chromatography (SiO₂, CH₂Cl₂/acetone 1 – 5% gradient). The product was obtained as a pale yellow sticky oil (184 mg, 92%). Rf (CH₂Cl₂/CH₃OH 9:1) = 0.50; IR (neat): 2934 (m), 2109 (s), 1814 (w), 1784 (w), 1735 (s), 1581 (w), 1519 (s), 1338 (m), 1275 (s), 1201 (s), 1074 (m); ¹H NMR (400 MHz, CDCl₃): 7.60 (s, 1H), 7.03 (s, 1H), 6.63 (q, 3J_H-H = 6.4 Hz, 1H), 4.18 (t, 3J_H-H = 6.0 Hz, 2H), 3.96 (s, 3H), 3.94 – 3.84 (m, 2H), 2.88 (t, 3J_H-H = 7.3 Hz, 2H), 2.84 (bs, 4H), 2.35 – 2.24 (m, 2H), 1.68 (d, 3J_H-H = 6.4 Hz, 3H); ¹³C NMR (101 MHz, CDCl₃): 169.2 (C), 168.2 (C), 167.1 (C), 154.4 (C), 147.5 (C), 139.9 (C), 132.3 (C), 109.6 (CH), 108.5 (CH), 70.2 (CH), 67.6 (CH₂), 56.5 (CH₃), 50.8 (CH₂), 27.7 (CH₂), 25.7 (CH₂), 24.3 (CH₂), 22.2 (CH₃); MS (ESI+): 981.3 (50, [2M+Na]⁺), 534.0 (60, [M+CH₃OH+Na]⁺), 502.3 (100, [M+Na]⁺).

Compound 15. To a solution of 13 (8.9 µL, 0.62 mmol) in dry CH₂Cl₂ (0.5 mL), a solution of 14 (69 mg, 0.59 mmol) and triethylamine (9.0 µL, 0.65 mmol) in dry CH₂Cl₂ (1.0 mL) was added dropwise, at rt. The solution was stirred at rt for 2 h. The mixture was diluted in more CH₂Cl₂ (10 mL) and washed with saturated NH₄Cl (2 × 10 mL), brine (2 × 10 mL), dried over Na₂SO₄ and concentrated under vacuum. Pure compound was obtained as a colorless liquid without further purification (68 mg, 85%). IR (neat): 3336 (w), 2867 (m), 1710 (w), 1648 (w), 1518 (w), 1502 (m), 1350 (w), 1324 (w), 1250 (m), 1097 (s), 991 (m), 948 (w); ¹H NMR (400 MHz, CDCl₃): 7.01 (bs, 1H), 5.01 (bs, 1H), 3.66 – 3.52 (m, 2H), 2.88 (t, 3J_H-H = 6.4 Hz, 3H), 1.40 (s, 9H); ¹³C NMR (101 MHz, CDCl₃): 157.5 (C), 156.0 (C), 145.3
(C), 142.8 (C), 140.8 (C), 138.8 (C), 136.8 (C), 79.1 (C), 70.6 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 69.4 (CH₂), 40.2 (CH₂), 28.5 (CH₃); ¹⁹F NMR (282 MHz, CDCl₃): -140.5 - -140.7 (m, 2F), -151.9 (t, ³J_{F-F} = 20.9 Hz, 1F), -160.5 - -160.8 (m, 2F); MS (ESI+): 1366.7 (20, [M+NH₄]⁺), 701.8 (100, [M+2NH₄]²⁺).

**Compound 16.** Compound 15 (60 mg, 0.44 mmol) was dissolved in CH₂Cl₂ (2.0 mL) and TFA (0.5 mL) and the mixture was stirred for 1 h. Then, the solvent was removed under vacuum, obtaining 16 (55 mg, quant.) as a colorless oil without further purification. IR (neat): 2870 (m), 1680 (m), 1518 (w), 1502 (m), 1350 (m), 1199 (w), 1097 (s), 991 (m), 948 (w); ¹H NMR (500 MHz, CD₃OD): 3.81 – 3.59 (m, 92H), 3.35 – 3.32 (m, 2H), 3.25 – 3.20 (m, 2H); ¹³C NMR (126 MHz, CD₃OD): 159.6 (C), 146.1 (C), 144.1 (C), 142.3 (C), 139.8 (C), 137.8 (C), 71.6 (CH₂), 71.5 (CH₂), 71.5 (CH₂), 71.4 (CH₂), 71.4 (CH₂), 71.3 (CH₂), 71.3 (CH₂), 71.2 (CH₂), 71.2 (CH₂), 71.1 (CH₂), 71.0 (CH₂), 70.8 (CH₂), 70.2 (CH₂), 68.0 (CH₂), 41.2 (CH₂), 40.7 (CH₂); ¹⁹F NMR (282 MHz, CD₃OD): -77.0 (s, 3F), -143.8 - -143.9 (m, 2F), -155.40 (t, ³J_{F-F} = 20.4, 1F), -163.7 - -164.0 (m, 2F); MS (ESI+): 1267.7 (10, [M+H]⁺), 643.5 (100, [M+H+NH₄]²⁺).

**Compound 17.** To a solution of 12 (12 mg, 0.25 mmol) in dry CH₂Cl₂ (2.0 mL), 16 (32 mg, 0.25 mmol) and triethylamine (14 µL, 1.0 mmol) were added, controlling the pH above 8. After 2 h, the reaction mixture was washed with HCl (0.1 M, 1 × 10 mL), and extracted in CH₂Cl₂ (3 × 10 mL), dried over Na₂SO₄ and the solvent was removed. Then, the crude product was directly purified by flash column chromatography (SiO₂, CH₂Cl₂/CH₃OH 1 – 4% gradient). The product was obtained as a pale yellow oil (27 mg, 66%). Rᵣ (CH₂Cl₂/CH₃OH 9:1) = 0.58; IR (neat): 3334 (w), 2868 (m), 2109 (m), 1747 (w), 1681 (m), 1518 (m), 1276 (m), 1098 (s), 991 (m), 949 (w); ¹H NMR (500 MHz, CDCl₃): 7.59 (s, 1H), 7.07 (bs, 1H), 7.02 (s, 1H), 6.62 (q, ³J_{H-H} = 6.4 Hz, 1H), 6.27 (bs, 1H), 4.12 (t, ³J_{H-H} = 6.3 Hz, 2H), 3.96 (s, 3H), 3.93
- 3.86 (m, 2H), 3.69 – 3.57 (m, 92H), 3.57 – 3.53 (m, 2H), 3.47 – 3.44 (m, 2H), 2.40 (t, $^3J_{H-H} = 7.6$ Hz, 2H), 2.24 – 2.15 (m, 2H), 1.68 (d, $^3J_{H-H} = 6.4$ Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): 172.1 (C), 167.1 (C), 157.6 (C), 154.2 (C), 147.8 (C), 145.2 (C), 143.2 (C), 141.2 (C), 140.0 (C), 138.7 (C), 136.7 (C), 131.9 (C), 109.2 (CH), 108.4 (CH), 70.7 (CH$_2$), 70.7 (CH$_2$), 70.7 (CH$_2$), 70.6 (CH$_2$), 70.6 (CH$_2$), 70.6 (CH$_2$), 70.5 (CH$_2$), 70.4 (CH$_2$), 70.2 (CH), 70.0 (CH$_2$), 69.5 (CH$_2$), 68.8 (CH$_2$), 56.5 (CH$_3$), 50.2 (CH$_2$), 40.3 (CH$_2$), 39.4 (CH$_2$), 32.6 (CH$_2$), 24.9 (CH$_2$), 22.2 (CH$_3$); $^{19}$F NMR (282 MHz, CDCl$_3$): -140.3 – -140.8 (m, 2F), -151.8 (t, $^3J_{F-F} = 20.7$ Hz, 1F), -160.4 – -160.8 (m, 2F); HRMS (ESI+) calcd for C$_{70}$H$_{115}$F$_5$N$_6$O$_3$1 [M+2H]$^{2+}$: 816.3837, found: 816.3835.

