The Dynamic Range of Acidity: Tracking Rules for the Unidirectional Penetration of Cellular Compartments

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1. Materials and Methods

As in ref [S1]. Briefly, reagents for synthesis were purchased from Fluka, Sigma-Aldrich, TCI, and Across. Salts of the best grade available from Fluka or Sigma-Aldrich were used as received. Alexa Fluor® 647 EGF, MitoTracker™ Green FM, Phosphate buffered saline (PBS, pH = 7.4), DMEM (GlutaMAX, 4.5 g/L D-glucose, pyruvate, with phenol red) 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 Fluorobrite™ DMEM were from ThermoFisher Scientific. 35 mm glass-bottom dishes were obtained from MatTek (P35G-0.170 14-C).

Column chromatography was carried out on silica gel 60 (SilicaFlash P60, 40-63 μm). Analytical chromatographies (TLC) were performed on silica gel 60 (Merck, 0.2 mm), respectively. Reverse phase flash chromatography was performed on a Biotage® Isolera Spektra using pre-packed 12 g Biotage® SNAP Ultra C18 cartridge. LCMS were recorded using a Thermo Scientific Accela HPLC equipped with a Thermo C18 Hypersil GOLD column (50 x 2.1 mm, 1.9 μm particles size) coupled with a LCQ Fleet three-dimensional ion trap mass spectrometer (ESI, Thermo Scientific) with a linear elution gradient from 95% H2O / 5% CH3CN + 0.1% TFA to 10% H2O / 90% CH3CN + 0.1% TFA in 4.0 minutes at a flow rate of 0.75 mL/min (B5), 70% H2O / 30% CH3CN + 0.1% TFA to 10% H2O / 90% CH3CN + 0.1% TFA in 4.0 minutes at a flow rate of 0.75 mL/min (B30) or 40% H2O / 60% CH3CN + 0.1% TFA to 10% H2O / 90% CH3CN + 0.1% TFA in 4.0 minutes at a flow rate of 0.75 mL/min (B60). IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer (ATR, Golden Gate) and are reported as wavenumbers υ in cm⁻¹ with band intensities indicated as s (strong), m (medium), w (weak), or 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), quartet (q), and quintet (p) with
coupling constants ($J$) given in Hz, or multiplet (m). Broad peaks are marked as br. $^1$H and $^{13}$C 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 and 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]).

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, 488 nm (white light laser) and collecting the fluorescence between 550 and 650 nm or a Nikon Eclipse Ti A1R microscope upgraded with a FLIM kit from PicoQuant, equipped with a 485 nm laser (PicoQuant, LDH-D-C-485) at 20 MHz.

**Abbreviations.** CLSM: Confocal laser scanning microscopy; DAPI: 4’,6-Diamidino-2-phenylindole; DMEM: Dulbecco’s modified eagle medium; DMSO: Dimethyl sulfoxide; EGF: Epidermal growth factor; EGF-FR: Alexa Fluor® 647 EGF; FBS: Fetal bovine serum; FLIM: Fluorescence lifetime imaging microscopy; PBS: Phosphate-buffered saline; PS: Penicillin/Streptomycin; $R_f$: Retention factor; TBTA: Tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine.
2. **Synthesis**

![Chemical structures of compounds 1-5](image)

**Figure S1.** Structure of the final flippers used in this study.

**Compounds 1 and 5** were prepared following previously reported procedures.[S1]

**Compound 2** was prepared following previously reported procedures.[S2]

![Chemical reaction](image)

**Scheme S1.** a) CuSO$_4$·5H$_2$O, Na-ascorbate, TBTA, CH$_2$Cl$_2$, H$_2$O, MeOH, 1.5 h, 46%.
Compound 6 was prepared following previously reported procedures.\textsuperscript{[S3]}

Compound 9 was prepared following previously reported procedures.\textsuperscript{[S4]}

