Supplementary information

Cell-permeant large Stokes shift dyes for transfection-free multicolor nanoscopy

Alexey N. Butkevich[#*], Gražvydas Lukinavičius[#*], Elisa D'Este and Stefan W. Hell

Max Planck Institute for Biophysical Chemistry, Department of NanoBiophotonics
Am Fassberg 11, 37077 Göttingen, Germany

*Corresponding authors' e-mails: alexey.butkevich@mpibpc.mpg.de and grazvydas.lukinavicius@mpibpc.mpg.de.

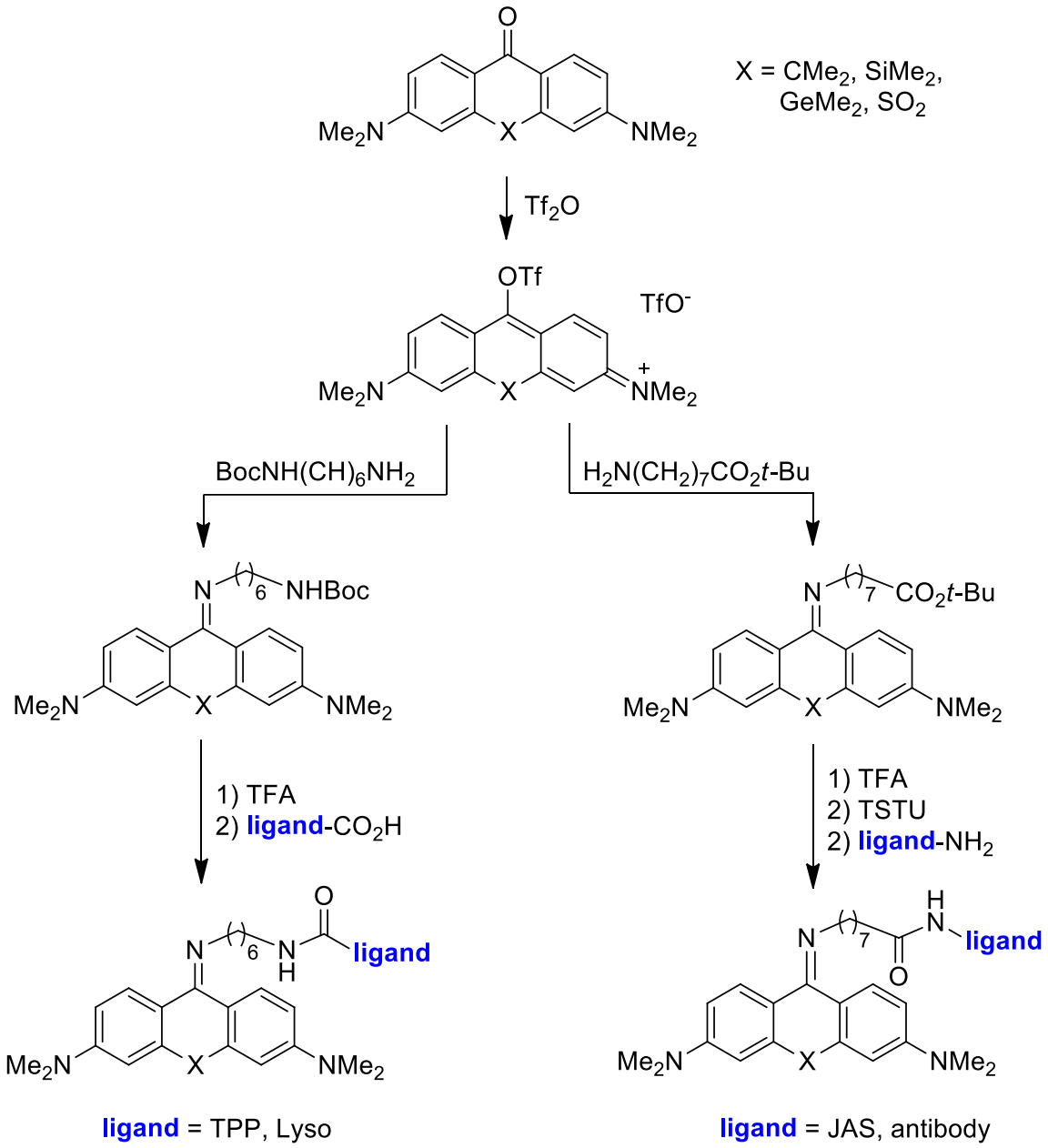
[#] A.N.B. and G.L. contributed equally to this work.

Supplementary inventory

Supplementary Video

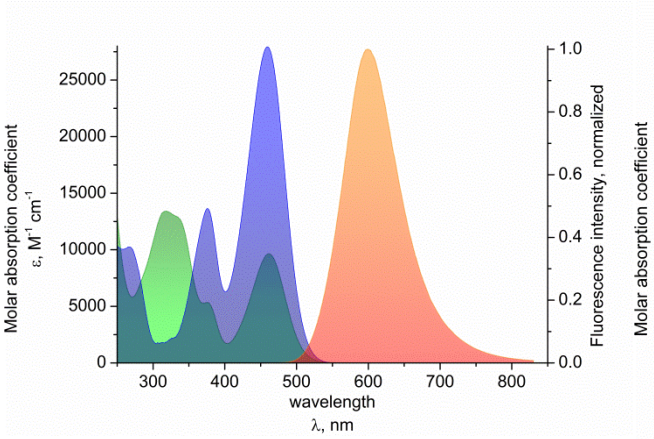
Supplementary Video S1. Three-color STED movie of living human fibroblasts stained with 2 μM SiX-lyso, 5 μM 580CP-actin and 5 μM SiR-tubulin. Lysosome movement along the microtubules is visible. Note that prolonged exposure to STED beam results in phototoxicity. However, because of the large Stokes shift of the SiX probe a single STED de-excitation light source (the highest intensity beam) is positioned at 775 nm, resulting in minimal possible photoinduced damage to the living cells. Scale bar 1 μm.

Supplementary Figures

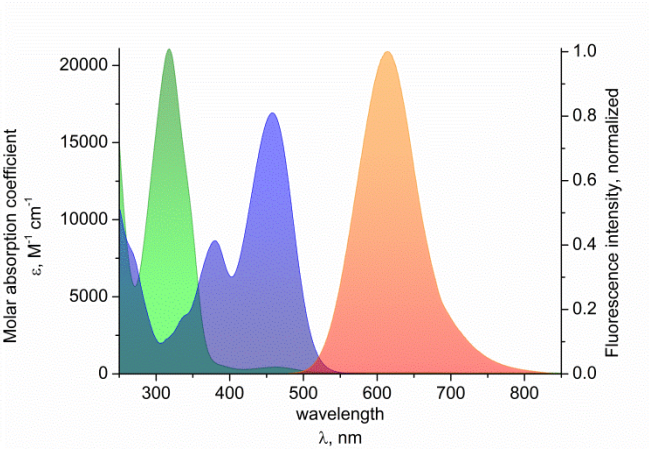


Supplementary Figure S1. General synthetic approach to iminoanthrone probes (for experimental details, see Supplementary Methods). Ligands (target): TPP (inner mitochondrial membrane) – propyltriphenylphosphonium, Lyso (lysosome) – pepstatin A, JAS (actin) – desbromo-desmethyl-jasplakinolide. TFA – trifluoroacetic acid, TSTU – *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.

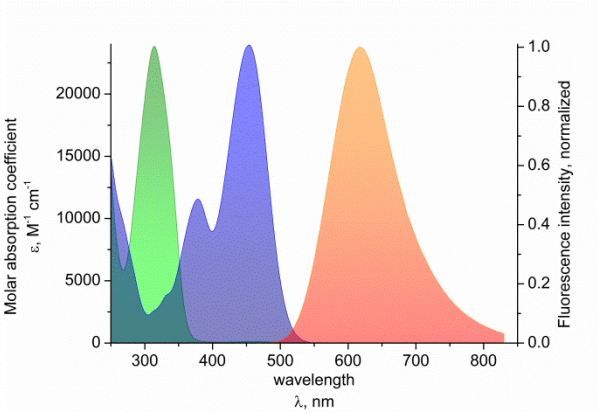
a (CX)



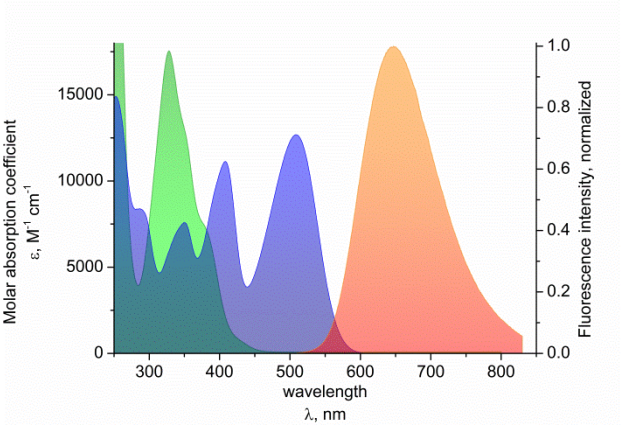
b (GeX)



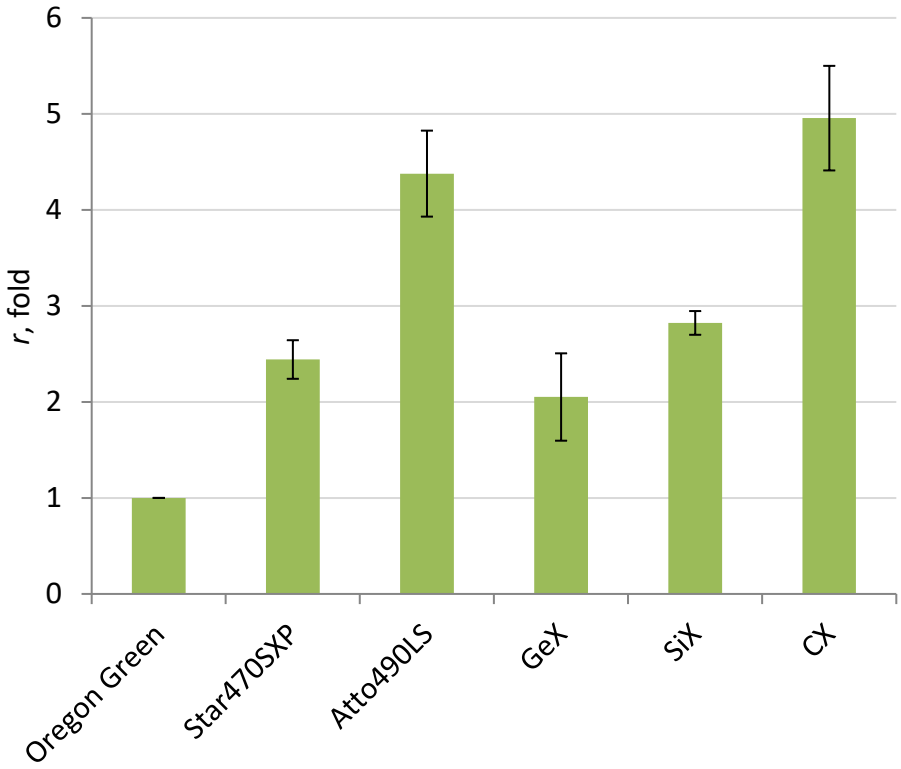
c (SiX)



d (SO_2X)

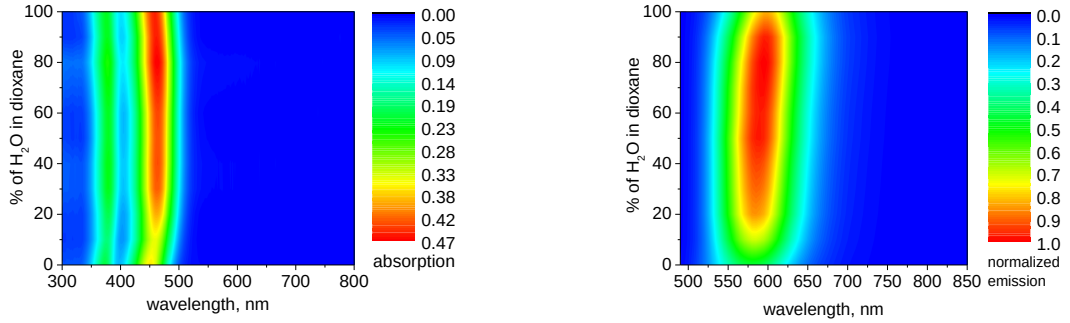


Supplementary Figure S2. Absorption and fluorescence emission spectra of dyes 1-4 in aqueous and organic media. Absorption and fluorescence emission spectra of dyes a) CX (**1**), b) GeX (**2**), c) SiX (**3**) and d) SO_2X (**4**). The absorption spectra have been recorded on a Varian Cary 4000 UV-Vis spectrophotometer in acetonitrile (green), 10% methanol in PBS at pH 7.4 (blue, **a-c**) or in 10% acetonitrile in PBS at pH 7.4 (blue, **d**). The fluorescence emission spectra have been recorded on a Varian Cary Eclipse fluorescence spectrophotometer in 10% methanol in PBS at pH 7.4 (orange, **a-c**) or in 10% acetonitrile in PBS at pH 7.4 (orange, **d**).

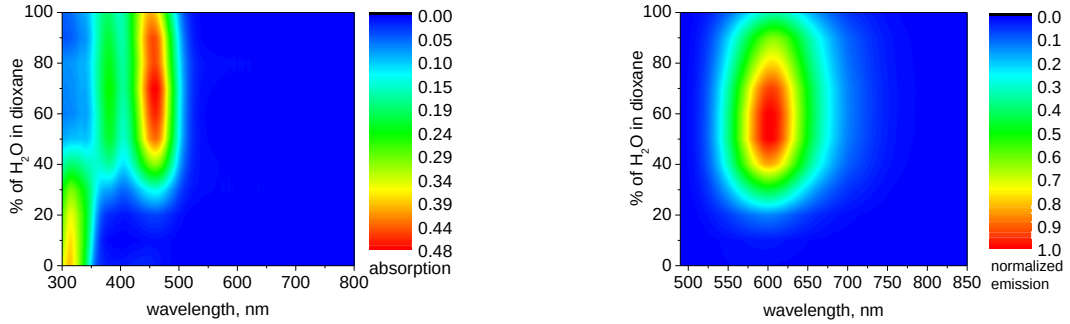


Supplementary Figure S3. Relative photostability of LSS dyes upon irradiation at 470 nm in 10% methanol – PBS 7.4 (reference: Oregon Green). The results are presented as mean with standard deviation, N = 2 (see section ***In vitro* characterization of the probes**).