**Compound 3.** To a solution of 17 (26.9 mg, 16.5 µmol), 18 (7.3 mg, 13 µmol) and TBTA (2.0 mg, 3.8 µmol) in CH$_2$Cl$_2$ (1.0 mL), a solution of CuSO$_4$·5H$_2$O (3.2 mg, 13 µmol) and sodium ascorbate (2.5 mg, 13 µmol) in water (50 µL) was added. The mixture was vigorously stirred at rt and under nitrogen atmosphere for 1 h. Then, it was diluted with CH$_2$Cl$_2$ (10 mL) and dried over Na$_2$SO$_4$. The crude product was concentrated under reduced pressure and purified by flash column chromatography (SiO$_2$, CH$_2$Cl$_2$/CH$_3$OH 99:1 – 88:12 gradient) and reverse phase flash column chromatography (Scorpius C18e-HP, H$_2$O + 0.1% TFA/MeCN +0.1% TFA 90:5 – 20:80 gradient) to yield 3 (25.0 mg, 89%) as a red oil. $R_f$ (CH$_2$Cl$_2$/CH$_3$OH 9:1) = 0.55; IR (neat): 2869 (m), 2212 (w), 1754 (w), 1675 (m), 1518 (m), 1318 (w), 1276 (w), 1096 (s), 991 (m), 948 (w); $^1$H NMR (500 MHz, CDCl$_3$): 7.73 (s, 1H), 7.56 (s, 1H), 7.53 – 7.37 (b, 1H), 6.85 (s, 1H), 6.59 (q, $^3J_{H-H} = 6.3$ Hz, 1H), 6.53 – 6.45 (bs, 1H), 5.25 – 5.16 (m, 2H), 4.78 (s, 2H), 4.73 (s, 2H), 4.09 (t, $^3J_{H-H} = 6.3$ Hz, 2H), 3.95 (s, 3H), 3.79 – 3.47 (m, 94H), 3.47 – 3.41 (m, 2H), 2.60 (s, 3H), 2.44 – 2.37 (m, 5H), 2.34 (s, 6H), 2.21 – 2.13 (m, 2H), 1.62 (d, $^3J_{H-H} = 6.3$ Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): 172.3 (C), 165.1 (C), 157.8 (C), 154.3 (C), 147.8 (C), 145.5 (C), 145.3 (C), 145.2 (C), 143.8 (C), 143.2 (C), 143.2 (C), 142.5 (C), 141.4 (C), 141.1 (C), 140.3 (C), 139.8 (C), 138.7 (C), 138.2 (C), 135.7 (C), 132.6 (C), 132.5 (C), 24.9 (CH$_2$), 22.2 (CH$_3$); $^{19}$F NMR (282 MHz, CDCl$_3$): -140.3 – -140.8 (m, 2F), -151.8 (t, $^3J_{F-F} = 20.7$ Hz, 1F), -160.4 – -160.8 (m, 2F); HRMS (ESI+) calcd for C$_{70}$H$_{115}$F$_5$N$_6$O$_3$1 [M+2H]$^{2+}$: 816.3837, found: 816.3835.
Compound 20. To a solution of 19 (33.6 mg, 104 µmol) in dry DMF (1.0 mL), HATU (39.2 mg, 103 µmol) and DIPEA (13.2 mg, 102 µmol) were added. After 30 seconds, 14 (99.3 mg, 84.6 µmol) was added and the reaction mixture was stirred at rt for 1 h. The reaction progress was monitored by LCMS. After the completion of the reaction was detected, the mixture was diluted with ethyl acetate (10 mL), washed with saturated NaHCO₃ (2 × 5 mL), citric acid (10%, 2 × 5 mL), H₂O (1 × 5 mL), LiCl (5%, 2 × 5 mL), and brine (1 × 5 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated. The remaining solid was purified by flash column chromatography (SiO₂, CH₂Cl₂/CH₃OH 0 – 20%) to yield 20 as a colorless oil (103 mg, 82%). Rₜ (CH₂Cl₂/CH₃OH 9:1) = 0.43; IR (neat): 3303 (w), 2883 (m), 1631 (m), 1553 (w), 1466 (w), 1343 (m), 1280 (m), 1103 (s), 961 (m), 852 (m); ¹H NMR (400 MHz, CDCl₃): 6.47 (t, 3 J_H-H = 5.7 Hz, 1H), 6.38 (t, 3 J_H-H = 5.7 Hz, 1H), 5.10 – 4.95 (bs, 1H), 3.81 – 3.34 (m, 106H), 3.28 – 3.21 (m, 2H), 2.46 (s, 4H), 1.77 – 1.68 (m, 2H), 1.60 – 1.51 (m, 2H), 1.46 – 1.40 (m, 2H), 1.39 (s, 9H), 1.36 – 1.29 (m, 2H); ¹³C NMR (101 MHz, CDCl₃): 172.1 (C), 156.0 (C), 79.1 (C), 71.3 (CH₂), 70.6 (CH₂), 70.3 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 70.0 (CH₂), 69.8 (CH₂), 45.0 (CH₂), 40.4 (CH₂) 39.3 (CH₂), 39.3 (CH₂), 32.5 (CH₂), 31.6 (CH₂), 31.6 (CH₂), 29.4 (CH₂), 28.4 (CH₃), 26.7 (CH₂), 25.4 (CH₂); MS (ESI+): 1495.7 (10, [M+NH₄⁺]), 757.1 (80, [M+2NH₄]²⁺), 505.2 (100, [M+H+2NH₄]³⁺).
**Compound 21.** Compound 20 (93.6 mg, 63.3 µmol) was dissolved in CH₂Cl₂ (2.0 mL) and TFA (0.4 mL) and the mixture was stirred for 1 h. Then, the solvent was removed under vacuum, to give 21 (104 mg, *quant.*) as a colorless oil without further purification. IR (neat): 2866 (m), 1662 (m), 1542 (w), 1454 (w), 1345 (m), 1298 (w), 1250 (w), 1200 (m), 1101 (s), 948 (m); ¹H NMR (500 MHz, CDCl₃): 8.11 – 7.86 (bs, 3H), 7.00 (t, 3J_H-H = 5.4 Hz, 1H), 6.69 (t, 3J_H-H = 5.6 Hz, 1H), 3.81 – 3.37 (m, 106H), 3.19 – 3.14 (m, 2H), 2.51 – 2.47 (m, 4H), 1.79 – 1.72 (m, 2H), 1.62 – 1.55 (m, 2H), 1.47 – 1.40 (m, 2H), 1.38 – 1.31 (m, 2H); ¹³C NMR (126 MHz, CDCl₃): 172.5 (C), 71.4 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.3 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 70.2 (CH₂), 70.2 (CH₂), 70.1 (CH₂), 70.1 (CH₂), 70.1 (CH₂), 70.0 (CH₂), 70.0 (CH₂), 69.9 (CH₂), 69.9 (CH₂), 67.1 (CH₂), 45.1 (CH₂), 40.1 (CH₂), 39.4 (CH₂), 39.3 (CH₂), 32.6 (CH₂), 31.8 (CH₂), 31.6 (CH₂), 29.5 (CH₂), 26.7 (CH₂), 25.5 (CH₂); MS (ESI+): 1378.8 (10, [M+H]+), 698.6 (90, [M+H+NH₄]²⁺), 690.1 (60, [M+2H]²⁺), 466.5 (100, [M+2H+NH₄]³⁺).