Compound 3. To a mixture of 6 (4 mg, 7 μmol) and 9 (1 mg, 7 μmol) in CH$_2$Cl$_2$ (0.5 mL) was added TBTA (0.5 mg, 1 μmol) followed by a solution of CuSO$_4$·5H$_2$O (2 mg, 8 μmol) and sodium ascorbate (2 mg, 9 μmol) in H$_2$O (0.2 mL). The reaction mixture was stirred vigorously for 1.5 h at rt. All volatiles were removed under reduced pressure and the resulting crude product was purified flash column chromatography (CH$_2$Cl$_2$/MeOH 0-15% gradient) and subsequently by reverse phase flash column chromatography (H$_2$O + 0.1% TFA/MeCN + 0.1% TFA 50-100% gradient). The fractions containing the product were combined and diluted with CH$_2$Cl$_2$. The organic phase was collected and washed with sat. aq. NaHCO$_3$ (2x). The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure to give 3 as a red solid (2.3 mg, 46%). The product purity was confirmed by LC-MS analysis (Figure S26). IR (neat): 2927 (m), 2860 (m), 2216 (w), 1681 (m), 1503 (w), 1462 (w), 1437 (w), 1414 (m), 1383 (m), 1314 (s), 1202 (m), 1178 (m), 1142 (s), 1092 (m), 1060 (m), 966 (w), 835 (w), 800 (w), 722 (w), 660 (w); $^1$H NMR (400 MHz, DMSO-\textit{d}_6): 8.14 (s, 1H), 7.57 (s, 3H), 4.77 (s, 2H), 4.62 (s, 2H), 4.35 (t, $^3$J$_{H-H} = 7.0$ Hz, 2H), 2.80 – 2.70 (m, 2H), 2.48 (s, 3H) 2.36 (s, 3H), 2.34 (s, 3H), 2.32 (s, 3H), 1.85 – 1.76 (m, 2H), 1.56 – 1.45 (m, 2H), 1.37 – 1.19 (m, 4H); $^{13}$C NMR (126 MHz, DMSO-\textit{d}_6): 143.9 (C), 143.6 (C), 142.2 (C), 141.7 (C), 140.8 (C), 139.9 (C), 138.0 (C), 136.9 (C), 133.3 (C), 131.5 (C), 130.5 (C), 130.2 (C), 128.5 (C), 128.0 (C), 127.9 (C), 125.9 (C), 124.0 (CH), 113.0 (C), 109.9 (C), 64.5 (CH$_2$), 62.7 (CH$_2$), 49.1 (CH$_2$), 38.7 (CH$_2$), 29.5 (CH$_2$), 26.8 (CH$_2$), 25.3 (CH$_2$), 25.1 (CH$_2$), 13.8 (CH$_3$), 12.6 (CH$_3$), 12.5 (CH$_3$), 12.0 (CH$_3$); HRMS (ESI+) calcd for C$_{31}$H$_{31}$N$_5$O$_3$S$_6$[M+H]$^+$: 714.0824, found: 714.0795.
Scheme S2. a) Et₃N, CH₂Cl₂, 16 h, 73%. b) TFA, CH₂Cl₂, quant. c) CuSO₄·5H₂O, Na-ascorbate, TBTA, CH₂Cl₂, H₂O, 1.5 h, 38%.

**Compound 11.** To a solution of aminohexylazide 9 (120 mg, 0.84 mmol) and 1,3-Bis(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea 10 (244 mg, 0.84 mmol) were dissolved in CH₂Cl₂ (3 mL) and then Et₃N (130 µL, 0.93 mmol) was added. The reaction mixture was stirred at ambient temperature overnight. All volatiles were removed under reduced pressure and the remaining brown oil purified by column chromatography (SiO₂, CH₂Cl₂, Rᵣ = 0.5) to afford compound 11 as a colorless oil (236 mg, 73%). IR (neat): 3336 (w), 2979 (w), 2935 (w), 2863 (w), 2094 (m), 1719 (m), 1635 (m), 1613 (s), 1573 (m), 1414 (m), 1365 (m), 1326 (m), 1279 (m), 1251 (m), 1228 (m), 1228 (m), 1154 (s), 1129 (s), 1052 (s), 1026 (m), 879 (w), 853 (w), 809 (m), 771 (m), 621 (w); ¹H NMR (400 MHz, CDCl₃): 11.48 (s, 1H), 8.28 (t, ³JH-H = 5.2 Hz, 1H), 3.44 – 3.34 (m, 2H), 3.24 (t, ³JH-H = 6.9 Hz, 2H), 1.63 – 1.53 (m, 4H), 1.48 (s, 18H), 1.40 – 1.32 (m, 4H); ¹³C NMR (101 MHz, CDCl₃): 163.7 (C), 156.2 (C), 153.4 (C), 83.1 (C), 79.3 (C), 51.4 (CH₂), 40.8 (CH₂), 28.9 (CH₂), 28.8 (CH₂), 28.4 (CH₃), 28.1 (CH₃), 26.4 (CH₂), 26.4 (CH₂); MS (ESI+): 385 (100, [M+H]⁺).
**Compound 12.** To a solution of compound 11 (2.4 mg, 6.7 µmol) in CH$_2$Cl$_2$ (200 µL) was added TFA (200 µL) at rt. After 1 h when full deprotection was detected by TLC, all volatiles were removed under reduced pressure. The product was obtained without further purification as a colorless oil (2 mg, *quant.*). The spectroscopic data are in agreement with those reported in literature.$^{[S7]}$