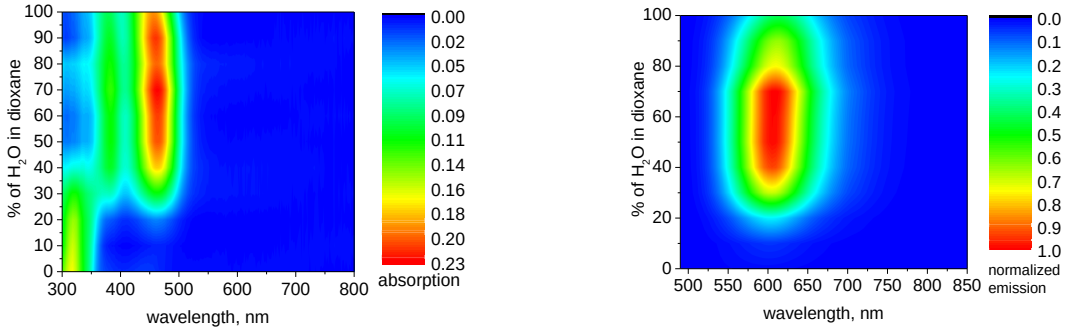
a (CX)



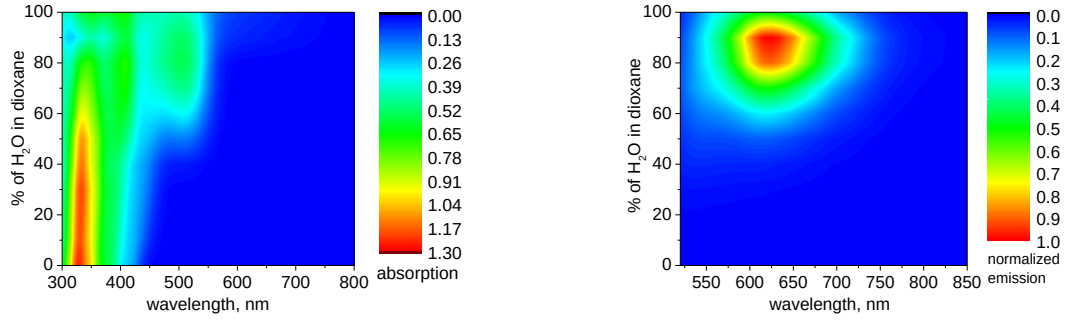
b (GeX)



c (SiX)

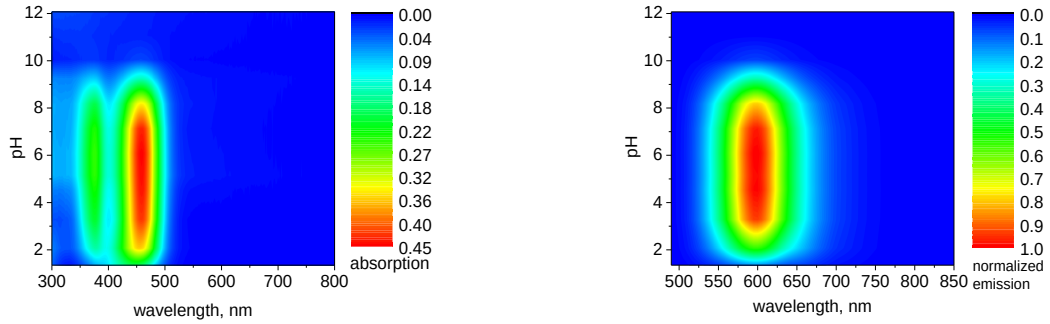


d (SO2X)

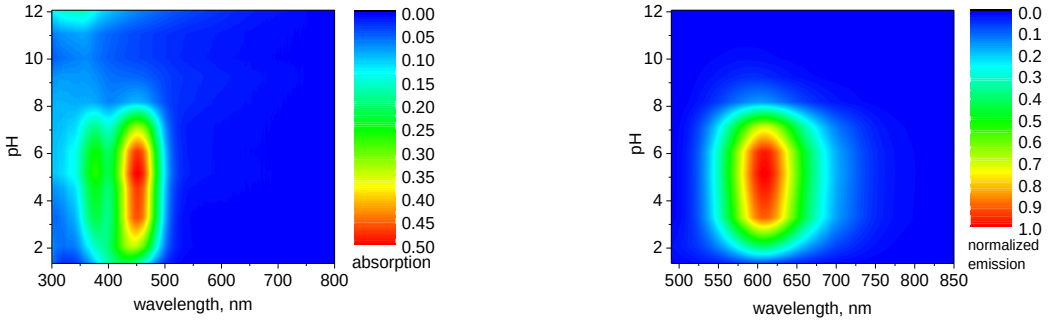


Supplementary Figure S4. Absorption and fluorescence emission spectra of dyes 1-4 in dioxane/water mixtures. Absorption and fluorescence emission spectra of dyes **a**) CX (**1**), **b**) GeX (**2**), **c**) SiX (**3**) and **d**) SO2X (**4**) in 1,4-dioxane/water mixtures (v/v%, +1% DMSO). The spectra have been recorded in triplicate in polypropylene 96 well plates on a Spark 20M (Tecan) microplate reader at 25 °C and are background corrected.

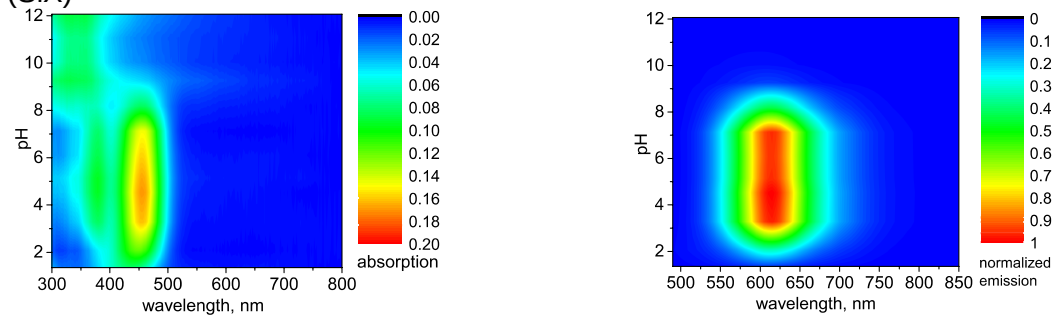
a (CX)



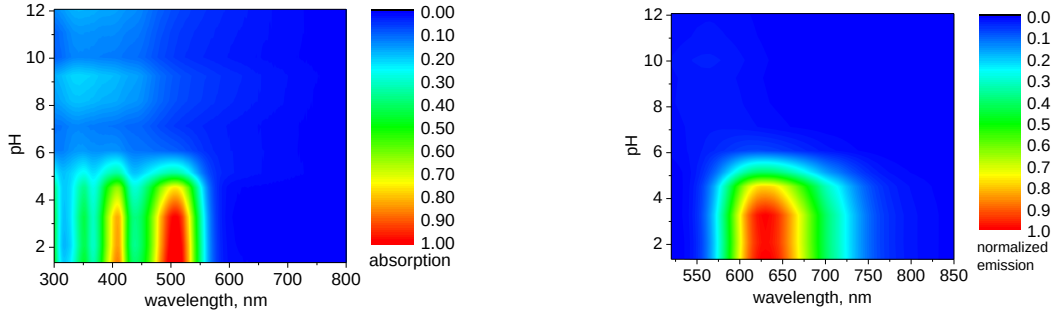
b (GeX)



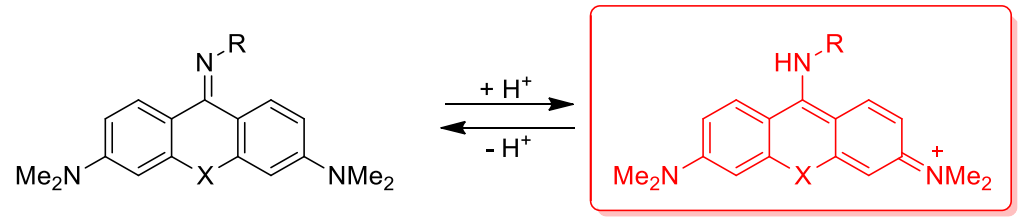
c (SiX)



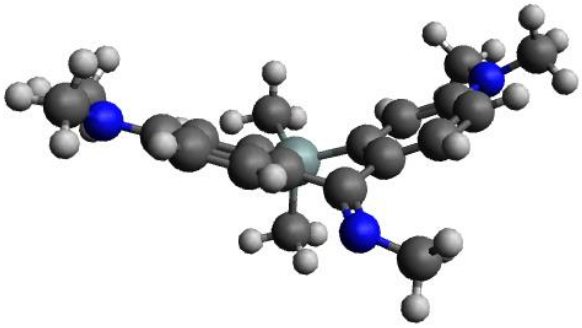
d (SO2X)

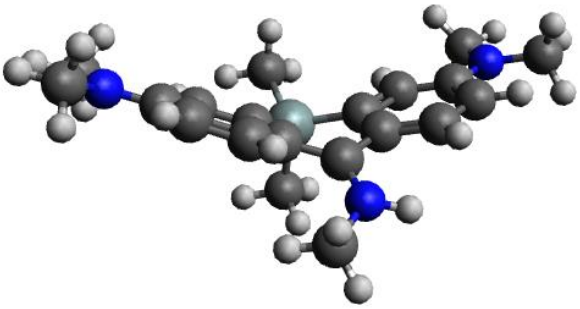


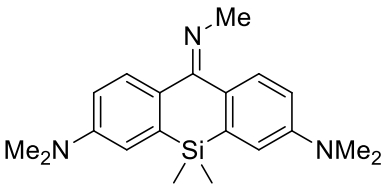
Supplementary Figure S5. Absorption and fluorescence emission spectra of dyes 1-4 in phosphate buffer at different pH values. Absorption and fluorescence emission spectra of dyes **a**) CX (**1**), **b**) GeX (**2**), **c**) SiX (**3**) and **d**) SO2X (**4**) in 0.2 M Na-phosphate buffer (pH 1-12, +1 v/v% DMSO). The spectra have been recorded in triplicate in polypropylene 96 well plates on a Spark 20M (Tecan) microplate reader at 25 °C and are background corrected.

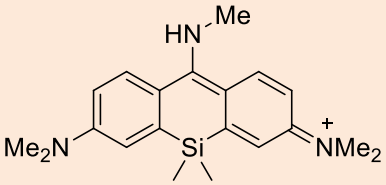


Model structures used in TD-DFT calculations, on example of X = $SiMe_2$:

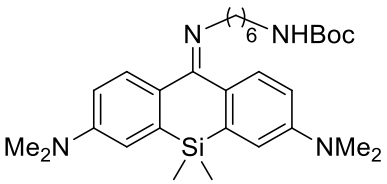






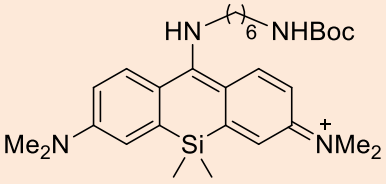


corresponding to the actual compound SiX (**3**) :



SiX (**3**)

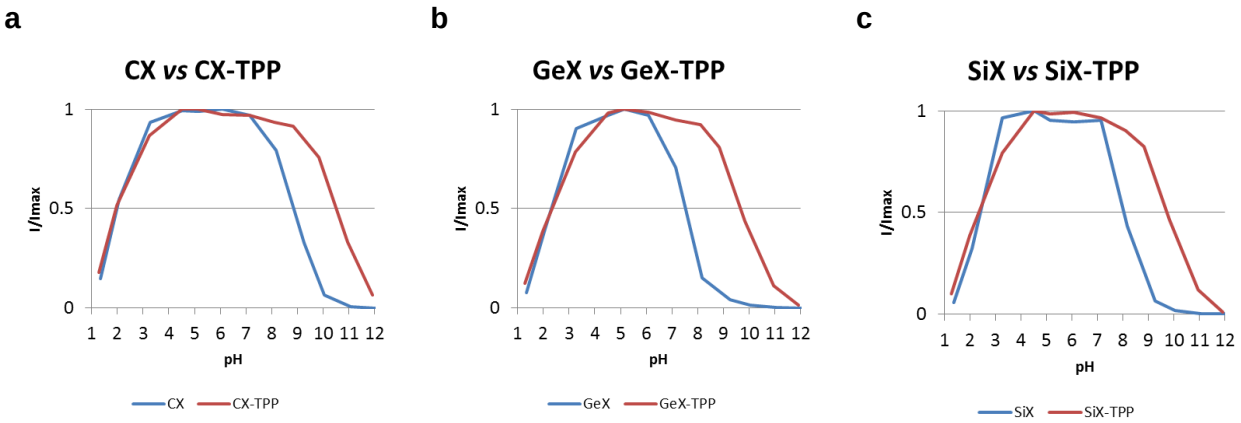
(250 … 350 nm absorption,
low water content or high pH)