**Compound 22.** To a solution of 12 (32.4 mg, 67.6 µmol) in dry CH₂Cl₂ (4.0 mL), 21 (100 mg, 67.0 µmol) and then triethylamine (20.0 µL, 145 µmol) were added, controlling the pH above 8. Then, the reaction was stirred at rt for 3 h. The reaction mixture was washed with saturated NH₄Cl (3 × 5 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated. The residue was purified by flash column chromatography (SiO₂, CH₂Cl₂/CH₃OH 0 – 12%), to give 22 as a colorless oil (57 mg, 50%). Rᵣ (CH₂Cl₂/CH₃OH 9:1) = 0.43; IR (neat): 3343 (w) 2866 (m), 2108 (m), 1745 (w), 1654 (m), 1521 (m), 1455 (w), 1348 (w), 1277 (m), 1098 (s), 949 (m); ¹H NMR (500 MHz, CDCl₃): 7.58 (s, 1H), 7.02 (s, 1H), 6.62 (q, 3J_H-H = 6.4 Hz, 1H), 6.59 – 6.54 (bs, 1H), 6.44 – 6.36 (m, 2H), 4.11 (t, 3J_H-H = 6.3 Hz, 2H), 3.95 (s, 3H), 3.93 – 3.85 (m, 2H), 3.68 – 3.40 (m, 108H), 2.52 – 2.50 (m, 4H), 2.40 (t, 3J_H-H = 6.9 Hz, 2H), 2.23 – 2.15 (m, 2H), 1.81 – 1.73 (m, 2H), 1.68 (d, 3J_H-H = 6.4 Hz, 3H), 1.64 – 1.57 (m, 2H), 1.49 – 1.42 (m, 2H), 1.40 – 1.34 (m, 2H); ¹³C NMR (126 MHz, CDCl₃): 172.3 (C),
Compound 4. To a solution of 22 (27.3 mg, 15.7 µmol), 18 (6.0 mg, 11 µmol) and in CH₂Cl₂ (1.0 mL), a solution of CuSO₄·5H₂O (2.6 mg, 11 µmol) and sodium ascorbate (2.1 mg, 11 µmol) in water (100 µL) was added. After adding TBTA (1.8 mg, 3.4 µmol), the mixture was vigorously stirred at rt and under nitrogen for 1 h. Then, more CuSO₄·5H₂O (1.3 mg, 5.3 µmol) and sodium ascorbate (1.1 mg, 5.4 µmol) in H₂O (50 µL) were added, and the mixture was stirred for extra 1 h. The crude product was concentrated under reduced pressure and purified by flash column chromatography (SiO₂, CH₂Cl₂/CH₃OH 100:0 – 88:12 gradient) and reverse phase flash column chromatography (Scorpius C18e-HP, H₂O + 0.1% TFA/MeCN +0.1% TFA 90:5 – 10:90 gradient) to yield 4 (23.6 mg, 98%) as a red oil. Rᵣ (CH₂Cl₂/CH₃OH 9:1) = 0.46; IR (neat): 2866 (m), 2213 (w), 1753 (w), 1655 (m), 1521 (m), 1449 (w), 1276 (m), 1202 (m), 1093 (s), 948 (m); ¹H NMR (500 MHz, CDCl₃): 7.73 (s, 1H), 7.56 (s, 1H), 6.95 – 6.87 (bs, 1H), 6.85 (s, 1H), 6.62 – 6.56 (m, 2H), 6.52 (t, 3JH-H = 5.4 Hz, 1H), 5.24 – 5.16 (m, 2H), 4.78 (s, 2H), 4.73 (s, 2H), 4.09 (t, 3JH-H = 6.3 Hz, 2H), 3.95 (s, 3H), 3.68 – 3.39 (m, 108H), 2.60 (s, 3H), 2.51 (s, 4H), 2.44 – 2.37 (m, 5H), 2.34 (s, 6H), 2.20 – 2.13 (m, 2H), 1.80 – 1.73 (m, 2H), 1.62 (d, 3JH-H = 6.5 Hz, 3H), 1.61 – 1.56 (m, 2H), 1.48 – 1.41 (m, 2H), 1.40 – 1.34 (m, 2H); ¹³C NMR (126 MHz, CDCl₃): 172.6 (C), 172.4 (C), 172.4 (C), 165.1 (C), 154.3 (C), 147.8 (C), 145.5 (C), 145.3 (C), 143.8 (C), 143.2 (C), 142.5 (C), 141.4 (C), 140.3 (C), 139.8 (C), 138.2 (C), 135.7 (C), 132.6 (C), 132.5 (C), 132.1 (C), 131.4 (C), 131.3 (C), 129.5 (C), 128.9 (C), 125.7 (C), 124.3 (CH), 112.6 (C), 109.1 (CH), 109.0 (C), 108.2 (CH), 71.4 (CH₂), 172.2 (C), 172.1 (C), 167.1 (C), 154.2 (C), 147.8 (C), 140.0 (C), 131.9 (C), 109.2 (CH), 108.4 (CH), 71.4 (CH₂), 70.7 (CH₂), 70.7 (CH₂), 70.6 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 70.0 (CH₂), 69.9 (CH₂), 68.8 (CH), 56.5 (CH₃), 50.8 (CH₂), 45.2 (CH₂), 39.4 (CH₂), 39.4 (CH₂), 32.7 (CH₂), 32.6 (CH₂), 31.8 (CH₂), 31.7 (CH₂), 29.6 (CH₂), 26.8 (CH₂), 25.5 (CH₂), 24.9 (CH₂), 22.2 (CH₃); HRMS (ESI⁺) calcd for C₇₇H₁₄₀ClN₇O₃₄ [M+2H]²⁺: 871.9638, found: 871.9622.
70.8 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 70.1 (CH₂), 70.1 (CH₂), 69.8 (CH₂), 68.8 (CH), 65.5 (CH₂), 63.3 (CH₂), 56.7 (CH₃), 51.2 (CH₂), 45.2 (CH₂), 39.4 (CH₂), 39.3 (CH₂), 32.6 (CH₂), 32.5 (CH₂), 31.8 (CH₂), 31.7 (CH₂), 29.6 (CH₂), 26.8 (CH₂), 25.5 (CH₂), 24.9 (CH₂), 22.0 (CH₃), 14.2 (CH₃), 13.3 (CH₃), 13.0 (CH₃), 12.5 (CH₃); HRMS (ESI+) calcd for C₁₆₂H₁₅₇ClN₈O₃₇S₆ [M+2H]²⁺: 1157.4404, found: 1157.4380.