**Compound 4.** To a mixture of 6 (3.5 mg, 6.1 µmol) and 12 (2.0 mg, 6.7 µmol) in CH$_2$Cl$_2$ (750 µL) was added TBTA (0.35 mg, 0.60 µmol) followed by a solution of CuSO$_4$·5H$_2$O (1.6 mg, 6.7 µmol) and sodium ascorbate (1.3 mg, 6.7 µmol) in H$_2$O (250 µL). The reaction mixture was stirred vigorously for 1.5 h at rt. All volatiles were removed under reduced pressure and the resulting crude product was purified by flash column chromatography (CH$_2$Cl$_2$/MeOH, 0-20% gradient) and subsequently by a reverse phase flash column chromatography (H$_2$O + 0.1% TFA/MeCN + 0.1% TFA 40-100% gradient). The fractions containing the product were combined and diluted with CH$_2$Cl$_2$. The organic phase was collected and washed with sat. aq. NaHCO$_3$ (2x). The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure to give 4 as red solid (1.9 mg, 38%). The product purity was confirmed by LC-MS analysis (Figure S27). IR (neat): 3364 (w), 3306 (w), 3199 (w), 3069 (w), 2922 (s), 2853 (m), 2215 (w), 1670 (m), 1581 (w), 1462 (m), 1378 (m), 1315 (m), 1200 (m), 1144 (s), 1092 (m), 1009 (w), 832 (w), 721 (w), 668 (w); $^1$H NMR (400 MHz, DMSO-d$_6$): 8.15 (s, 1H), 7.50 – 7.42 (m, 1H), 7.33 – 6.53 (bs, 4H), 4.78 (s, 2H), 4.63 (s, 2H), 4.35 (t, $^3$J$_{H-H}$ = 7.0 Hz, 2H), 3.06 (q, $^3$J$_{H-H}$ = 6.5 Hz, 2H), 2.48 (s, 3H), 2.36 (s, 3H), 2.34 (s, 3H), 2.32 (s, 3H), 1.86 – 1.76 (m, 2H), 1.50 – 1.38 (m, 2H), 1.32 – 1.22 (m, 4H); $^{13}$C NMR (126 MHz, DMSO-d$_6$): 156.6 (C), 143.9 (C), 143.6 (C), 142.2 (C), 141.7 (C), 140.8 (C), 139.9 (C), 138.0 (C), 136.9 (C), 133.3 (C), 131.5 (C), 130.5 (C), 130.2 (C), 128.5 (C), 127.9 (C), 126.6 (C), 125.9 (C), 124.0 (CH), 113.1 (C), 110.0 (C), 64.5 (CH$_2$), 62.7 (CH$_2$), 49.2 (CH$_2$), 40.7 (CH$_2$), 29.6 (CH$_2$), 28.3 (CH$_2$), 25.4 (CH$_2$), 25.4 (CH$_2$), 13.8 (CH$_3$), 13.6 (CH$_3$), 12.5 (CH$_3$), 12.0 (CH$_3$); HRMS (ESI+) calcd for C$_{32}$H$_{33}$N$_7$O$_3$S$_6$ [M–X]$^+$: 756.1042, found: 756.1075.