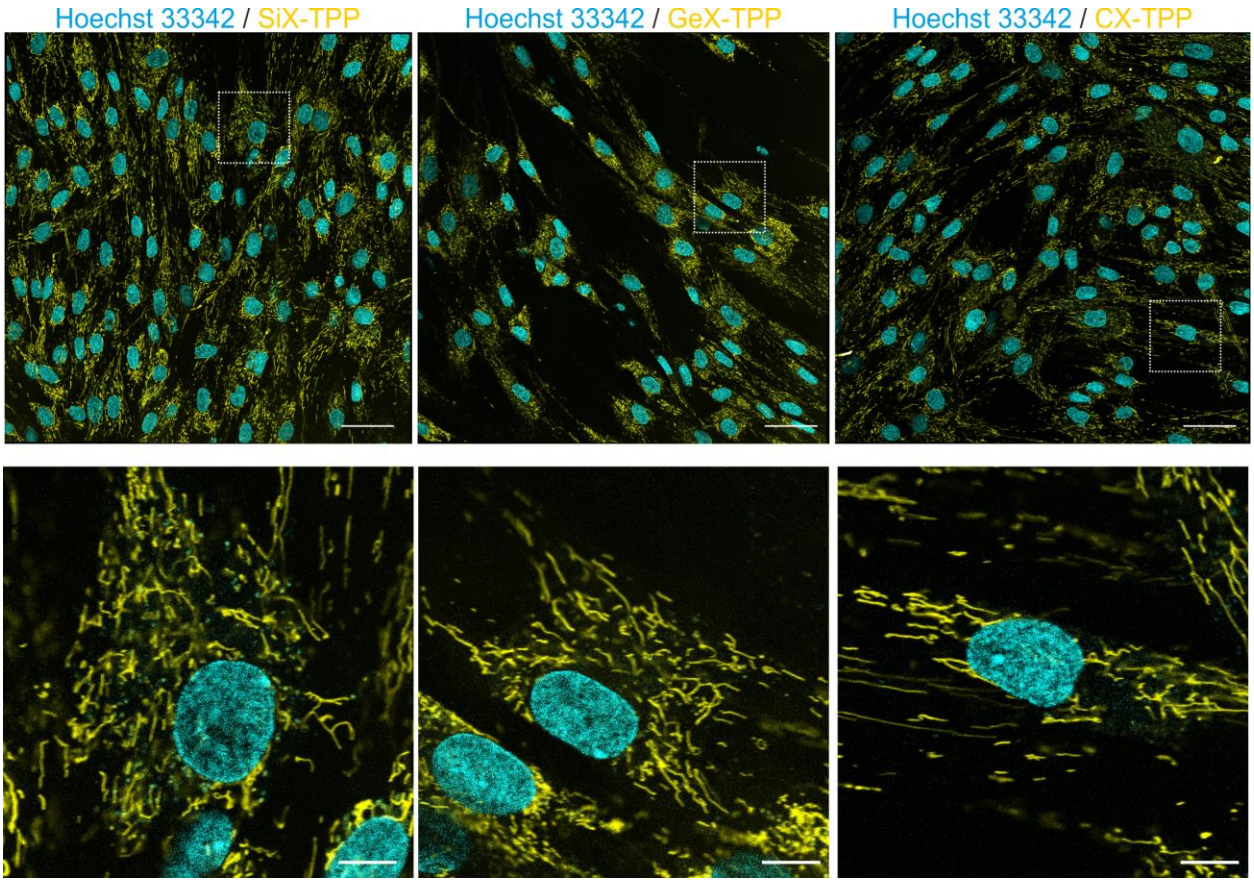
SiX, protonated (**3**H^+)

(400 … 500 nm absorption,
high water content or low pH)

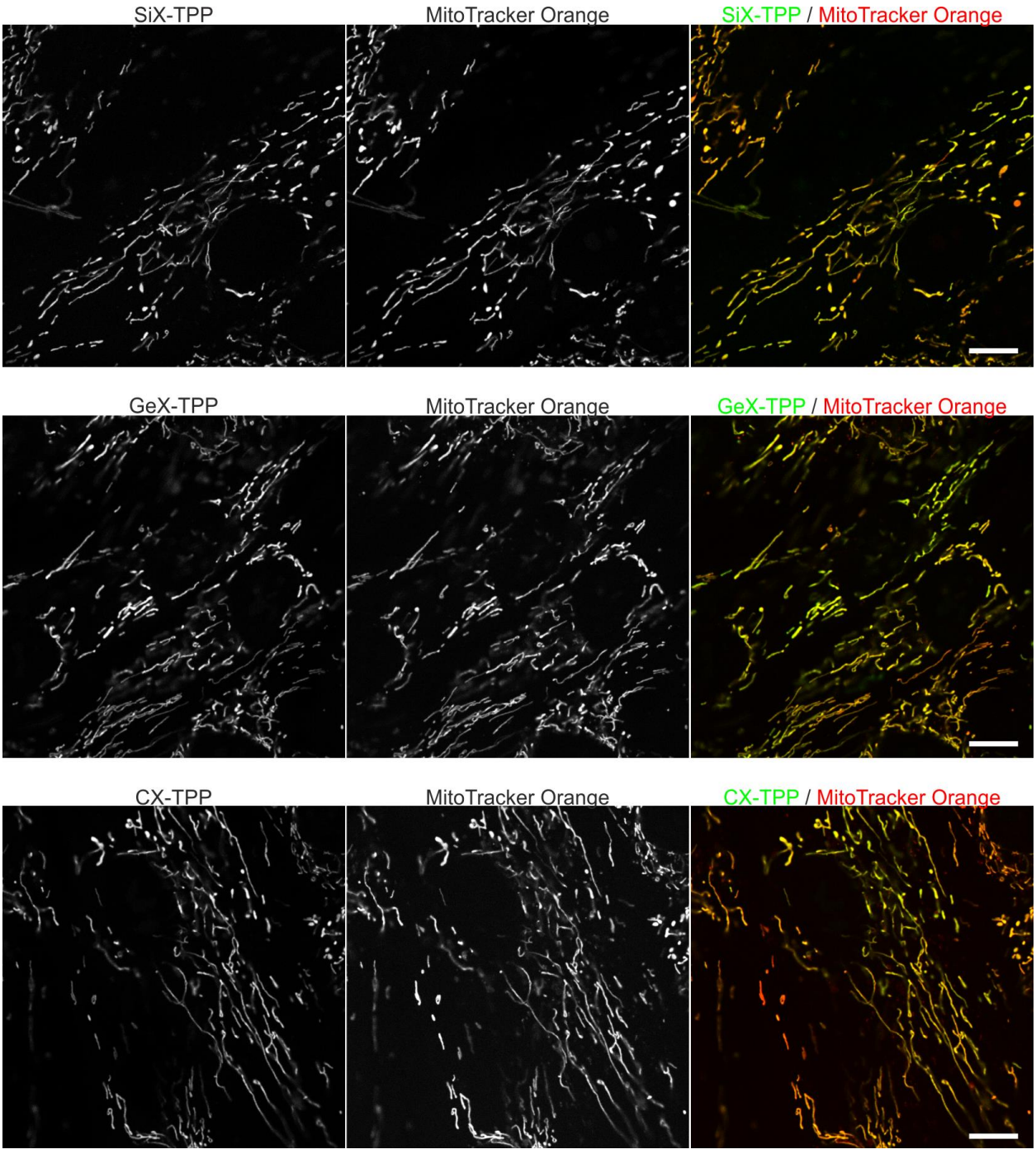
Supplementary Figure S6. Model structures of iminoanthrone dyes used in TD-DFT calculations. The flexible ω-(Boc-amino)alkyl chain was truncated in model structures to a methyl group to avoid the necessity of using hybrid two-layered methods (ONIOM).



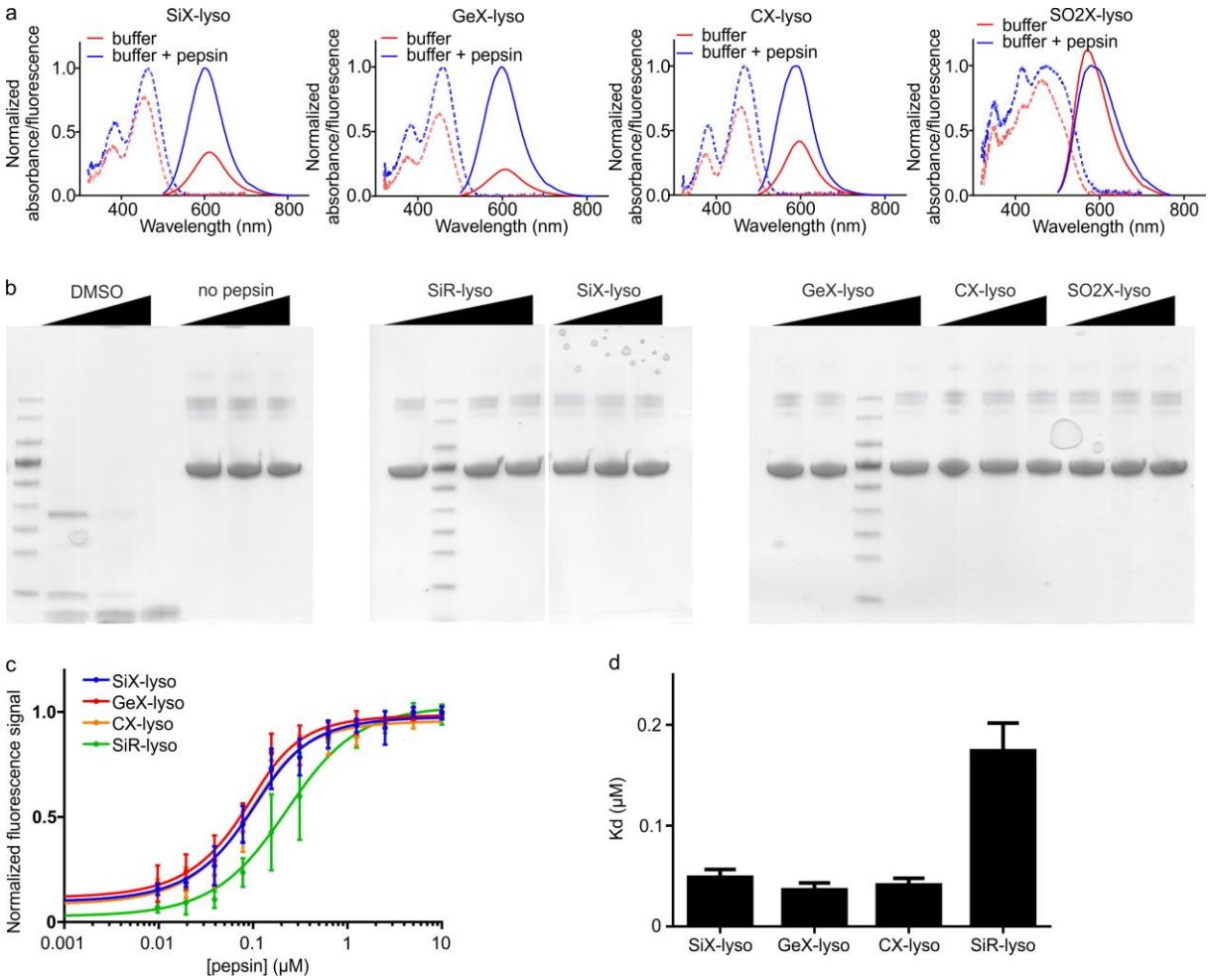
Supplementary Figure S7. Normalized fluorescence emission intensity (I/I_{max}) of dyes 1-3 and mitochondrial probes 1a-3a in phosphate buffer at different pH values. Fluorescence emission spectra of dyes **a**) CX (**1**) and CX-TPP (**1a**), **b**) GeX (**2**) and GeX-TPP (**2a**), **c**) SiX (**3**) and SiX-TPP (**3a**) have been recorded in triplicate in 0.2 M Na-phosphate buffer (pH 1-12, +1 v/v% DMSO) in glass bottom 96-well plates on a Spark 20M (Tecan) microplate reader at 25 °C. The spectra have been background corrected, and for each dye the intensities I at emission λ_{max} have been normalized to a highest observed value I_{max} across the entire pH gradient. The normalized emission intensity plots of the *N*-Boc-protected dyes (**1-3**) and TPP probes (**1a-3a**) are overlaid demonstrating the extended fluorescence range of TPP probes at basic pH as compared to the free dyes.



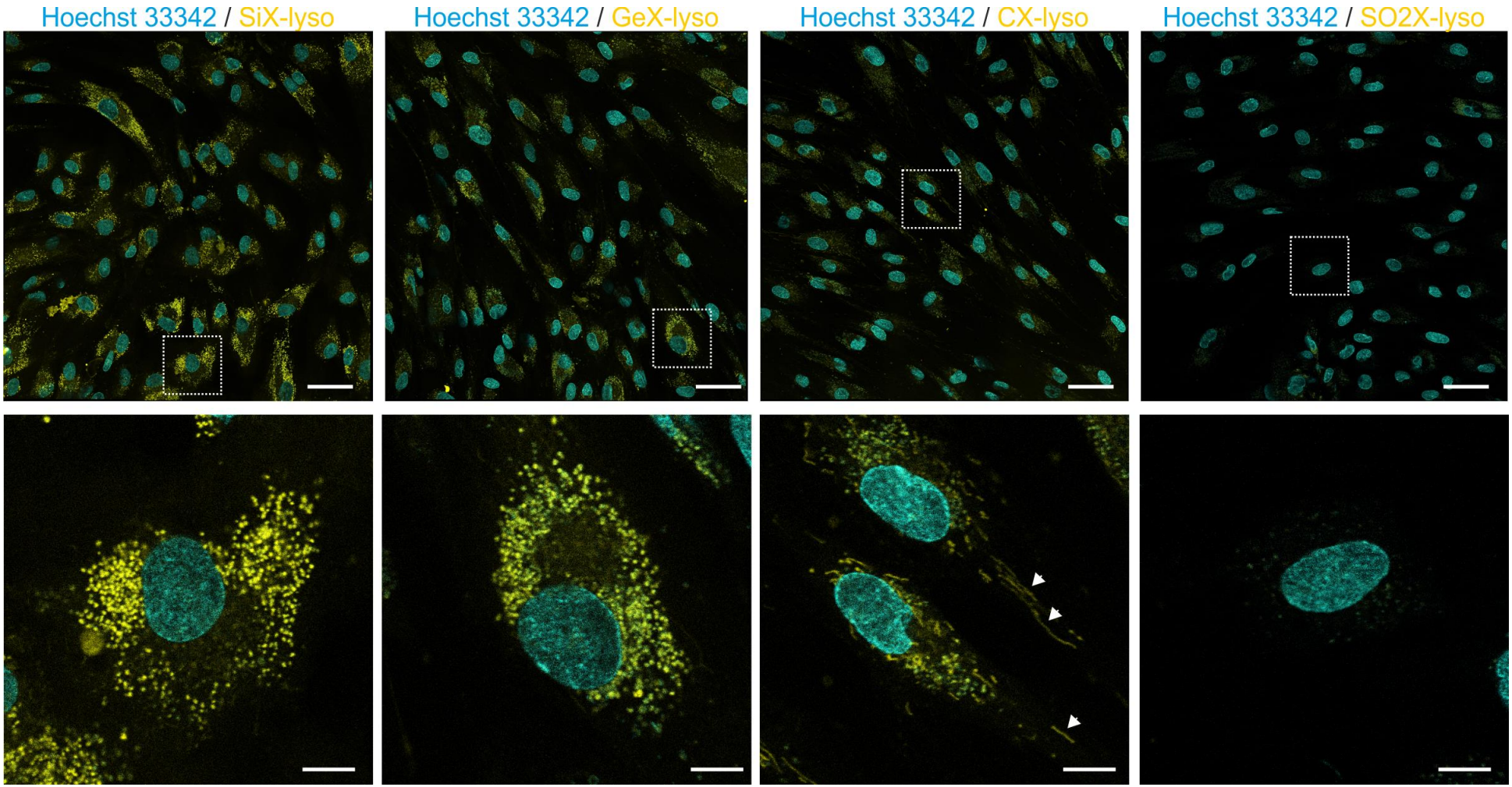
Supplementary Figure S8. Confocal microscopy images of human fibroblasts stained with mitochondrial probes. The upper row shows large field of view of stained human fibroblasts. Dotted squares correspond to the zoomed-in areas shown in the lower row. Cells were stained with 2 μM probes **1a-3a** and Hoechst 33342 (0.1 μg/ml) for 1 h at 37°C in the growth medium, washed twice with HBSS and imaged immediately in the growth medium. Scale bars are 50 μm (upper row) and 10 μm (lower row).