3. **Photo-Release of the Probes in Presence of LUVs**

   **LUVs preparation.** As in reference S7, LUV stock solutions were prepared by evaporating a solution of DOPC (23 mg) or SM/CL (14.8 mg and 3.5 mg, respectively) in CHCl₃/CH₃OH 9:1 (1 mL) on a rotary evaporator and then under vacuum overnight. The resulting film was hydrated with a buffer solution (1.0 mL, 10 mM Tris, 100 mM NaCl, pH 7.4) for 30 min at rt, subjected to freeze-thaw cycles (10×, liquid N₂, 55 °C water bath) and extruded (15×) through a polycarbonate membrane (pore size, 100 nm). Hydration and extrusion were performed at rt for DOPC LUVs and at 65 °C for SM/CL LUVs.

   **Fluorescence spectroscopy.** LUVs (5 μL of 30 mM lipid) were added to a gently stirred buffer solution (2.0 mL, 10 mM Tris, 100 mM NaCl, 5 mM GSH, 2 mM Trolox, pH 7.4) at 25 °C in a quartz cuvette. Emission and excitation spectra (λₑₓ = 450 nm, λₑₘ = 600 nm) were recorded to serve as backgrounds. Slits of 5 nm were used for both excitation and emission in all experiments. Afterwards, the probe 3 (2 μL of 100 μM stock solution in DMSO) was added and, after 10 min of equilibration, emission and excitation spectra were recorded. Then, the stirred solution was irradiated with direct bulk illumination (λ = 365 nm, 100% power) in different time intervals and emission and excitation spectra were recorded afterwards.
Figure S1. (a,b) Evolution of the emission spectra of 100 nM probe 3 in solutions of 75 µM DOPC (a) and SM/CL (b) LUVs upon bulk irradiation from 0 to 20 min. (c) Kinetics of partitioning of probe 3 in SM/CL LUVs as a function of irradiation time, extracted from excitation spectrum in Figure 2B.

HPLC analysis. A solution of probe 3 or 4 (500 nM) in buffer (1.0 mL, 10 mM Tris, 100 mM NaCl, 5 mM GSH, 300 µM DOPC LUVs, 2 mM Trolox, pH 7.4) in a glass vial was irradiated with direct bulk illumination (λ = 365 nm, 100% power) at different time intervals. Before irradiation and after each irradiation cycle, an aliquot (100 µL) is injected in the HPLC for analysis using the following conditions: flow 1.0 mL/min with a linear elution gradient from 50% H₂O/50% CH₃CN + 0.1% TFA to 100% CH₃CN + 0.1% TFA in 4 min, then 100% CH₃CN + 0.1% TFA for 1 min and finally return to 50% H₂O/50% CH₃CN + 0.1% TFA, λabs at 410 nm.

Figure S2. From bottom to top, HPLC traces of probe 3 (a, 500 nM) and probe 4 (b, 500 nM) before, 2 min, 4 min and 6 min after bulk irradiation.
4. **Cell Lines, DNA Transfection and Preparation Protocols**

All cells were used until maximum 25\textsuperscript{th} passage.

**HeLa Kyoto (HK) cells.** Cells were cultured in 25 cm\textsuperscript{2} cell culture flasks with a vent cap and grew in high glucose, pyruvate-supplemented DMEM (with GlutaMAX) + 10% FBS + 1% Pen/Strep.

**HeLa cells stably expressing the HaloTag-GFP-Mito fusion protein (HGM).** The cells were originally designed by the Chenoweth lab\textsuperscript{S8} and cultured in 25 cm\textsuperscript{2} cell culture flasks with a vent cap and grew in high glucose, pyruvate-supplemented DMEM (with GlutaMAX) + 10% FBS + 1% Pen/Strep. A selection was performed by complementing the media with puromycin (1 µg/mL) during the first 2–3 passages.

**HeLa cells stably expressing the HaloTag-NLS fusion protein.**

*Preparation of pMCB320-HaloTag-2xNLS plasmid.* To produce a lentiviral plasmid encoding HaloTag-2xNLS, the HaloTag sequence was amplified by PCR from pTRIPZ (M)-HT-NLS plasmid (Addgene #82518), to include a 5’AgeI restriction site, and a 3’ 2xNLS and EcoRI restriction site. pMCB320 plasmid (Addgene #89359) was digested two times independently, using EcoRI and NheI (to yield linear vector lacking PuroR-T2A-mCherry, 7500 bp) or NheI and AgeI (to yield PuroR-T2A, 1100 bp) restriction sites, respectively. The corresponding fragments were recovered and the full plasmid reconstructed by insertion of PuroR-T2A and the HaloTag-2xNLS fragments through ligation of the three fragments using T4 DNA ligase. The final plasmid was verified by sequencing.

*Production of lentivirus.* HEK293T cells (grown in same media as HK) were co-transfected with lentiviral plasmid pMCB320-HaloTag-2xNLS and packaging plasmids (pMD2.G, pRSV-Rev, pMDLg/RRE; Addgene #12259, #12253 and #12251, respectively) to produce VSVG-pseudotyped lentiviral particles. Following transfection with polyethyleneimine, fresh medium was added after 24h, and virus-containing supernatant
collected after 48h. The supernatant was filtered through a 0.45 µm polyethersulfone membrane filter and either used directly or stored at -80 °C.