Note, this compound was labeled 4m when in micellar form under certain conditions.
3. **Cell Culture and Imaging**

The HeLa Kyoto cells were seeded at $8 \times 10^4$ cell/mL on 35 mm glass bottom dishes (Mattek Corporation, P35G-0.170 14-C) and grew in DMEM medium (Gibco, 10013CM), enriched with 10% FBS, 1% PS for 24 h. For imaging, the cells were washed three times and replaced with Leibovitz’s medium (Gibco, 21083027).

For measurements in ‘inverted mode’, only 200 µL of medium were kept in each dish and covered by a glass cover. The dish was then flipped upside down to enable upright imaging without drying out the cells.

4. **Tracking Early Endosomes**

*Co-localization with EGF-FR.* HeLa Kyoto cells were incubated with Leibovitz’s medium (1 mL) containing EGF-FR (500 ng/mL) and Lys-Flipper 3 (2.5 µM from 2.5 µL of 1 mM solution in DMSO), or monomeric Arg-Flipper 4 (2.5 µM from 5 µL of 0.5 mM solution in DMSO) for 10 min, and then washed three times with Leibovitz’s medium (1 mL). The cells were directly imaged at 37 °C in ‘inverted mode’. Argon laser (20 MHz pulse) was used as light source. EGF-FR: $\lambda_{\text{ex}} = 647$ nm, LP = 10%; $\lambda_{\text{em}} = 665–769$ nm. Flipper: $\lambda_{\text{ex}} = 488$ nm, LP = 30%; $\lambda_{\text{em}} = 550–640$ nm.

To co-localize micellar Arg-Flipper, HeLa Kyoto cells were incubated with Leibovitz’s medium containing EGF-FR (500 ng/mL) and Arg-Flipper 4m (3.8 µM from 2.5 µL of 1.5 mM solution in DMSO) for 10 min, and then washed three times with Leibovitz’s medium (1 mL). The cells were directly imaged at 37 °C in ‘inverted mode’. Argon laser was used as light source. EGF-FR: $\lambda_{\text{ex}} = 633$ nm, LP = 4%; $\lambda_{\text{em}} = 640–670$ nm. Flipper: $\lambda_{\text{ex}} = 488$ nm, LP = 30%; $\lambda_{\text{em}} = 550–625$ nm.
**Figure S2.** CLSM (A-C, F-H) and FLIM (D, E, I, J) images of HeLa Kyoto cells stained with EGF-FR (red) for 10 min and Lys-Flipper 3 (green). Flipper channel (C, D, H, I); EGF-FR channel (B, E, G, J); overlay (A, F). Scale bars: 10 μm.

**Figure S3.** CLSM (A-C, F-H) and FLIM (D, E, I, J) images of HeLa Kyoto cells stained with EGF-FR (red) for 10 min and Arg-Flipper 4 (green). Flipper channel (C, D, H, I); EGF-FR channel (B, E, G, J); overlay (A, F). Scale bars: 10 μm.
Figure S4. CLSM images of HeLa Kyoto cells stained with EGF-FR (red) for 10 min and Arg-Flipper 4m (green). Flipper channel (C, F); EGF-FR channel (B, E); overlay (A, D). Scale bars: 10 μm.

Automated microscopy analysis. As in [S2]. Namely, approximately 10000 Hela MZ cells were plated in 96 wells imaging plates from Ibidi (ref: 89626) for 24 h. Cells were incubated for 30 min in Fluorobrite™ containing Lys-Flipper 3 (1 μM), washed with the same medium, then incubated for 15 min with 1 μg/mL EGF-FR in Fluorobrite™ medium and DAPI 1/1000 at 37 °C, and then wash 3 times with Fluorobrite™ before imaging at room temperature.

In all conditions at least 9 images per well were acquired using IXM confocal automatic microscope (Molecular Device) using 60X water immersion objective 1.3NA.