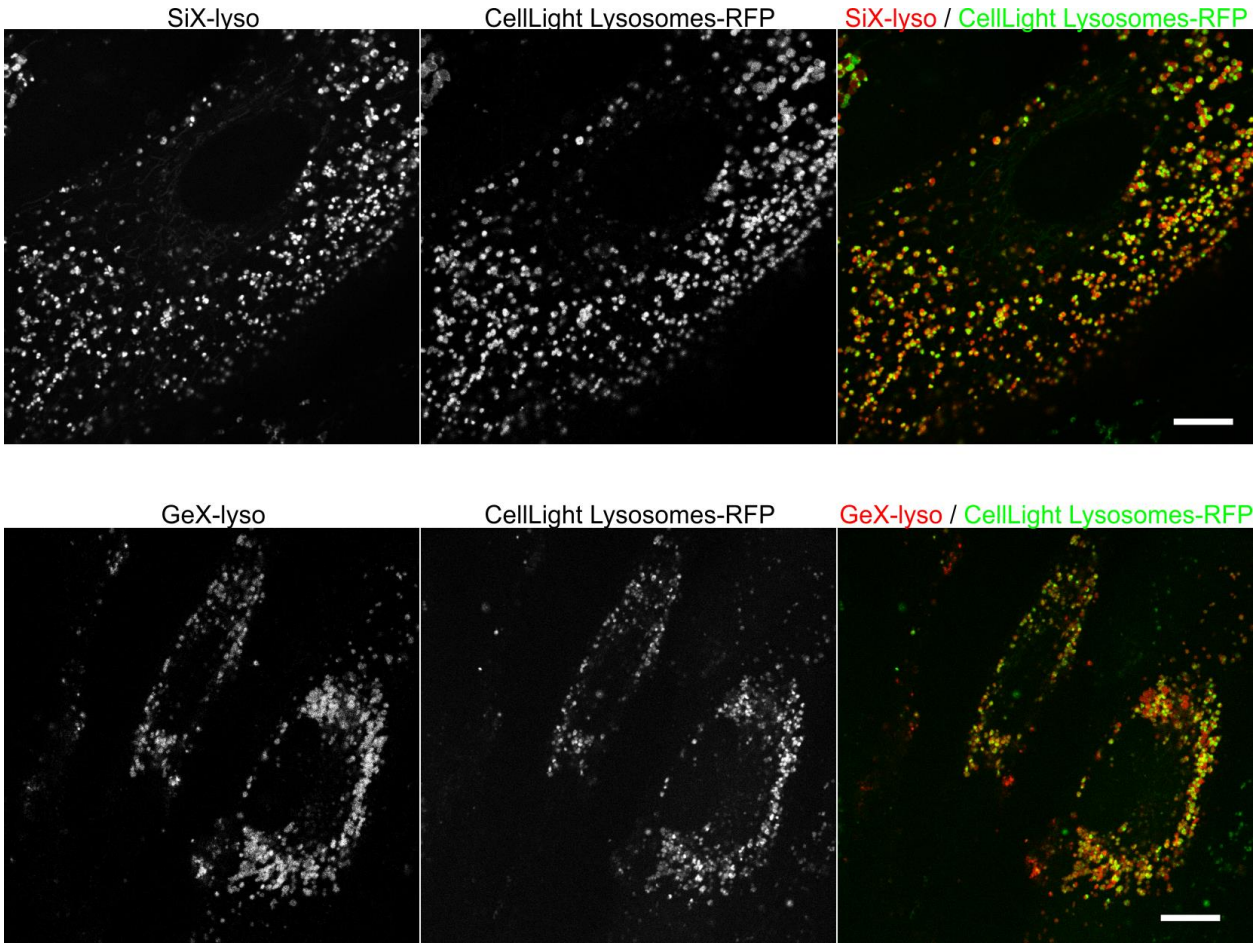
Supplementary Figure S9. Colocalization of LSS mitochondrial probes with MitoTracker Orange. Images show human fibroblasts stained with 0.5 µg/ml MitoTracker Orange CMTMRos (red; ThermoFisher) and 1 µM LSS mitochondrial probes (CX-TPP, GeX-TPP, SiX-TPP; green) for 1 h at + 37°C. Cells were washed twice with HBSS and imaged in DMEM growth media. Scale bar 10 µm.



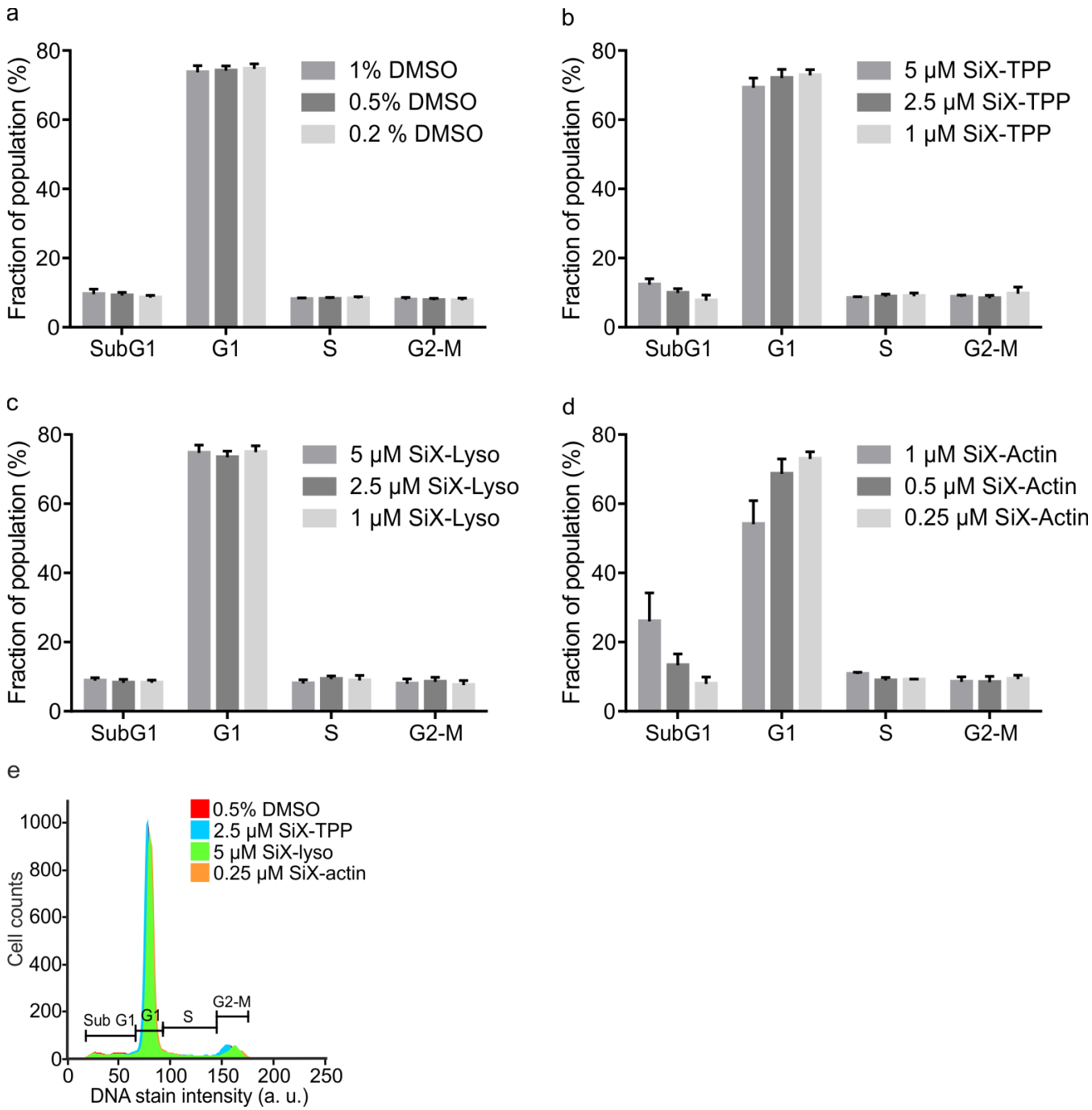
Supplementary Figure S10. Properties of lysosomal probes. (a) Absorbance and fluorescence spectra of 2 μM lysosomal probes **1b-4b** upon addition of 6 μM target protein – pepsin in 50 mM $HCOONH_4$ pH 4.0 buffer. (b) Inhibition of BSA hydrolysis by pepsin: SDS-PAGE gel showing degradation of BSA (1mg/ml) by 1 μM pepsin. Addition of 3 μM lysosomal probes **1b-4b** effectively inhibits degradation. 20, 40 and 80 min time points of the reactions are shown. (c) Fluorescence increase of lysosomal probes **1b-3b** and SiR-lysosome titrated with increasing concentrations of pepsin. Probe concentrations are kept constant at 0.1 μM. Fluorescence recorded after incubation at room temperature for 2 h. Experimental results are represented as mean with standard deviation (SD) of technical duplicates repeated three times. (d) Apparent K_d, obtained by fitting to equation (1) (see section ***In vitro* characterization of the probes**) and presented as mean with standard error of the mean.



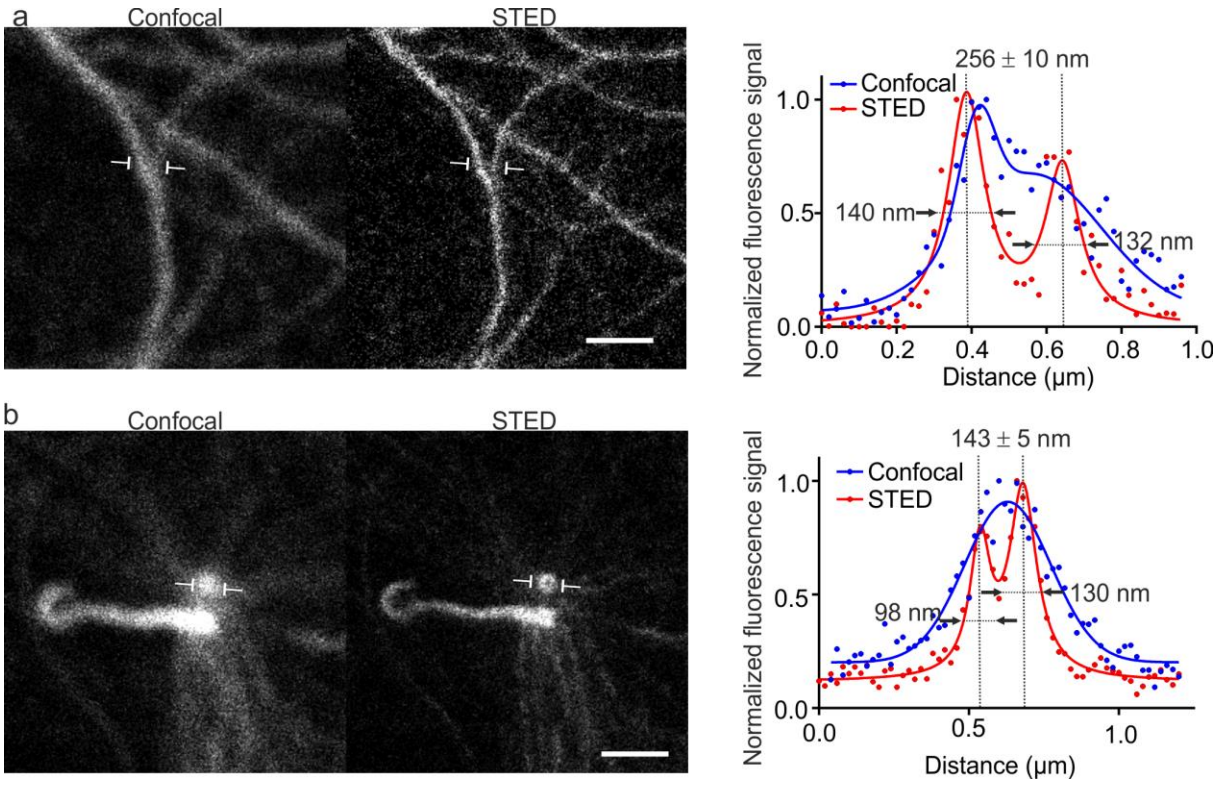
Supplementary Figure S11. Confocal microscopy images of human fibroblasts stained with lysosomal probes. The upper row shows large field of view of stained human fibroblasts. Dotted squares correspond to the zoomed-in areas shown in the lower row. White arrows indicate off-target mitochondrial staining of CX-lyso probe (**1b**). Cells were stained with 2 μM probes **1b-4b** and Hoechst 33342 (0.1 μg/ml) for 1 h at 37°C in the growth medium, washed twice with HBSS and imaged immediately in the growth medium. Scale bars are 50 μm (upper row) and 10 μm (lower row).