PCR primers:
5’-CGTCAGATCGACC GGTC-3’
5’-GAATTCCTCAGTCCACTTTCCGCTTTTTCTTAGATCTTCCACCTTGCGTTTTTTC TTGGGCCTGCAAGGC CGC GG AAATCTCGAGCG-3’

Transduction. HK cells were transduced with the lentivirus-containing supernatant diluted in polybrene-complemented medium (4 µg/mL) at the appropriate titer. Following 24h incubation at 37 °C, lentivirus-containing medium was removed. Selection of transduced cells was started 48 hours after transduction by complementing the medium with puromycin (1.75 µg/mL). Multiplicity of infection was determined by flow cytometry-based analysis of unselected cells. For this, cells were incubated for 15 min prior to the flow cytometry analysis with 23 (1 µM). Transduced HK were grown with media complemented with puromycin (1 µg/mL) to ensure continuous HaloTag-2xNLS expression.

General procedure for transient transfection. As described in ref. S1, HK cells were seeded at 5 ×10^4 cells/mL in DMEM + 10% FBS + 1% Pen/Strep on 35 mm glass bottom dishes (2 mL per dish) and kept at 37 °C with 5% CO2 overnight. The next day, transient transfection was performed by diluting the cells with Lipofectamine® 2000 – DNA complexes prepared as follows: Lipofectamine® 2000 (2 μL) was diluted with Opti-MEM/GlutaMAX reduced serum medium (150 μL) and left incubate at rt for 5 min. DNA (0.6 – 1.0 µg) was diluted with Opti-MEM/GlutaMAX reduced serum medium (150 μL), gently mixed and added to the diluted Lipofectamine solution. The resulting solution was gently mixed and incubated for 20 min at rt. From each dish, 900 μL of the original medium was kept. The solution of DNA-Lipofectamine complex (300 μL each) was added to the dish. The cells were kept at 37 °C with 5% CO2. After 4 h, the medium was exchanged with fresh DMEM + 10% FBS + 1%
Pen/Strep and the cells were incubated overnight. Plasmid encoding for ST-HaloTag-HA was a gift from D. Toomre\textsuperscript{89} and plasmid encoding for Halo-TNFa-RUSH\textsuperscript{810} was a gift from J. Lippincott-Schwartz and acquired in Addgene (plasmid # 166901).

**General procedure for microscopy experiments.** For CLSM and FLIM experiments, cells were seeded at $8 \times 10^4$ cells/mL in DMEM (with GlutaMAX) + 10% FBS + 1% Pen/Strep on 35 mm glass bottom dishes (2 mL per dish) the day before the experiment (or $5 \times 10^4$ cells/mL two days before the experiment for transient transfection the day in between) and kept at 37 °C with 5% CO$_2$ overnight. For CAPA, HGM cells were seeded at $8 \times 10^4$ cells/mL in FluoroBrite DMEM + 10% FBS on μ-Plates 96-well ibiTreat sterile and kept at 37 °C with 5% CO$_2$ overnight.

5. **Labeling and Release in Living Cells**

Media supplemented with neutralized Trolox (1 mM) are marked “+Trx”.

5.1. **Colocalization of Probe 3 in ER**

Seeded HK cells were washed with Leibovitz's medium +Trx (3 × 1 mL) and incubated with 3 (1 μM) in Leibovitz's medium +Trx (1 mL) for 15 min. Then, cells were washed with Leibovitz’s medium and incubated with ER tracker Blue-White (0.5 μM) in Leibovitz's medium +Trx (1 mL) for extra 15 min. Finally, the cells were washed with Leibovitz's medium +Trx (3 × 1 mL).

PCC was calculated using the Coloc2 tool from ImageJ using the flipper channel and the ER tracker channel.
Figure S3. (a,b) CLSM images of HK cells after incubation with 3 (1 μM, 15 min) and ER-Tracer Blue-White (0.5 μM, 15 min). Left, green: ER-Tracer Blue-White channel; middle, red: flipper channel; right, green+red: merged channels. ER tracker: \( \lambda_{ex} = 405 \text{ nm}, \ LP = 2\%; \ \lambda_{em} = 436 - 496 \text{ nm}. \) Flipper: \( \lambda_{ex} = 488 \text{ nm}, \ LP = 30\%; \ \lambda_{em} = 550 - 700 \text{ nm}. \) Scale bar: 10 μm.

5.2. Release of Probe 3 from ER

On the day of the experiment, HK cells were washed with DMEM medium (with 10% FBS) +Trx (3 × 1 mL) and kept in the same medium at 37 °C with 5% CO₂ for 2 h. Then, cells were washed with Leibovitz's medium +Trx (3 × 1 mL) and incubated with 3 (2.5 μM, 15 min). Afterwards, cells were washed with Leibovitz's medium (with 10% FBS) +Trx (3 × 1 mL) and kept in Leibovitz's medium +Trx (1 mL) for FLIM imaging. A set of five images were acquired in different locations of the dish. Then, the dish was irradiated with bulk illumination (\( \lambda = 365 \text{ nm}, \ 100\% \text{ power} \)) for 2.5 min and cells were washed again with Leibovitz's medium +Trx (with 10% FBS) (3 × 1 mL). Cells were kept in Leibovitz's medium +Trx (1 mL) for imaging. Up to five images were acquired in different locations of the dish. Then, 1 (1 μL, 1 mM stock solution in DMSO, 1 μM in the dish) was added and the images at same positions in the dish were acquired after 2 min. Finally, the medium was carefully exchanged with Leibovitz's medium.
+Trx (with 10% FBS, 5 × 1 mL), and images were acquired again in the same previous positions.

For analysis, a pixel binning to two points and a threshold was applied to increase the number of photons per pixel and to exclude background pixels during the analysis, respectively. A biexponential reconvolution model was used for the fitting. The values of \( \tau_1 \) and \( \tau_{av} \) reported in table S1 are those directly obtained from the fitted curve, presented as the mean ± standard deviation from 5 pictures containing at least 6 cells each. Then, a FLIM fit of one of the images under each condition was performed to generate the histogram and images corresponding to the fitting of \( \tau_1 \) (Figure 3). To guide the eye, the reconvoluted histograms were fitted using Lorentzian regression curves.