To analyze the co-localization of the Lys-Flipper 3 with endosomes, MetaXpress Custom Module editor software was used to first segment the image and generate relevant masks, which were then applied on the fluorescent images to extract relevant measurements. In the first step, the cell was segmented using the nuclei (DAPI) channel. To facilitate segmentation of EGF-FR or flipper labeled
or flipper endosomes, a top hat deconvolution method was applied to reduce the background noise and highlight bright granules. Endosomes were then segmented from the deconvoluted image. The final masks are then applied to the original fluorescent images. Number of EGF-FR/Lys-Flipper 3 copositive dots were normalized by the number of EGF-FR dots per cell to give colocalization ratio (Figure 6C).

*Hela MZ and RPE-1 staining with Lys-Flipper 3.* 10³ Hela MZ or RPE-1 cells were plated in 96 wells imaging plates from Ibidi (ref:89626) in Fluorobrite™ medium supplemented with 10% FCS and 1 mg/mL L-glutamine for 24 h. Cells were then incubated 2 h with 100 nM Bafilomycin A1 (ref:1334, Tocris) or DMSO in Fluorobrite™ medium supplemented with 1 mg/mL L-glutamine. Cells were further incubated 20 min. in the same mixture supplemented with 1 μM Lys-Flipper 3 and 5 μg/mL Hoechst 33342 (H-3570, Molecular Probes). Images were acquired at 37 °C with IXM confocal automatic microscope (Molecular Device) using 60X water immersion objective 1.3NA.

5. Tracking Golgi Apparatus

*Transfection with GTS-HaloTag-meGFP targeting Golgi apparatus.* As described in Ref. [S8]. HeLa cells were seeded at 5 ×10⁴ 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. On the next day, transient transfection was performed by diluting the cells with Lipofectamine® 2000 – DNA complexes prepared as follows (conditions per glass bottom dish): Lipofectamine® 2000 (1.5 μL) was diluted with Opti-MEM/GlutaMAX reduced serum medium (150 μL) and left incubate at rt for 5 min. DNA plasmid (Hensel group,[S9] 1.5 μL of 500 ng/μL solution in water) 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, half of the original medium (1 mL) was removed. The solution of DNA-Lipofectamine complex (300 μL each) was added to each well. The cells were kept at 37 °C with 5% CO₂. After 3 h,
the medium was exchanged with fresh DMEM + 10% FBS + 1% Pen/Strep or FluoroBrite DMEM + 10% FBS and the cells were incubated overnight.

**Co-localization with HeLa Kyoto cells expressing GFP in Golgi apparatus.** On the day of the experiment, seeded transiently transfected HeLa cells were washed with Leibovitz medium (3 × 1 mL) and incubated with Lys-Flipper 3 (2.5 µM, 2.5 µL of 1 mM solution in DMSO) in Leibovitz medium (1 mL) for 15 min at 37 °C under 5% CO₂. The cells were washed with Leibovitz’s medium (3 × 1 mL) and finally kept in clean Leibovitz’s medium. The distribution of fluorescent compounds was analyzed without fixing. The cells were directly measured in ‘inverted mode’ at 37 °C. Argon laser was used as light source with excitation wavelength 488 nm and emission recorded at 618 – 737 nm in the flipper channel, LP: 30% and 492 – 534 nm for the GFP channel, LP: 8%.

**Figure S5.** CLSM images of transiently transfected HeLa Kyoto cells stained with Lys-Flipper 3 (green). Flipper channel (C, F); GFP channel (B, E); overlay (A, D). Scale bars: 10 µm.
6. Tracking Mitochondria

Co-localization with MitoTracker Green™. HeLa Kyoto cells were incubated with Leibovitz’s medium containing MitoTracker Green™ (100 nM) for 5 min and then Arg-Flipper 4m (1.5 µM – 1 µL of 1.5 mM solution in DMSO) was added, and the cells were incubated for further 10 min. The cells were washed two times with Leibovitz’s medium (1 mL) and directly imaged in the same medium in ‘inverted mode’. Argon laser was used as light source with excitation wavelength 488 nm and emission recorded: Mitotracker Green™: LP = 15%; λ_{em} = 492 – 540 nm. Flipper: LP = 40%; λ_{em} = 615 – 730 nm.

PCC was calculated using the Coloc2 tool from ImageJ using the flipper channel and the MitoTracker channel.