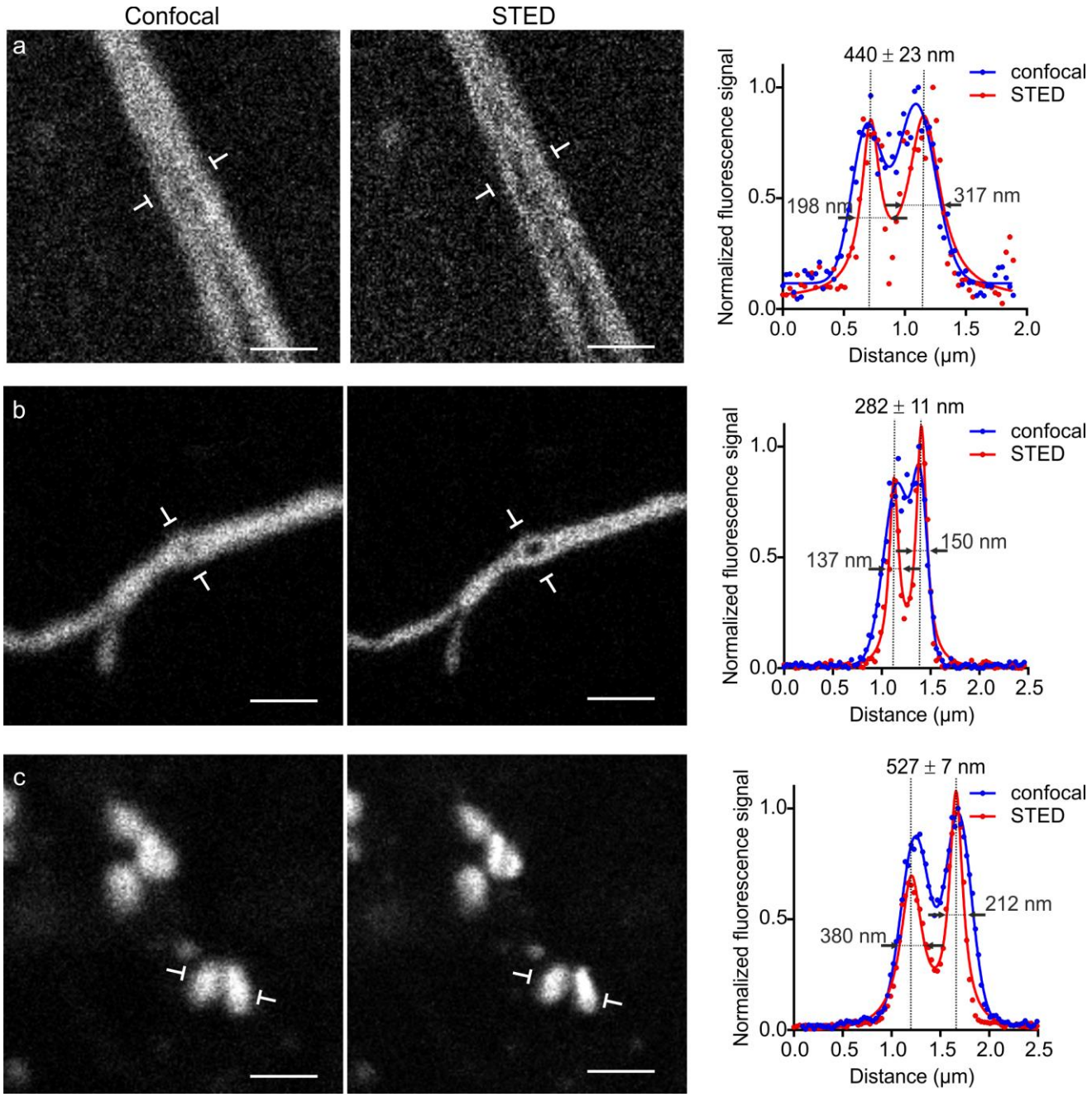
Supplementary Figure S12. Colocalization of LSS lysosomal probes with lysosomal marker Lamp1-tagRFP. Images show human fibroblasts 48 h after transduction with CellLight Lysosomes-RFP (green, ThermoFisher) marker and stained with 1 µM LSS lysosomal probes (red) for 1 h at + 37°C. Cells were washed twice with HBSS and imaged in DMEM growth media. Note that full colocalization is not expected because lysosomal associated membrane protein 1 (Lamp1) is localized to lysosomal membranes while LSS probes are localized inside lysosomes. Scale bar 10 µm.



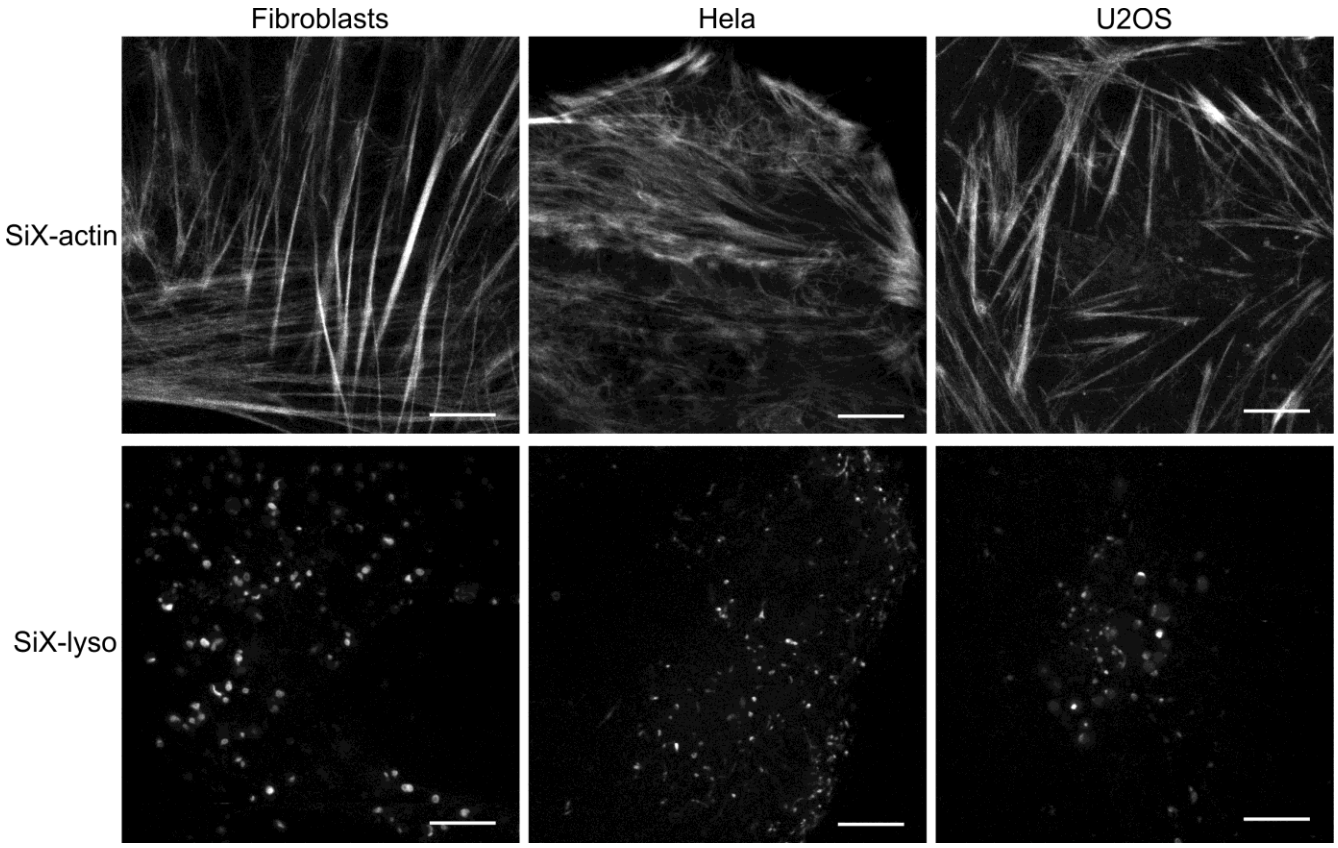
Supplementary Figure S13. Cell population distributions over cell cycle stages upon treatment with SiX probes. Cells were treated with DMSO (a), SiX-TPP **3a** (b), SiX-lyso **3b** (c) and SiX-actin **3c** (d) at different concentrations in growth media for 24 h (in a humidified 5% CO_2 incubator at 37°C). Afterwards, the cells were lysed, the nuclear DNA stained with DAPI and counted using NucleoCounter NC-3000 advanced image cytometer (Chemometec). 10000 cells per condition were counted and DNA staining intensity profile analysed to identify populations of the cells at different cell cycle stages. The experimental results are represented as mean with standard deviation (SD; N ≥ 3). (e) Representative fluorescence intensity histogram of nuclear DNA staining with DAPI. HeLa cells were treated with the indicated probes for 24 h, fixed and stained. Measured DNA content is corresponding to cell cycle phases: G1, S, G2 and M. Cells containing less DNA than haploid (SubG1) are considered non-viable.



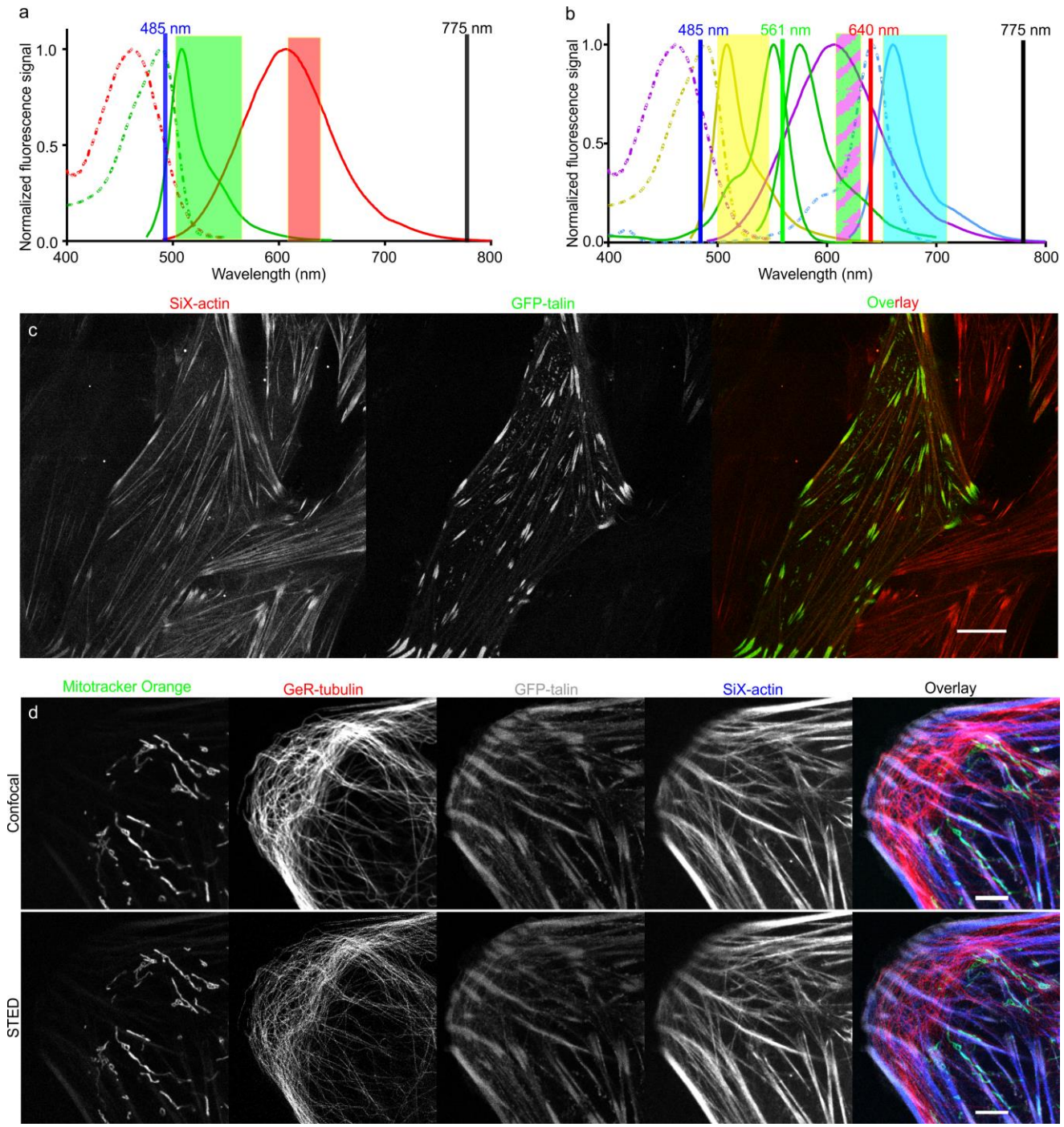
Supplementary Figure S14. Confocal and nanoscopy (STED at 775 nm) images of acetylated tubulin in human fibroblasts. (a) Images of cytosolic acetylated microtubules. Intensity profile of microtubules on the right clearly shows improvement in resolution. Human fibroblasts were stained with mouse anti-acetylated tubulin (Sigma Aldrich, cat. No. T7451), labeled with SiX-NHS. (b) Images of primary cilium growing from a centrosome. Intensity profile of the perpendicular centriole is shown on the right. Cells were grown for 1 week past confluence without passaging to increase the amount of acetylated tubulin and the percentage of cells forming primary cilium. Human fibroblasts were stained with mouse anti-acetylated tubulin primary antibody, labeled with SiX-NHS (**3d**). Confocal profiles are fitted to Gaussian and STED profiles are fitted to Lorentz distributions. The numbers next to arrows indicate full width at half maximum (FWHM) of fitted dual Lorentz distribution. The distances between the Lorentz distribution maxima are shown as values with fitting error. Scale bar 1 µm.