**Table S1.** Lifetime values of 3, in the presence or absence of 1, under the different conditions tested.

| Conditions             | \( \tau_{av} / \text{ns} \) | \( \tau_1 / \text{ns} \) |
|------------------------|-----------------------------|-------------------------|
| before irradiation     | 3.71 ± 0.06                 | 4.56 ± 0.05             |
| after irradiation      | 4.71 ± 0.05                 | 5.37 ± 0.02             |
| after addition of 1     | 4.94 ± 0.02                 | 5.52 ± 0.01             |
| after washing with FBS | 4.74 ± 0.06                 | 5.38 ± 0.04             |

### 5.3. Control Experiment for the Intermembrane Transfer of Probe 1

Excitation spectrum of an equilibrated solution of 1 (100 nM) in buffer (2.0 mL, 10 mM Tris, 100 mM NaCl, pH 7.4) containing SM/CL 7:3 LUVs (75 µM) was recorded (\( \lambda_{em} = 600 \text{ nm}, 5 \text{ nm slit} \)). Then, a stock solution of DOPC LUVs (5 µL of 30 mM lipid) was added and the spectra were recorded over a period of 5 min, in intervals of 30 s. Excitation spectra of the LUV solutions without the probe were used as their corresponding backgrounds.
Time \((t)\) after addition of DOPC LUVs was plotted against the excitation intensity at 485 nm \((I_t)\) and the values were fitted using equation S1 with \(I_0\) being excitation intensity at \(t = 0\) min, \(I_\infty\) the excitation intensity at very long times and \(k\) the rate constant.

\[
I_t = (I_0 - I_\infty) \cdot e^{(-k \cdot t)} + I_\infty \quad (S1)
\]

Calculated \(k = 1.51 \text{ min}^{-1}\).

**Figure S4.** Kinetics of equilibration of 1 (100 nM), illustrating the evolution of the excitation intensity of the probe at \(\lambda = 485\) nm in SM/CL LUVs (75 \(\mu\)M) upon addition of DOPC LUVs (75 \(\mu\)M).

**5.4. Control Experiment for the Extraction of Probe 1 with BSA**

*Extraction of 1 with BSA from LUVs.* Excitation spectrum of an equilibrated solution of 1 (40 nM) in buffer (2.0 mL, 10 mM Tris, 100 mM NaCl, pH 7.4) containing SM/CL 7:3 LUVs (75 \(\mu\)M) was recorded \((\lambda_{em} = 600\) nm, 5 nm slit). Then, increasing volume of a stock solution of BSA in distilled water (0.5 mM, 0 to 80 \(\mu\)L) was added to the solution, and excitation spectra were recorded 2 min after each addition. Excitation spectra \((\lambda_{em} = 600\) nm) of buffer solution (2.0 mL, 10 mM Tris, 100 mM NaCl, pH 7.4) at 25 °C was recorded to serve as background.

Concentration of BSA \((c_{BSA})\) in the solution was plotted against the excitation intensity at 485 nm \((I)\) and the values were fitted using equation S2 with \(I_{max}\) being excitation intensity.
at maximum binding; $K_d$ the equilibrium dissociation constant; NS the slope of linear nonspecific binding and $I_0$ $I$ with no added BSA.

\[ I = I_{\text{max}} \cdot \frac{c_{\text{BSA}}}{(K_d + c_{\text{BSA}})} + \text{NS} \cdot c_{\text{BSA}} + I_0 \]  

(S2)

Calculated $K_d = 6.73 \times 10^{-7}$ M.

**Figure S5.** Isotherm curve fitting the extraction of 1 from SM/CL LUVs upon binding to BSA.

*Extraction of 1 with BSA in FBS from living cells.* On the day of the experiment, HK cells were washed with Leibovitz’s medium +Trx (3 × 1 mL) and kept in the same medium at 37 °C with 5% CO$_2$ for 45 min. Then, cells were incubated with 3 (5 µM, 15 min) or 1 (1 µM, 15 min). Afterwards, cells incubated with 1 were washed with Leibovitz's medium with or without 10% FBS (3 × 1 mL) and kept in Leibovitz's medium (1 mL) for FLIM imaging. Cells incubated with 3 were irradiated with bulk illumination ($\lambda = 365$ nm, 100% power) for 3 min and then washed with Leibovitz's medium with 10% FBS (3 × 1 mL) and kept in Leibovitz's medium (1 mL) for FLIM imaging. Cells incubated with 3 without irradiation were used for comparison of fluorescence intensity. Four images per condition were acquired in different locations of the dishes.
Figure S6. FLIM histograms corresponding to probe 1 (a, 1 µM) without (blue) and with (red) washing with FBS and 3 (b, 5 µM) before (blue) and after (red) bulk light irradiation and washing with FBS. Solid lines represent the mean values and bands in light colours the standard deviations; values calculated from 4 images with at least 4–6 cells per image.

5.5. CAPA for Probe 4

As in ref. S1, the day of the experiment, seeded HGM cells were washed with PBS (3 × 3 mL/well) and the media was exchanged to Leibovitz’s (4 × 150 µL/well) using a plate washer, keeping a final volume of 135 µL/well. Then, serial dilutions of probe 4 in PBS were prepared in a 96-well V-bottom plate and added to the µ-Plate containing the cells (15 µL/well, 10x final concentration in PBS) to reach a final volume of 150 µL/well (0 to maximum 1 µM). Duplicates were performed for each condition. Cells were incubated for 1 h at 37 °C with 5% CO₂. After this, cells were washed again and 23 was added (15 µL/well, 50 µM in PBS) to reach a final volume of 150 µL/well (5 µM of 23), except for the control wells, where only PBS was added (15 µL/well). After 15 min of incubation at 37 °C with 5% CO₂, the plate was washed again. Then, Hoechst 33342 was added (15 µL/well, 170 µM in PBS) to reach a final volume of 150 µL/well (17 µM). After 15 min of incubation at 37 °C with 5% CO₂, the plate was washed one last time and the cells were kept in clean Leibovitz’s media.
For signal subtraction of the residual fluorescence of the probes in the red channel (rhodamine channel), the same experiment was carried out, without the addition of 23 and incubating instead with clean media.

During imaging, samples were kept at 37 °C with 5% CO₂. A total of 16 images/well at 40x were recorded, using three channels: blue (excitation filter: 377/50 nm, emission filter: 477/60 nm, exposure time: 10 ms), green (excitation filter: 475/34 nm, emission filter: 536/40 nm, exposure time: 20 ms) and red (excitation filter: 531/40 nm, emission filter: 593/40 nm, exposure time: 40 ms).

Nuclei segmentation of the blue channel image and cell body segmentation and top-hat transform of the green channel image were applied. Mitochondria mask was applied to extract the integrated intensity value for each condition in the red channel image, which was corrected by subtraction of the integrated intensity value without addition of 23. The corrected integrated intensity values for each condition were then normalized.