Figure S6. CLSM images of HeLa Kyoto cells stained with MitoTracker Green™ (red) flipper 4m (green). Flipper channel (C, F); MitoTracker channel (B, E); overlay (A, D). Scale bars: 10 µm. PCC = 0.89 ± 0.01.
**Dependence on temperature.** HeLa Kyoto cells were incubated with Leibovitz’s medium containing MitoTracker Green™ (100 nM) for 10 min at 37 °C and cooled to 4 °C. Then, Arg-Flipper 4m (1 µM – 1 µL of 1.5 mM solution in DMSO) was added and the cells were incubated for further 20 min at 4 °C. The cells were washed two times with Leibovitz’s medium (1 mL). Images were directly acquired in ‘inverted mode’ at room temperature. Argon laser was used as light source with excitation wavelength 488 nm and emission recorded: Mitotracker Green™: LP = 15%; \(\lambda_{em} = 492 – 540\) nm; Flipper: LP = 40%; \(\lambda_{em} = 615 – 730\) nm.

PCC was calculated using the Coloc2 tool from ImageJ using the flipper channel and the MitoTracker channel.

**Figure S7.** CLSM images of HeLa Kyoto cells stained with MitoTracker Green™ (red) and Arg-Flipper 4m (green). Flipper channel (C, F); MitoTracker channel (B, E); overlay (A, D) after incubation at 4 °C. Scale bars: 10 µm. PCC = 0.89 ± 0.01.
Dependence on membrane potential. HeLa Kyoto cells were washed with Leibovitz’s medium (3 x 1 mL) and incubated with Leibovitz’s medium containing FCCP (2.5 µM) for 10 min. Afterwards Arg-Flipper 4m (3.8 µM from 2.5 µL of 1.5 mM solution in DMSO) was added and the cells were incubated for 5 min at 37 °C and directly imaged in ‘inverted mode’ at 37 °C.

Argon laser was used as light source with excitation wavelength 488 nm and emission recorded at 550 – 730 nm in the flipper channel, LP: 15%.

Figure S8. CLSM images of HeLa Kyoto cells incubated with FCCP for 10 min and then Arg-Flipper 4m. Scale bars: 10 µm.

Dependence on concentration. HeLa Kyoto cells were washed with Leibovitz’s medium (3 x 1 mL) and incubated with Leibovitz’s medium (1 mL) containing Arg-Flipper 4 (1.25, 3.8, or 7.5 µM – 2.5, 7.5, or 15 µL of 0.5 mM solution in DMSO), incubated for 10 min and then imaged in ‘inverted mode’ at 37 °C.
Figure S9. CLSM (A-C) and FLIM (D-F) images of HeLa Kyoto cells treated with 1.25 µM (A, D), 3.8 µM (B, E) and 7.5 µM (C, F) Arg-Flipper 4 and fluorescence lifetime (τ₂ (G), τav (H)) at the different concentrations. Scale bars: 10 µm. Comments: This experiment shows how with increasing concentration, Arg-Flipper changes targeting from plasma membrane (and the onset of endocytosis) to mitochondria. This concentration dependence supports that monomeric Arg-Flipper 4 targets plasma membrane and self-assembled micellar Arg-Flipper 4m targets mitochondria, for the reasons outlined in Figure 5. As expected for self-assembly of cationic amphiphiles, this switch from plasma membrane to mitochondria targeting depends on other parameters beyond concentration that can be...
difficult to control and understand. For images showing monomeric 4 optimized to target plasma membrane (including onset of endocytosis), see Figures 9C, 10, S3, S13A, S16; for micellar 4m optimized to target mitochondria, see Figures 9A, B, D, E, S4, S6, S7, S17, S19.

*Dependence on pH.* HeLa Kyoto cells were washed with Leibovitz’s medium (3 x 1 mL) and then incubated with Leibovitz’s medium at different pH containing Arg-Flipper 4m (3.8 µM from 2.5 µL of 1.5 mM solution in DMSO) for 10 min at 37 °C. The cells were then directly imaged in ‘inverted mode’.