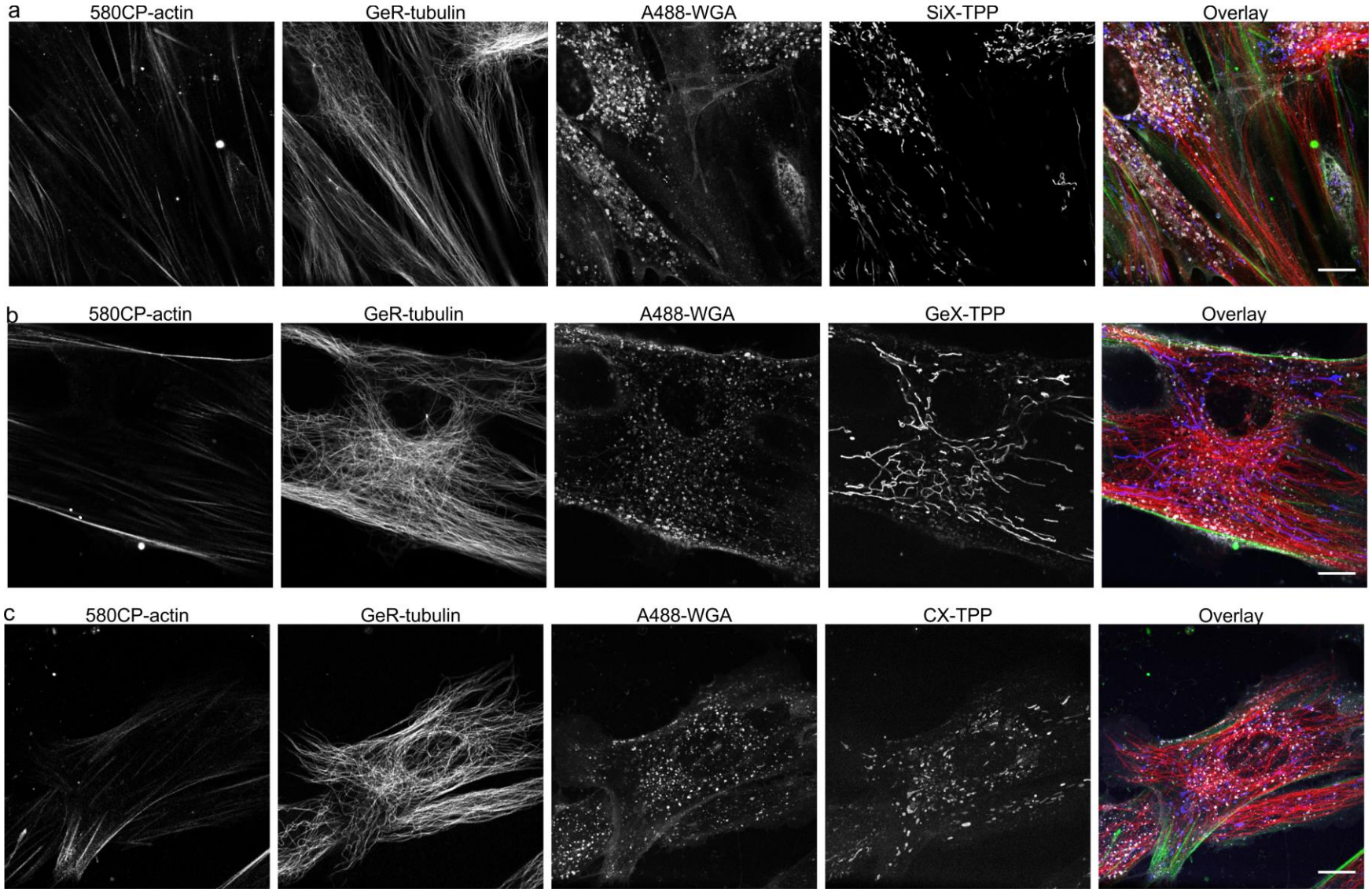
Supplementary Figure S15. Confocal and nanoscopy (STED at 775 nm) images of human fibroblasts stained with SiX probes. (a) Images of actin stress fibers in human fibroblasts stained with 0.25 µM SiX-actin **3c**. (b) Images of mitochondria in human fibroblasts stained with 1 µM SiX-TPP **3a**. (c) Images of lysosomes in human fibroblasts stained with 3 µM SiX-lyso **3b**. In all cases the intensity profiles shown on the right are taken at the position indicated in the corresponding images on the left. Confocal profiles (blue) and STED profiles (red) are fitted to Gaussian and Lorentz distributions, respectively. The distances between Lorentz distribution maxima are shown as values with fitting error. The numbers next to arrows indicate full widths at half maximum (FWHM) of fitted dual Lorentz distributions. Scale bars 1 µm.



Supplementary Figure S16. Nanoscopy (STED at 775 nm) images of human fibroblasts, HeLa and U2OS cell lines stained with SiX-actin and SiX-lyso as indicated. Cells were stained with 1 µM SiX-actin **3c** or 3 µM SiX-lyso **3b** for 1 h at 37°C in the growth medium, washed twice with HBSS and imaged in the growth medium. Scale bars 5 µm.



Supplementary Figure S17. Multicolour images of human fibroblasts expressing GFP-talin. (a) Two color microscopy setup used for imaging SiX and GFP simultaneously with 485 nm single laser excitation. Note that STED depletion laser at 775 nm is only able to affect the fluorescence of SiX. (b) Four color microscopy setup used for imaging SiX, GFP, MitoTracker Orange CM-H2TMRos (Thermofisher) and GeR-tubulin with three excitation lasers at 485 nm, 561 nm and 640 nm. Note that STED depletion laser at 775 nm is affecting fluorescence of SiX, MitoTracker Orange and GeR-tubulin but not of GFP. (c) Two colour confocal image of human fibroblasts expressing GFP-talin stained with 0.25 µM SiX-actin for 1 h at 37°C in the growth medium. Both channels can be read simultaneously as shown in (a) panel. Scale bar 10 µm. (d) Four color image of human fibroblasts expressing GFP-talin stained with 0.25 µM SiX-actin, 4 µM MitoTracker Orange CM-H2TMRos (Thermofisher) and 2 µM GeR-tubulin. Scale bar 5 µm.



Supplementary Figure S18. Four-color confocal images of human fibroblasts. Cells were stained with a mixture of probes in DMEM growth media: 1 µM 580CP-actin, 2 µM GeR-tubulin, 5 µg/ml Alexa Fluor 488 - Wheat germ agglutinin (WGA) conjugate and 1 µM large Stokes shift mitochondrial probes CX-TPP **1a**, GeX-TPP **2a** or SiX-TPP **3a.** Scale bars 10 µm.

Supplementary Tables.

Supplementary Table S1. Calculated excitation λ_{max} values for protonated and deprotonated forms of model structures vs. observed absorption λ_{max} values for iminoanthrone dyes 1-4 in protic and aprotic solvents.

Dye/model structure	Calculated excitation λ_{max} in MeOH, nm (corresponding oscillator strength)	Absorption λ_{max} measured in MeCN, nm	Absorption λ_{max} measured in 10% MeOH – PBS 7.4, nm	Calculation error $\Delta\lambda_{max}$, nm (energy difference, eV)
CX (**1**)	344 (0.4630)	318	–	+26 (-0.29)
CX, protonated (**1**H$^+$)	440 (0.7124)	461	459	-19 (+0.12)
GeX (**2**)	336 (0.4048)	314	–	+22 (-0.26)
GeX, protonated (**2**H$^+$)	446 (0.6758)	458	454	-9 (+0.05)
SiX (**3**)	342 (0.3536)	317	–	+25 (-0.29)
SiX, protonated (**3**H$^+$)	449 (0.6648)	463	458	-9 (+0.05)
SO2X (**4**)	317 (0.2645) 349 (0.1998) 385 (0.1760)	328 360 396	–	-11 (+0.13) -11 (+0.11) -11 (+0.09)
SO2X, protonated (**4**H$^+$)	485 (0.5223) 384 (0.2250) 327 (0.1194)	–	509* 408* 349*	-24 (+0.12) -24 (+0.19) -22 (+0.24)

* measured in 10% acetonitrile – PBS 7.4.

Supplementary Table S2. Apparent dissociation constants of lysosomal probes.

	Apparent K_d^{app} (nM)*	Number of experimental replicates
SiX-lyso	49 ± 8	6
GeX-lyso	36 ± 7	6
CX-lyso	41 ± 7	6
SiR-lysosome	174 ± 27	6

* apparent K_ds are presented as mean with standard error of the mean (SEM).

Supplementary Table S3. Results of t-test of population distribution over cell cycle (data presented in Figure 3d,e).

	t-test p-value of probe sample as compared to 0.5% DMSO		
	2.5 µM SiX-TPP	5 µM SiX-lyso	0.25 µM SiX-actin
Sub G1	0.346	0.608	0.258
G1	0.144	0.679	0.313
S	0.164	0.836	0.007*
G2/M	0.308	0.809	0.032

* - indicates significant difference.

Supplementary Methods

General experimental information and synthesis

NMR spectra were recorded at 25 °C with an Agilent 400-MR spectrometer at 400.06 MHz (^{1}H), 376.40 MHz (^{19}F) and 100.60 MHz (^{13}C) and are reported in ppm. All ^{1}H spectra are referenced to tetramethylsilane (δ = 0 ppm) using the signals of the residual protons of $CHCl_3$ (7.26 ppm) in $CDCl_3$, CHD_2CN (1.94 ppm) in CD_3CN or DMSO-d_5 (2.50 ppm) in DMSO-d_6. ^{13}C spectra are referenced to tetramethylsilane (δ = 0 ppm) using the signals of the solvent: $CDCl_3$ (77.16 ppm), $\underline{C}D_3CN$ (1.32 ppm) or DMSO-d_6 (39.52 ppm). Multiplicities of signals are described as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet or overlap of non-equivalent resonances; br = broad signal. Coupling constants (J) are given in Hz.

ESI-MS were recorded on a Varian 500-MS spectrometer (Agilent). **ESI-HRMS** were recorded on a MICROTOF spectrometer (Bruker) equipped with ESI ion source (Apollo) and direct injector with LC autosampler Agilent RR 1200.

Liquid chromatography: Analytical HPLC was performed on a Knauer Azura liquid chromatography system with a binary P 6.1L pump (Article No. EPH35, Knauer), UV diode array detector DAD 6.1L (Article No. ADC11, Knauer), an injection valve with a 20 µL loop and two electrical switching valves V 2.1S with 6-port multiposition valve head (Article No. EWA10, Knauer). Analytical columns: Knauer Eurospher II 100-5 C18, 5 µm, 150×4 mm (Article No. 15DE181E2J, Knauer) or Interchim Uptisphere Strategy C18-HQ, 10 µm, 250×4.6 mm (Article No. US10C18HQ-250/P46, Interchim), typical flow rate: 1.2 mL/min, unless stated otherwise.

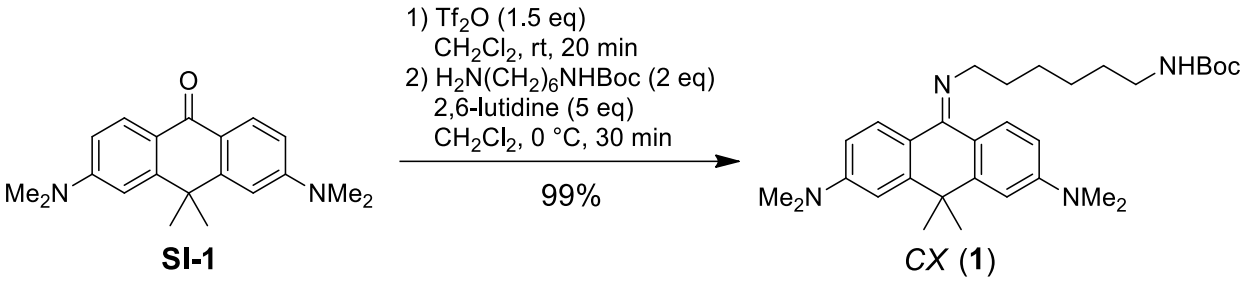
Preparative HPLC was performed on an Interchim puriFlash 4250 2X preparative HPLC/Flash hybrid system (Article No. 1I5140, Interchim) with a 2 mL injection loop, a 200-600 nm UV-Vis detector and an integrated ELSD detector (Article No. 1A3640, Interchim). Preparative column: Interchim Uptisphere Strategy C18-HQ, 10 µm, 250×21.2 mm (Article No. US10C18HQ-250/212, Interchim), typical flow rate: 20 mL/min, unless specified otherwise.

Analytical TLC was performed on Merck Millipore ready-to-use plates with silica gel 60 (F_{254}) (Cat. No. 1.05554.0001). Preparative TLC was performed on precoated thin-layer plates with silica gel for high performance TLC (HPTLC Silica gel 60 F_{254} 10×10 cm, layer thickness 150-200 µm, with concentrating zone 10 x 2.5 cm), purchased from Merck Millipore (Darmstadt, Germany; Cat. No. 1.13727.0001).

Flash chromatography was performed on Biotage Isolera flash purification system using the type of cartridge and solvent gradient indicated.

Chemical synthesis of iminoanthrone dyes and probes

tert-Butyl 6-(3,6-bis(dimethylamino)-10,10-dimethylanthracen-9(10*H*)-ylideneamino)hexylcarbamate (CX) (1)



SI-1 *CX* (**1**)

Trifluoromethanesulfonic anhydride (42 µL, 0.25 mmol, 1.5 eq) was added dropwise to a solution of ketone **SI-1** (prepared according to reported procedure[1]; 51 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (2 mL). The resulting intense blue reaction mixture was stirred at rt for 20 min and then added dropwise to a solution of *N*-Boc-1,6-diaminohexane (71 mg, 0.33 mmol, 2 eq) and 2,6-lutidine (97 µL, 0.83 mmol, 5 eq) in anhydrous CH_2Cl_2 (2 mL), cooled in ice-water bath. The resulting solution was stirred for 30 min in ice-water bath, sat. aq. $NaHCO_3$ (20 mL) was added, the mixture was extracted with ethyl acetate (4×20 mL), and the combined extracts were dried over Na_2SO_4. The filtrate was evaporated on Celite and the product was isolated by flash column chromatography (Büchi Sepacore Silica HP 12 g; gradient 0% to 100% A:B, A = 80:20:2 CH_2Cl_2 – ethanol – 25% aq. NH_3, B = CH_2Cl_2) and freeze-dried from 1,4-dioxane to give fluffy orange-yellow solid, yield 87 mg (99%).

^{1}H NMR (400 MHz, CD_3CN): δ 8.59 (br.s, 1H), 7.76 (d, *J* = 9.0 Hz, 2H), 6.98 (br.s, 2H), 6.78 (dd, *J* = 9.1, 2.6 Hz, 2H), 5.27 (br.s, 1H), 3.92 (t, *J* = 7.2 Hz, 2H), 3.14 (s, 12H), 2.98 (q, *J* = 6.5 Hz, 2H), 1.86 (p, *J* = 7.2 Hz, 2H), 1.65 (s, 6H), 1.48 – 1.24 (m, 6H), 1.37 (s, 9H).

^{13}C NMR (101 MHz, CD_3CN): δ 166.1, 154.8, ~132 (132.5), ~128 (128.3), ~112, ~111 (111.1), ~109 (108.8), 50.4, 41.8, 40.7, 40.4, 32.3, 30.6, 29.9, 28.6, 26.8, 26.7; rotation around the C=N imine bond results in significant signal broadening in the anthrone fragment. The values in brackets have been obtained from an ASAPHMQC experiment.