The resulting dependence of the relative intensity values ($I_{HRO}$) to the concentration of probe 4 ($c$) was fitted with equation S3 to retrieve the half maximal cell penetration concentration (CP₅₀) value as 16 ± 2 nM.

$$I_{HRO} = \frac{1}{1 + (c / CP_{50})} \quad (S3)$$

**Figure S7.** CAPA dose-response curves after 1 h incubation of 4. Duplicates were performed for each condition.
5.6. Release of Probe 4 Inside GA

On the day of the experiment, HK cells transiently expressing ST-HaloTag-HA protein were washed with Leibovitz’s medium +Trx (3 × 1 mL) and incubated with 4 or 2 (100 nM) at 37 °C with 5% CO₂ for 1 h. Then, cells were washed with Leibovitz’s medium +Trx (3 × 1 mL) and kept at 37 °C with 5% CO₂ for extra 15 min. Up to five images were acquired in different locations and, afterwards, the dish was irradiated with bulk illumination (λ = 365 nm, 100% power) for 2.5 min. Cells were washed again with Leibovitz’s medium +Trx (3 × 1 mL) and kept in Leibovitz’s medium for image acquisition. For imaging both before and after release, an upright microscope was used. To measure in ‘inverted mode’, around 200 µL of medium were kept on each dish and a cover slit was placed on top of the surface, then the dish was flipped upside down to image upright without drying out the cells.

For analysis, a threshold is applied to exclude background pixels and a biexponential reconvolution model was used for the fitting. The values of $\tau_1$ and $\tau_{av}$ reported in table S2 are those directly obtained from the fitted curve, presented as the mean ± standard deviation from 4 pictures containing at least 4-6 cells each. Histograms in Figure 4 represent the mean ± standard deviation of $\tau_{av}$.

**Table S2.** Lifetime values of probes 4 and 2 in HK cells transiently expressing ST-HaloTag-HA, under the different conditions tested.

| Conditions          | $\tau_{av}$ / ns | $\tau_1$ / ns |
|---------------------|------------------|---------------|
| 4 before irradiation| 3.13 ± 0.06      | 3.85 ± 0.03   |
| 4 after irradiation | 4.3 ± 0.1        | 4.9 ± 0.1     |
| 2 before irradiation| 3.80 ± 0.03      | 4.37 ± 0.04   |
| 2 after irradiation | 3.82 ± 0.04      | 4.38 ± 0.05   |
5.7. Release of Probe 4 Inside ER

The day of the experiment, HK cells transiently expressing Halo-TNFa-RUSH protein were washed with Leibovitz’s medium (3 × 1 mL) and incubated with 4 (250 nM) at 37 °C with 5% CO₂ for 1 h. Then, cells were washed with full DMEM medium (10% FBS) +Trx (3 × 1 mL) and kept at 37 °C with 5% CO₂ for extra 2 h. Afterwards, cells were washed with Leibovitz’s medium +Trx (3 × 1 mL) and several images were acquired in different locations of the dish. Then, it was irradiated with bulk illumination (λ = 365 nm, 100% power) for 2 min 30 s. Cells were washed again with Leibovitz’s medium (with 10% FBS) +Trx (3 × 1 mL) and kept in Leibovitz’s medium (1 mL) for image acquisition after release.

For analysis, a threshold is applied to exclude background pixels and a biexponential reconvolution model is used for the fitting. The values of τ₁ and τ_avg reported in table S3 are those directly obtained from the fitted curve, presented as the mean ± standard deviation from 4 pictures containing 1–3 cells each.

Figure S8. Representative FLIM images of HK cells transiently expressing Halo-TNFa-RUSH protein, after incubation with 4 (250 nM, 1 h) before (a,c) and after (b,d) irradiation. Scale bar: 10 µm.
Table S3. Lifetime values of 4 in HK cells transiently expressing Halo-TNFα-RUSH, under the different conditions tested.

| Conditions         | \(\tau_{av}/\text{ns} \) | \(\tau_{1}/\text{ns} \) |
|--------------------|-----------------|-----------------|
| before irradiation | 2.95 ± 0.04     | 3.48 ± 0.02     |
| after irradiation  | 3.4 ± 0.1       | 4.2 ± 0.1       |

5.8. **Release of Probe 4 Inside Nucleus**

*Selective staining of nucleus.* On the day of the experiment, HK cells stably expressing HaloTag-NLS protein were washed with Leibovitz’s medium (3 × 1 mL) and incubated with 4 (250 nM) at 37 °C with 5% CO\(_2\) for 1 h or with 23 (1 µM) at 37 °C with 5% CO\(_2\) for 15 min. Then, cells were washed with Leibovitz’s medium (3 × 1 mL) and either directly imaged or washed with full DMEM medium +Trx (10% FBS, 3 × 1 mL) and kept at 37 °C with 5% CO\(_2\) for extra 2 h (only required for 4), and imaged afterwards in Leibovitz’s medium. For imaging, an upright microscope was used. To measure in ‘inverted mode’, around 200 µL of medium were kept on each dish and a cover slit was placed on top of the surface, then the dish was flipped upside down to image upright without drying out the cells.

*Release in nucleus.* The day of the experiment, HK cells stably expressing HaloTag-NLS protein were washed with Leibovitz’s medium (3 × 1 mL) and incubated with 4 (250 nM) at 37 °C with 5% CO\(_2\) for 1 h. Then, cells were washed with full DMEM medium +Trx (10% FBS, 3 × 1 mL) and kept at 37 °C with 5% CO\(_2\) for extra 2 h. Afterwards, cells were washed with Leibovitz’s medium +Trx (3 × 1 mL) and the dish was irradiated with bulk illumination (\(\lambda = 365 \text{ nm} \), 100% power) for 2 min 30 s. Cells were washed again with Leibovitz’s medium +Trx (with 10% FBS, 3 × 1 mL) and kept in Leibovitz’s medium (1 mL) for imaging. Up to five images were acquired in different locations of the dish. Then, an aqueous solution of sucrose (1 M, 1 mL) was added, to reach a final concentration of 0.5 M. After 10 min of
equilibration, images at the same positions in the dish were acquired again. As control, another set of cells was imaged excluding the irradiation step and the hyperosmotic shock, to compare the lifetime values of the probe in the nuclear envelope before and after release.

Figure S9. (a,b) Representative CLSM images of HK cells stably expressing HaloTag-NLS, after incubation with 23 (1 µM, 15 min); λ_{ex} = 561 nm, LP = 2%; λ_{em} = 570 – 800 nm. (c,f) Representative FLIM images of HK cells stably expressing HaloTag-NLS, after incubation with 4 (500 nM, 1 h) without (c,e) or with extraction step in the presence of FBS (10%, 2 h, d,f). Scale bar: 10 µm.