Argon laser was used as light source with excitation wavelength 488 nm and emission recorded at 550 – 730 nm in the flipper channel, LP: 15%.

![Figure S10. CLSM (A-F) and FLIM (G-L) images of HeLa Kyoto cells with Arg-Flipper 4m at 2.5 µM concentration in Leibovitz’s medium at pH 5.2 (A, G), 6.2 (B, H), 7.4 (C, I), 8.0 (D, J), 9.0 (E, K), or 9.8 (F, L). Scale bars: 10 µm.](image)

7. **Dependence on Time and Temperature**

*Time dependence at 37 °C.* HeLa Kyoto cells were washed with Leibovitz’s medium (3 x 1 mL) and incubated in Leibovitz’s medium containing Dmb-Flipper 2, Lys-Flipper 3 (2.5 µM – 2.5
µL of 1 mM solution in DMSO) or Arg-Flipper 4 (1.3 µM – 2.5 µL of 0.5 mM solution in DMSO) added to the cells and the dish directly imaged.

Figure S11. FLIM (A-C) and CLSM (D-F) images of HeLa Kyoto cells stained with Dmb-Flipper 2 (2.5 µM) and recorded after 5 min (A, D), 15 min (B, E) and 30 min (C, F), and fluorescence lifetimes ($\tau_2$ (G), $\tau_{av}$ (H)) recorded at the different times. Scale bars: 10 µm.

Figure S12. FLIM (A-C) and CLSM (D-F) images of HeLa Kyoto cells stained with Lys-Flipper 3 (2.5 µM) and recorded after 5 min (A, D), 15 min (B, E) and 30 min (C, F), and fluorescence lifetimes ($\tau_2$ (G), $\tau_{av}$ (H)) recorded at the different times. Scale bars: 10 µm.
Figure S13. CSLM (A-C) and FLIM (D-F) and images of HeLa Kyoto cells stained with Arg-Flipper 4 (2.5 µM) and recorded after 5 min (A, D), 15 min (B, E), and 30 min (C, F), and fluorescence lifetimes (τ₂ (G), τ_{av} (H)) recorded at the different times. Scale bars: 10 µm.

Time dependence at 4 °C. HeLa Kyoto cells were washed with cold Leibovitz’s medium (3 x 1 mL) and incubated with Leibovitz’s medium (1 mL) containing Dmb-Flipper 2 and Lys-Flipper 3 (2.5 µM – 2.5 µL of 1 mM solution in DMSO), Arg-Flipper 4 (2.5 µM – 2.5 µL at 0.5 mM concentration in DMSO) or Arg-Flipper 4m (2.5 µM – 2.5 µL at 1.5 mM concentration in DMSO) for 30 min at 4 °C. The cells were immediately imaged after being brought up to room temperature, in a 16 min timeframe, to prevent redistribution of the probes due to an increase in temperature.
**Figure S14.** CLSM (A-E) and FLIM (F-J) images of HeLa Kyoto cells treated with Dmb-Flipper 2 at 4 °C and recorded at 6 min (A, F), 7 min (B, G), 8 min (C, H), 9 min (D, I) 10 min (E, J), and fluorescence lifetimes ($\tau_2$ (K), $\tau_{av}$ (L)) recorded at the different times. Scale bars: 10 µm.
**Figure S15.** CLSM (A-E) and FLIM (F-J) images of HeLa Kyoto cells treated with Lys-Flipper 3 at 4 °C and recorded at 6 min (A, F), 8 min (B, G), 13 min (C, H), 15 min (D, I) and 16 min (E, J), and fluorescence lifetimes ($\tau_2$ (K), $\tau_{av}$ (L)) recorded at the different times. Scale bars: 10 μm.
Figure S16. CLSM (A-D) and FLIM (E-H) images of HeLa Kyoto cells treated with Arg-Flipper 4 at 4 °C and recorded at 7 min (A, E), 8 min (B, F), 9 min (C, G) and 10 min (D, H), and fluorescence lifetimes ($\tau_2$ (I), $\tau_{av}$ (J)) recorded at the different times. Scale bars: 10 μm.