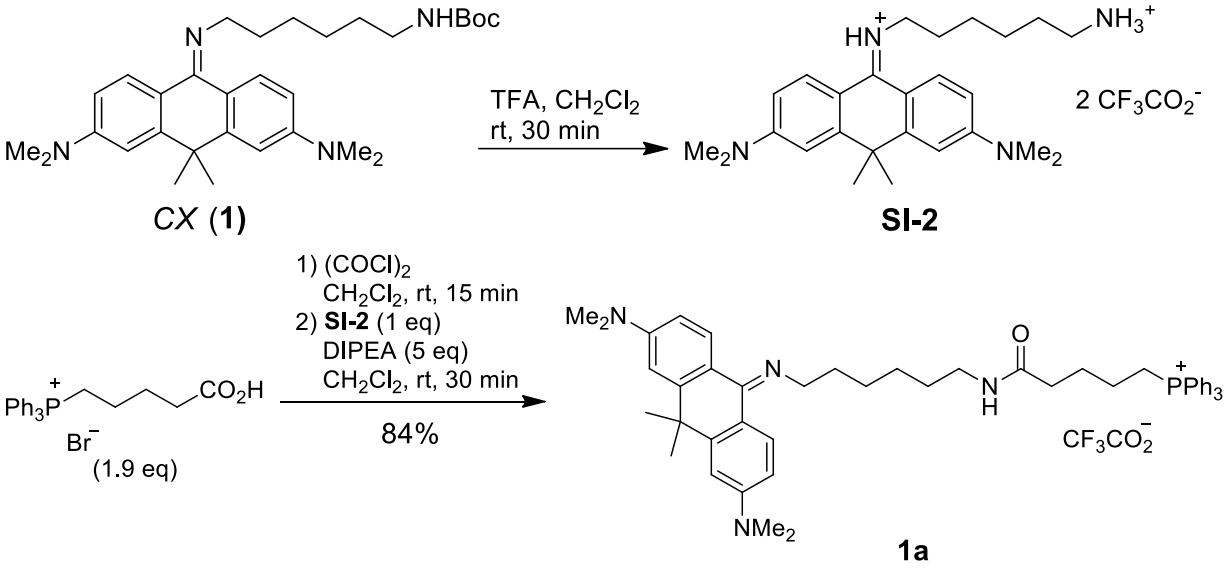
ESI-MS, positive mode: *m/z* (rel. int., %) = 507.4 $[M+H]^+$.

HR-MS (ESI, positive mode): 507.3694 $[M+H]^+$ (found), 507.3694 (calculated for $C_{31}H_{47}N_4O_2$, $[M+H]^+$).

UV-Vis (10% methanol – PBS, pH 7.4): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 459 (28000); emission, λmax, nm (Φ): 599 (0.55), excitation at 460 nm.

UV-Vis (acetonitrile): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 318 (13000), 461 (9600); emission, λmax, nm (Φ): 585 (0.74), excitation at 460 nm.

CX-TPP (**1a**)



Trifluoroacetic acid (300 µL) was added to a solution of the dye CX (**1**) (10 mg, 19.76 µmol) in CH_2Cl_2 (3 mL). The resulting yellow-orange solution was evaporated to dryness, re-evaporated with toluene (2×5 mL) and the residue of **SI-2** (trifluoroacetate salt) was dissolved in anhydrous CH_2Cl_2 (1 mL) to be used directly in the next step.

Oxalyl chloride (85 µL, 1 mmol) was added to the mixture of (4-carboxybutyl)triphenylphosphonium bromide (44 mg, 0.1 mmol) in CH_2Cl_2 (1 mL), cooled in ice-water bath. The reaction mixture was stirred at rt for 15 min, the resulting clear yellowish solution was evaporated to dryness, the residue was redissolved in anhydrous CH_2Cl_2 (0.6 mL), and the solution of the resulting acyl chloride was used in the following acylation.

A solution of trifluoroacetate salt **SI-2**, prepared as described above, was cooled in ice-water bath. DIPEA (20 µL, 110 µmol, ~5 eq) was added, followed immediately by the solution of acyl chloride (200 µL, 36 µmol, ~1.6 eq) dropwise. The cold bath was removed and the mixture was stirred at rt for 30 min, evaporated to dryness and the product was isolated by prep. HPLC (gradient 30/70 – 80/20 A:B over 20 min, A = acetonitrile, B = 0.1% TFA in water, t = 15.4 min) to give 11 mg (64%) of the product as viscous orange oil.

^{1}H NMR (400 MHz, DMSO-d_6): δ 10.30 (br.t, J = 5.8 Hz, 1H), 7.96 (br.d, J = 7.6 Hz, 1H), 7.92 – 7.85 (m, 3H), 7.83 – 7.72 (m, 12H), 7.03 (br.s, 1H), 6.96 (br.s, 1H), 6.83 (br.s, 1H), 6.81 (br.s, 1H), 3.92 (q, J = 6.7 Hz, 2H), 3.62 – 3.52 (m, 2H), 3.14 (br.s, 12H), 2.92 (q, J = 6.6 Hz, 2H), 2.08 (t, J = 7.2 Hz, 2H), 1.79 (p, J = 7.1 Hz, 2H), 1.67 (p, J = 7.4 Hz, 2H), 1.63 (s, 6H), 1.56 – 1.43 (m, 2H), 1.33 – 1.14 (m, 4H).

^{13}C NMR (101 MHz, DMSO-d_6): δ 134.8, 133.5, 130.1, 127.7, 110.2, 109.3, 107.8, 107.1, 48.9, 40.0, 39.6, 38.1, 34.2, 31.5, 28.9, 28.3, 26.1, 25.7, 21.2, 19.6 (indirect detection from the gHSQC experiment, only H-coupled carbons are resolved).

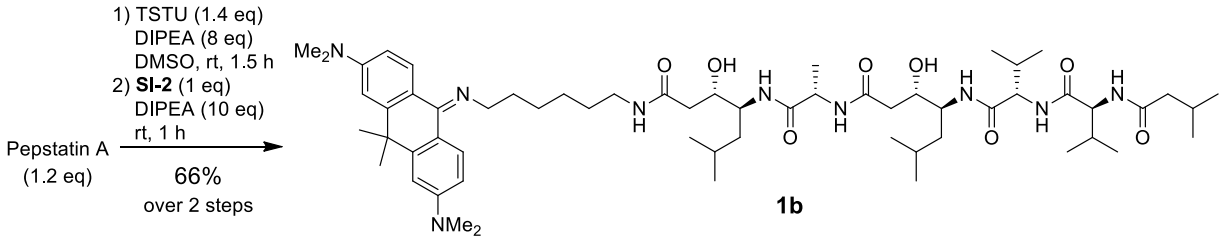
^{19}F NMR (376 MHz, DMSO-d_6): δ -74.26.

^{31}P NMR (162 MHz, DMSO-d_6): δ 23.9.

ESI-MS, positive mode: *m/z* (rel. int., %) = 751.5 (2) $[M]^+$, 376.2 (100) $[M+H]^{2+}$.

HR-MS (ESI, positive mode): 751.4500 $[M]^+$ (found), 751.4499 (calculated for $C_{49}H_{60}N_4OP$, $[M]^+$).

CX-**lyso** (**1b**)



Trifluoroacetic acid (300 µL) was added to a solution of the dye CX (**1**) (10 mg, 19.76 µmol) in CH_2Cl_2 (3 mL). The resulting yellow-orange solution was evaporated to dryness, re-evaporated with toluene (2×5 mL) and the residue of **SI-2** (trifluoroacetate salt) was dissolved in anhydrous DMSO (0.25 mL) to be used directly in the next step.

TSTU (*O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; 43 µL of 20 mg/100 µL stock solution in DMF, 28.5 µmol, 1.4 eq) was added to a mixture of Pepstatin A (16.3 mg, 23.71 µmol, 1.2 eq) and DIPEA (35 µL, 200 µmol, 8 eq) in anhydrous DMSO (1 mL). TLC control (silica, 10% methanol – CH_2Cl_2) showed complete conversion: R_f = 0.06 (starting material), 0.32 (product), both stained light-brown with vanillin stain. DIPEA (38 µL, 240 µmol, 10 eq), followed by the resulting solution of the NHS ester, was added dropwise to a solution of **SI-2** in DMSO (0.25 mL), and the reaction mixture was stirred at rt for 1 h. TLC control (silica, 90:10:2 CH_2Cl_2 – methanol – 25% aq. NH_3): R_f = 0.32 (CX), 0.13 (product). The reaction mixture was evaporated with CH_2Cl_2, the remaining DMSO solution was freeze-dried, and the product was isolated by preparative HPLC (gradient 30/70 – 80/20 A:B over 20 min, A = acetonitrile, B = 0.1% TFA in water, t = 18.7 min). Yield 14 mg (66% over 2 steps) of fluffy orange-yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ 10.25 (t, *J* = 5.8 Hz, 1H), 7.94 (d, *J* = 7.4 Hz, 2H), 7.83 (d, *J* = 8.8 Hz, 1H), 7.78 (d, *J* = 9.0 Hz, 1H), 7.67 (t, *J* = 5.6 Hz, 1H), 7.47 (d, *J* = 8.8 Hz, 1H), 7.31 (d, *J* = 9.1 Hz, 1H), 7.03 (br.s, 1H), 6.96 (br.s, 1H), 6.84 (s, 1H), 6.82 (s, 1H), 4.26 – 4.09 (m, 3H), 3.93 (q, *J* = 6.7 Hz, 2H), 3.87 – 3.70 (m, 2H), 3.14 (br.s, 12H), 3.07 – 2.90 (m, 2H), 2.12

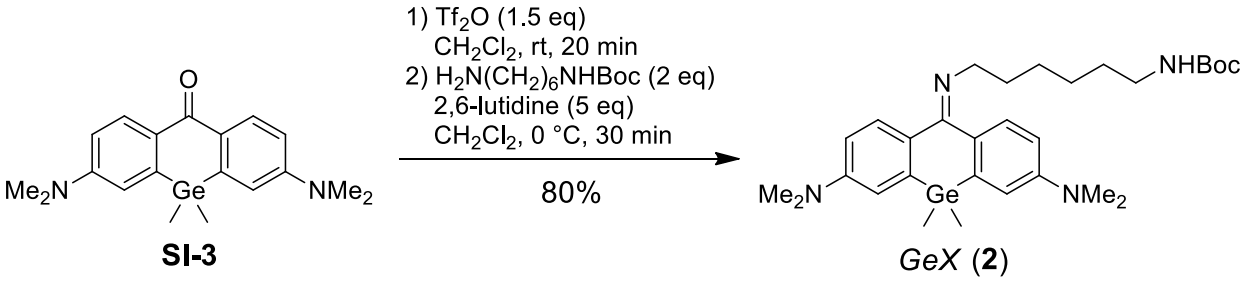
(d, *J* = 6.1 Hz, 2H), 2.07 – 2.00 (m, 3H), 2.00 – 1.88 (m, 2H), 1.81 (dt, *J* = 13.7, 6.2 Hz, 2H), 1.63 (s, 6H), 1.59 – 1.45 (m, 2H), 1.41 – 1.15 (m, 16H), 0.90 – 0.74 (m, 30H).

^{13}C NMR (101 MHz, DMSO-d_6): δ 131.0, 127.6, 110.2, 109.3, 107.8, 107.0, 69.1, 68.9, 57.9, 57.7, 50.6, 50.3, 48.9, 48.3, 44.3, 39.9, 39.6, 39.1, 38.2, 31.5, 30.3, 28.9, 28.3, 25.8, 24.1, 23.2, 22.2, 21.6, 19.2, 18.2, 18.1 (indirect detection from the gHSQC experiment, only H-coupled carbons are resolved).

ESI-MS, positive mode: *m/z* (rel. int., %) = 1074.8 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 1074.7686 $[M+H]^+$ (found), 1074.7689 (calculated for $C_{60}H_{100}N_9O_8$, $[M+H]^+$).

tert-Butyl 6-(3,7-bis(dimethylamino)-5,5-dimethyldibenzo[*b*,*e*]germin-10(5*H*)-ylideneamino)hexylcarbamate (GeX) (**2**)



SI-3 *GeX* (**2**)

Trifluoromethanesulfonic anhydride (42 µL, 0.25 mmol, 1.5 eq) was added dropwise to a solution of ketone **SI-3** (prepared according to reported procedure[2]; 61 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (2 mL). The resulting intense blue reaction mixture was stirred at rt for 20 min and then added dropwise to a solution of *N*-Boc-1,6-diaminohexane (71 mg, 0.33 mmol, 2 eq) and 2,6-lutidine (97 µL, 0.83 mmol, 5 eq) in anhydrous CH_2Cl_2 (2 mL), cooled in ice-water bath. The resulting solution was stirred for 30 min in ice-water bath, sat. aq. $NaHCO_3$ (20 mL) was added, the mixture was extracted with ethyl acetate (3×25 mL), and the combined extracts were dried over Na_2SO_4. The filtrate was evaporated on Celite and the product was isolated by flash column chromatography (Büchi Sepacore Silica HP 12 g; gradient 0% to 100% A:B, A = 85:15:2 CH_2Cl_2 – ethanol – 25% aq. NH_3, B = CH_2Cl_2) and freeze-dried from 1,4-dioxane to give fluffy yellow solid, yield 75 mg (80%).

^{1}H NMR (400 MHz, $CDCl_3$): 7.69 (d, *J* = 8.6 Hz, 1H), 7.27 (d, *J* = 8.6 Hz, 1H), 6.89 (d, *J* = 2.7 Hz, 1H), 6.78 (d, *J* = 2.7 Hz, 1H), 6.74 (dd, *J* = 8.6, 2.7 Hz, 1H), 6.67 (dd, *J* = 8.6, 2.8 Hz, 1H), 4.51 (br.s, 1H), 3.73 (t, *J* = 7.0 Hz, 2H), 3.12 – 3.03 (m, 2H), 3.01 (s, 6H), 2.96 (s, 6H), 1.72 (p, *J* = 7.0 Hz, 2H), 1.43 (s, 9H), 1.42 – 1.23 (m, 6H), 0.58 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 156.1, 149.9, 149.4, 141.9, 138.3, 129.7, 128.1, 115.7, 114.9, 113.7, 111.1, 79.0, 54.0, 40.7, 40.4, 32.0, 30.1, 28.5, 27.4, 26.8, -2.9.

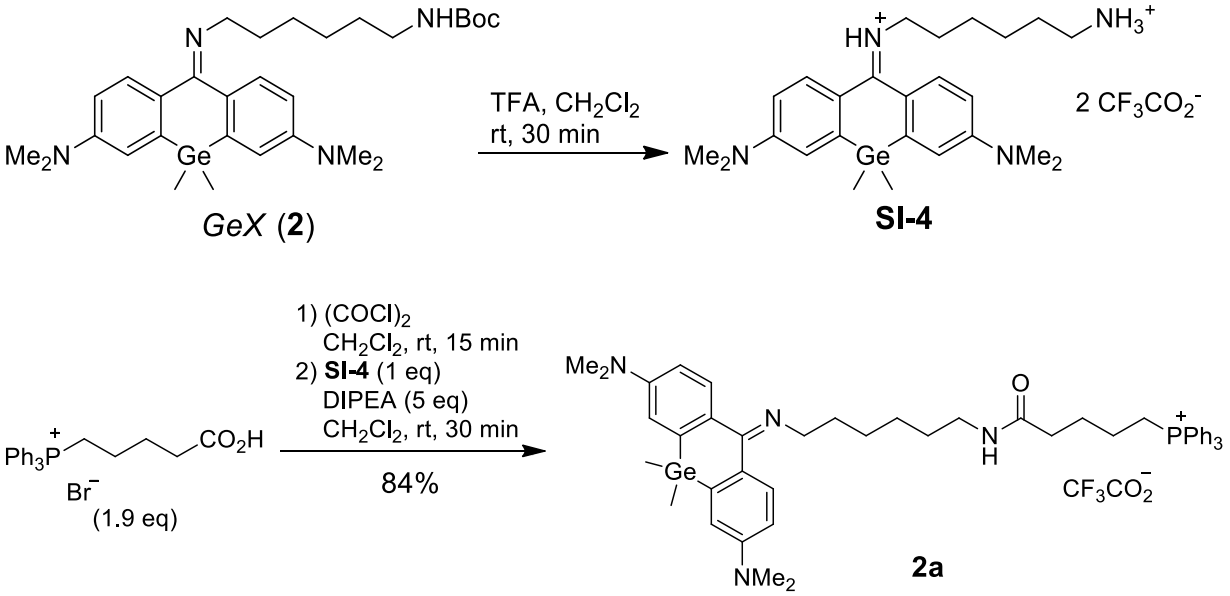
ESI-MS, positive mode: *m/z* (rel. int., %) = 565.3 [M+H, ^{70}Ge]$^+$.