For analysis, a pixel binning to two points and a threshold were applied to increase the number of photons per pixel and to exclude background pixels during the analysis, respectively. ROIs were manually selected from the images, outlining the nuclear envelope based on the signal of the probe and the nuclei shape. A biexponential deconvolution model was used for the fitting. The values of τ_1 and τ_{av} reported in table S4 are those directly obtained from the fitted curve, presented as the mean ± standard deviation from 4 pictures containing at least 4 cells each.
**Figure S10.** Representative original FLIM images* of HK cells stably expressing HaloTag-NLS labelled with 4 (250 nM, 1 h) a) before and b) after bulk irradiation and d) after addition of sucrose (0.5 M). Scale bar: 10 µm. *Images in Figure 5A in the main text are the same, showing in color only the masked area (nuclear envelop) for the calculation of lifetime values.

**Table S4.** Lifetime values of 4 in the ROI (nuclear envelope), under the different conditions tested.

| Conditions               | $\tau_{av}$ / ns | $\tau_1$ / ns |
|--------------------------|------------------|---------------|
| before irradiation       | 3.14 ± 0.03      | 3.92 ± 0.03   |
| after irradiation        | 3.46 ± 0.04      | 4.25 ± 0.04   |
| after addition of sucrose| 3.29 ± 0.02      | 4.08 ± 0.01   |

*Spatiotemporal Control on the Release in Nucleus. The day of the experiment, HK cells stably expressing HaloTag-NLS protein were washed with Leibovitz’s medium (3 × 1 mL) and incubated with 4 (250 nM) at 37 °C with 5% CO$_2$ for 1 h. Then, cells were washed with full DMEM medium + Trx (10% FBS, 3 × 1 mL) and kept at 37 °C with 5% CO$_2$ for extra 2 h. Afterwards, cells were washed with Leibovitz’s medium + Trx (3 × 1 mL) and the dish was placed in the microscope. After acquiring an image before irradiation, a ROI was selected using the FRAP mode from Leica LAS X and the region was irradiated ($\lambda_{ex} = 405$ nm, 0.5% power) for 30 s. Immediately afterward, another image was acquired in the same area. Several images were acquired in different locations of the dish.
6. NMR Spectra

Figure S11. 400 MHz $^1$H NMR spectrum of 12 in CDCl$_3$.

Figure S12. 101 MHz $^{13}$C NMR spectrum of 12 in CDCl$_3$. 
Figure S13. 400 MHz $^1$H NMR spectrum of 15 in CDCl$_3$.

Figure S14. 101 MHz $^{13}$C NMR spectrum of 15 in CDCl$_3$. 
Figure S15. 282 MHz $^{19}$F NMR spectrum of 15 in CDCl₃.

Figure S16. 500 MHz $^1$H NMR spectrum of 16 in CD₃OD.
Figure S17. 126 MHz $^{13}$C NMR spectrum of 16 in CD$_3$OD.

Figure S18. 282 MHz $^{19}$F NMR spectrum of 16 in CD$_3$OD.
**Figure S19.** 500 MHz $^1$H NMR spectrum of 17 in CDCl$_3$.

**Figure S20.** 126 MHz $^{13}$C NMR spectrum of 17 in CDCl$_3$. 
**Figure S21.** 282 MHz $^{19}$F NMR spectrum of 17 in CDCl$_3$.

**Figure S22.** 500 MHz $^1$H NMR spectrum of 3 in CDCl$_3$. 

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Figure S23. 126 MHz $^{13}$C NMR spectrum of 3 in CDCl$_3$.

Figure S24: 282 MHz $^{19}$F NMR spectrum of 3 in CDCl$_3$. 
Figure S25. 400 MHz $^1$H NMR spectrum of 20 in CDCl$_3$.

Figure S26. 101 MHz $^{13}$C NMR spectrum of 20 in CDCl$_3$. 
Figure S27. 500 MHz $^1$H NMR spectrum of 21 in CDCl$_3$.

Figure S28. 126 MHz $^{13}$C NMR spectrum of 21 in CDCl$_3$. 
Figure S29. 500 MHz $^1$H NMR spectrum of 22 in CDCl$_3$.

Figure S30. 126 MHz $^{13}$C NMR spectrum of 22 in CDCl$_3$. 
Figure S31. 500 MHz $^1$H NMR spectrum of 4 in CDCl$_3$.

Figure S32. 126 MHz $^{13}$C NMR spectrum of 4 in CDCl$_3$. 
7. **Supporting References**

[S1] K. Straková, J. López-Andarias, N. Jiménez-Rojo, J. E. Chambers, S. J. Marciniak, H. Riezman, N. Sakai, S. Matile, *ACS Cent. Sci.* **2020**, *6*, 1376–1385.

[S2] J. López-Andarias, K. Straková, R. Martinent, N. Jiménez-Rojo, H. Riezman, N. Sakai, S. Matile, *JACS Au* **2021**, *1*, 221–232.

[S3] S. Soleimanpour, A. Colom, E. Derivery, G. Gonzalez-Gaitan, A. Roux, N. Sakai, S. Matile, *Chem. Commun.* **2016**, *52*, 14450–14453.

[S4] J. H. Shrimp, J. Hu, M. Dong, B. S. Wang, R. MacDonald, H. Jiang, Q. Hao, A. Yen, H. Lin, *J. Am. Chem. Soc.* **2014**, *136*, 5656–5663.

[S5] L. Peraro, K. L. Deprey, M. K. Moser, Z. Zou, H. L. Ball, B. Levine, J. A. Kritzer, *J. Am. Chem. Soc.* **2018**, *140*, 11360–11369.

[S6] D. Liße, V. Wilkens, C. You, K. Busch, J. Piehler, *Angew. Chem. Int. Ed.* **2011**, *50*, 9352–9355.

[S7] J. García-Calvo, J. Maillard, I. Fureraj, K. Strakova, A. Colom, V. Mercier, A. Roux, E. Vauthey, N. Sakai, A. Fürstenberg, S. Matile, *J. Am. Chem. Soc.* **2020**, *142*, 12034–12038.

[S8] E. R. Ballister, C. Aonbangkhen, A. M. Mayo, M. A. Lampson, D. M. Chenoweth, *Nat Commun.* **2014**, *5*, 5475.

[S9] R. S. Erdmann, S. W. Baguley, J. H. Richens, R. F. Wissner, Z. Xi, E. S. Allgeyer, S. Zhong, A. D. Thompson, N. Lowe, R. Butler, J. Bewersdorf, J. E. Rothman, D. St Johnston, A. Schepartz, D. Toomre, *Cell Chem. Biol.* **2019**, *26*, 584–592.e6.

[S10] A. V. Weigel, C. L. Chang, G. Shtengel, C. S. Xu, D. P. Hoffman, M. Freeman, N. Iyer, J. Aaron, S. Khuon, J. Bogovic, W. Qiu, H. F. Hess, J. Lippincott-Schwartz, *Cell* **2021**, *184*, 2412–2429.e16.

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