Figure S17. CLSM (A-E) and FLIM (F-J) images of HeLa Kyoto cells treated with Arg-Flipper 4m at 4 °C and recorded at 8 min (A, F), 10 min (B, G), 12 min (C, H), 14 min (D, I), 16 min (E, J). Scale bars: 10 μm.
8. Response to Membrane Tension

*Measurements under isotonic conditions.* On the day of the experiment, HeLa Kyoto cells were washed with Leibovitz’s medium (2 × 1 mL, at 37 °C) and incubated with flipper 3 (2.5 μM from 2.5 μL of 1 mM solution in DMSO), or 4m (3.8 μM from 2.5 μL of 1.5 mM solution in DMSO) in Leibovitz’s medium (1 mL) at 37 °C under 5% CO₂. The cells were subsequently washed with Leibovitz’s medium (2 × 1 mL) and finally imaged in ‘inverted mode’ at 37 °C. Several images were acquired at different points of the dish.

*Measurements after hypertonic shock.* To samples previously imaged under isotonic conditions firstly 0.8 mL Leibovitz’s medium was added, followed by an aq. sucrose solution (1 M, 1 mL) to reach a final volume of 2 mL (0.5 M sucrose). After 10 min of equilibration, images were acquired in ‘inverted mode’ at 37 °C. Several images were acquired at different points of the dish.

Argon laser (20 MHz pulse) was used as light source with excitation wavelength 488 nm and emission recorded at 550 – 730 nm in the flipper channel, LP: 15%. For analysis Leica Application Suite Software LASX FLIM/FCS Version 3.5.6 was used to fit fluorescence decay data to a dual exponential model, where $\tau_2$ (longer lifetime) and $\tau_{av}$ were extracted.
Figure S18. FLIM images of HeLa Kyoto cells after incubation with Lys-Flipper 3 (5 µM) in Leibovitz’s medium under isotonic conditions (A, B) or under hypertonic conditions (C, D) and corresponding lifetimes ($\tau_2$ (E), $\tau_{av}$ (F)), where one data point corresponds to one image. Statistical significance was determined using Student’s t-test, ns $> 0.1234$, * $< 0.0332$, ** $< 0.0021$, *** $< 0.0002$, **** $< 0.0001$ (unpaired from isotonic to hypertonic).

Table S1. Lifetime values of Lys-Flipper 3 in HeLa Kyoto cells under the different conditions tested.

| Conditions   | $\tau_2$ / ns | $\tau_{av}$ / ns |
|--------------|---------------|------------------|
| isotonic     | 4.16 ± 0.06   | 3.53 ± 0.07      |
| hypertonic   | 4.03 ± 0.01   | 3.38 ± 0.02      |
**Figure S19.** FLIM images of HeLa Kyoto cells after incubation with Arg-Flipper 4m (3.8 µM) in Leibovitz’s medium under isotonic conditions (A, C) or under hypertonic conditions (B, D) and corresponding lifetimes ($\tau_2$ (E), $\tau_{av}$ (F)), where one data point corresponds to one image.

**Table S2.** Lifetime values of Arg-Flipper 4m in HeLa Kyoto cells under the different conditions tested.

| Conditions | $\tau_2$ / ns | $\tau_{av}$ / ns |
|------------|---------------|-----------------|
| isotonic   | 3.82 ± 0.09   | 3.26 ± 0.11     |
| hypertonic | 3.59 ± 0.02   | 3.07 ± 0.20     |
9. NMR Spectra

Figure S20. 400 MHz $^1$H NMR spectrum of 3 in DMSO-$d_6$.

Figure S21. 101 MHz $^{13}$C NMR spectrum of 3 in DMSO-$d_6$. 
**Figure S22.** 400 MHz $^1$H NMR spectrum of 11 in CDCl$_3$.

**Figure S23.** 101 MHz $^{13}$C NMR spectrum of 11 in CDCl$_3$. 
Figure S24. 400 MHz $^1$H NMR spectrum of 4 in DMSO-$d_6$.

Figure S25. 128 MHz $^{13}$C NMR spectrum of 4 in DMSO-$d_6$.  
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Figure S26. LCMS spectrum of 3 (ESI+, B30).
Figure S27. LCMS spectrum of 4 (ESI+, B30).
11. **Supporting References**

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