HR-MS (ESI, positive mode): 565.2931 [M+H, ^{70}Ge]$^+$ (found), 565.2936 (calculated for $C_{30}H_{47}N_4O_2Ge$, [M+H, ^{70}Ge]$^+$).

UV-Vis (10% methanol – PBS, pH 7.4): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 454 (24000); emission, λmax, nm (Φ): 618 (0.17), excitation at 450 nm.

UV-Vis (acetonitrile): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 314 (24000), 458 (120); emission, λmax, nm (Φ): 602 (0.42), excitation at 460 nm.

GeX-**TPP** (**2a**)



Trifluoroacetic acid (270 µL) was added to a solution of the dye GeX (**2**) (10 mg, 17.64 µmol) in CH_2Cl_2 (2.7 mL). The resulting yellow-orange solution was evaporated to dryness, re-evaporated with toluene (2×5 mL) and the residue of **SI-4** (trifluoroacetate salt) was dissolved in anhydrous CH_2Cl_2 (1 mL) to be used directly in the next step.

Oxalyl chloride (85 µL, 1 mmol) was added to the mixture of (4-carboxybutyl)triphenylphosphonium bromide (44 mg, 0.1 mmol) in CH_2Cl_2 (1 mL), cooled in ice-water bath. The reaction mixture was stirred at rt for 15 min, the resulting clear yellowish solution was evaporated to dryness, the residue was redissolved in anhydrous CH_2Cl_2 (0.6 mL), and the solution of the resulting acyl chloride was used in the following acylation.

A solution of trifluoroacetate salt **SI-4**, prepared as described above, was cooled in ice-water bath. DIPEA (20 µL, 110 µmol, ~5 eq) was added, followed immediately by the acyl chloride solution (200 µL, 36 µmol, ~1.8 eq) dropwise. The cold bath was removed and the mixture was stirred at rt for 30 min, evaporated to dryness and the product was isolated by prep. HPLC (gradient 40/60 – 80/20 A:B over 20 min, A =

acetonitrile, B = 0.1% TFA in water, t = 10.6 min) to give 14 mg (84%) of the product as viscous orange oil.

^{1}H NMR (400 MHz, DMSO-d_6): δ 11.12 (br.t, J = 6.3 Hz, 1H), 8.15 (d, J = 8.8 Hz, 1H), 7.92 – 7.84 (m, 3H), 7.82 – 7.70 (m, 12H), 7.62 (d, J = 9.0 Hz, 1H), 7.05 (d, J = 2.7 Hz, 1H), 6.99 (d, J = 2.7 Hz, 1H), 6.88 – 6.80 (m, 2H), 3.87 (q, J = 6.5 Hz, 2H), 3.62 – 3.50 (m, 2H), 3.09 (s, 6H), 3.07 (s, 6H), 2.89 (q, J = 6.6 Hz, 2H), 2.07 (t, J = 7.2 Hz, 2H), 1.76 – 1.62 (m, 4H), 1.56 – 1.43 (m, 2H), 1.29 – 1.19 (m, 2H), 1.18 – 1.07 (m, 4H), 0.62 (s, 6H).

^{13}C NMR (101 MHz, DMSO-d_6): δ 135.3, 134.0, 131.8, 131.6, 130.6, 116.6, 115.8, 112.3, 111.3, 50.0, 40.0, 38.6, 34.7, 29.4, 28.4, 26.5, 26.2, 21.8, 20.6, -2.2 (indirect detection from the gHSQC experiment, only H-coupled carbons are resolved).

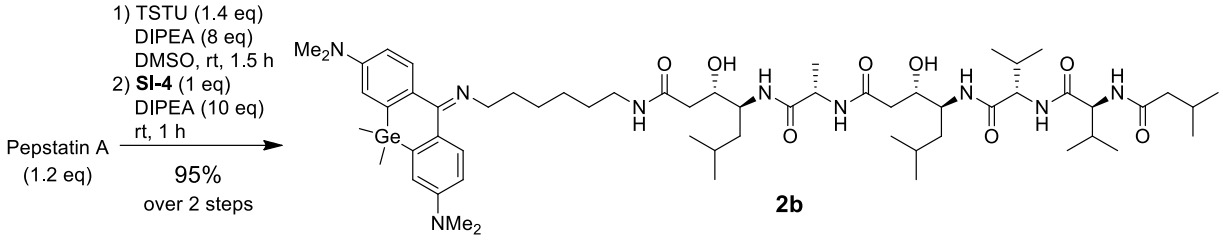
^{19}F NMR (376 MHz, DMSO-d_6): δ -74.63.

^{31}P NMR (162 MHz, DMSO-d_6): δ 23.9.

ESI-MS, positive mode: *m/z* (rel. int., %) = 813.4 (18) $[M]^+$, 407.2 (100) $[M+H]^{2+}$.

HR-MS (ESI, positive mode): 809.3722 [M, ^{70}Ge]$^+$ (found), 809.3742 (calculated for $C_{48}H_{60}N_4OPGe$, [M, ^{70}Ge]$^+$).

GeX**-lyso** (**2b**)



Trifluoroacetic acid (270 µL) was added to a solution of the dye GeX (**2**) (10 mg, 17.64 µmol) in CH_2Cl_2 (2.7 mL). The resulting yellow-orange solution was evaporated to dryness, re-evaporated with toluene (2×5 mL) and the residue of **SI-4** (trifluoroacetate salt) was dissolved in anhydrous DMSO (0.2 mL) to be used directly in the next step.

TSTU (*O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; 38 µL of 20 mg/100 µL stock solution in DMSO, 25.4 µmol, 1.4 eq) was added to a mixture of Pepstatin A (14.5 mg, 21.17 µmol, 1.2 eq) and DIPEA (31 µL, 178 µmol, 8 eq) in anhydrous DMSO (0.7 mL). TLC control (silica, 10% methanol – CH_2Cl_2) showed complete conversion: R_f = 0.06 (starting material), 0.32 (product), both stained light-brown with vanillin stain. DIPEA (38 µL, 218 µmol, 10 eq), followed immediately by the resulting solution of the NHS ester, were added dropwise to a solution of **SI-4** in DMSO (0.2 mL), and the reaction mixture was stirred at rt for 1 h. TLC control (silica, 90:10:2 CH_2Cl_2 – methanol – 25% aq. NH_3): R_f = 0.49 (GeX), 0.31 (product). The reaction mixture was evaporated with CH_2Cl_2, the remaining DMSO solution was freeze-dried, and the product was isolated by preparative HPLC (gradient 30/70 –

90/10 A:B over 20 min, A = acetonitrile, B = 0.1% TFA in water, t = 17.8 min). Yield 19 mg (95% over 2 steps) of fluffy yellow solid.

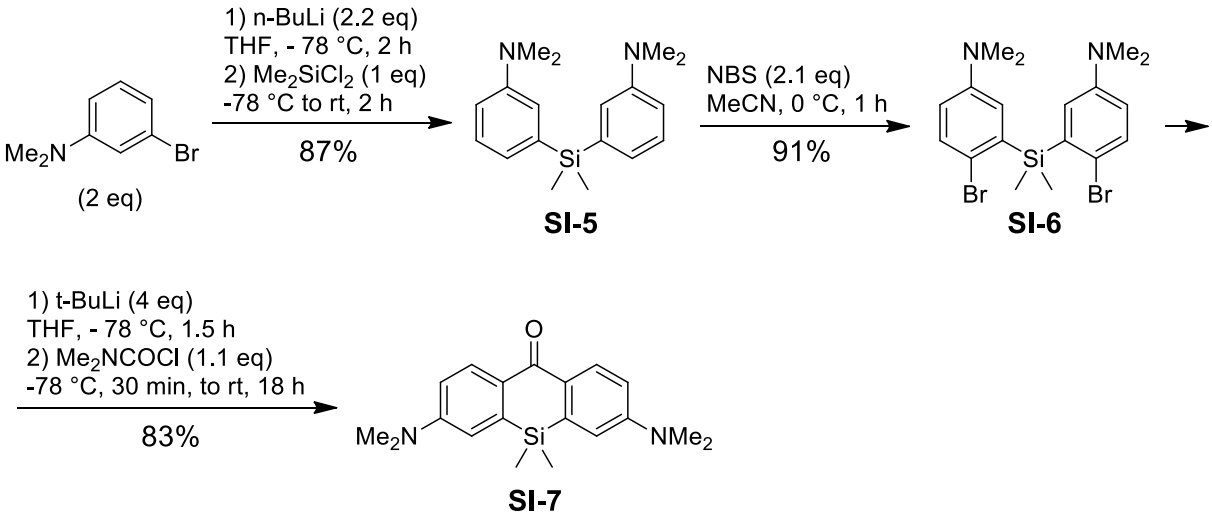
^{1}H NMR (400 MHz, DMSO-d_6): δ 11.06 (br.t, *J* = 5.9 Hz, 1H), 7.93 (d, *J* = 7.3 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 1H), 7.78 (d, *J* = 9.0 Hz, 1H), 7.73 (d, *J* = 9.0 Hz, 1H), 7.65 (t, *J* = 5.6 Hz, 1H), 7.62 (d, *J* = 9.0 Hz, 1H), 7.46 (d, *J* = 8.8 Hz, 1H), 7.30 (d, *J* = 9.1 Hz, 1H), 7.05 (d, *J* = 2.7 Hz, 1H), 6.98 (d, *J* = 2.7 Hz, 1H), 6.86 (dd, *J* = 9.4, 2.8 Hz, 1H), 6.84 (dd, *J* = 9.4, 2.9 Hz, 1H), 5.30 (br.s, 2H + H_2O), 4.28 – 4.09 (m, 3H), 3.91 – 3.71 (m, 4H), 3.09 (s, 6H), 3.07 (s, 6H), 3.03 – 2.86 (m, 2H), 2.12 (d, *J* = 6.1 Hz, 2H), 2.05 – 2.00 (m, 3H), 2.00 – 1.88 (m, 4H), 1.78 – 1.68 (m, 2H), 1.60 – 1.46 (m, 2H), 1.41 – 1.11 (m, 14H), 0.89 – 0.74 (m, 30H), 0.63 (s, 6H).

^{13}C NMR (101 MHz, DMSO-d_6): δ 131.1, 129.4, 115.9, 115.1, 111.6, 110.6, 68.9, 68.8, 57.7, 57.6, 50.5, 50.1, 49.3, 48.2, 44.2, 39.8, 39.3 (×3.07 ppm, ×3.09 ppm), 39.3 (×1.22 ppm, ×1.36 ppm), 39.0, 38.4 (×1.24 ppm, ×1.34 ppm), 38.0, 29.9, 28.7, 27.8, 25.6, 25.4, 23.9, 23.1, 22.0, 21.5, 19.0, 18.04, 18.02, -2.9 (indirect detection from the gHSQC experiment, only H-coupled carbons are resolved; ^{1}H correlations are indicated for the separate cross-peaks with indiscernible ^{13}C chemical shifts).

ESI-MS, positive mode: *m/z* (rel. int., %) = 579.9 (100) $[M+H+Na]^{2+}$, 1136.7 (78) $[M+H]^{+}$.

HR-MS (ESI, positive mode): 1136.6907 $[M+H]^{+}$ (found), 1136.6917 (calculated for $C_{59}H_{100}N_9O_8Ge$, $[M+H]^{+}$).

3,7-Bis(dimethylamino)-5,5-dimethyldibenzo[*b*,*e*]silin-10(5*H*)-one (SI-7)



Compound SI-5

n-Butyllithium (6.6 mL of 2.5 M in hexanes, 16.5 mmol, 2.2 eq) was added quickly dropwise to a cold (-78 °C) solution of 3-bromo-*N*,*N*-dimethylaniline (3.0 g, 15 mmol, 2 eq) in anhydrous THF (40 mL). The mixture was stirred at -78 °C for 2 h, and dichlorodimethylsilane (910 µL, 7.5 mmol, 1 eq) was added quickly dropwise at -78 °C. The resulting mixture was stirred at rt for 2 h. It was then quenched with water

(10 mL) and brine (40 mL), extracted with ethyl acetate (3×50 mL) and the combined extracts were dried over Na_2SO_4. The product was isolated by flash column chromatography (120 g RediSep Rf; gradient 0% to 30% ethyl acetate – hexane) and dried *in vacuo*. Yield 1.95g (87%), yellowish oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 (dd, *J* = 8.3, 7.1 Hz, 2H), 6.96 (d, *J* = 2.7 Hz, 2H), 6.94 (d, *J* = 7.1 Hz, 2H), 6.80 (br.d, *J* = 8.8 Hz, 1H), 2.94 (s, 12H), 0.54 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 149.9, 139.1, 128.6, 123.1, 118.6, 113.9, 41.0, -2.0.

Compound SI-6

N-Bromosuccinimide (2.44 g, 13.73 mmol, 2.1 equiv) was added in small portions to a solution of **SI-5** (1.95 g, 6.54 mmol) in acetonitrile (40 mL), cooled in ice-water bath. The mixture turned light yellow upon addition of each portion and faded quickly; at the end of addition, light purple color appeared. The mixture was stirred for further 1 h at 0 °C. Sat. aq. $NaHCO_3$ (40 mL) was then added, and the reaction mixture was extracted with CH_2Cl_2 (3×40 mL), the combined extracts were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (80 g RediSep Rf; gradient 0% to 80% CH_2Cl_2 – hexane), the fractions containing the product were pooled and evaporated to give light yellow solid. Yield 2.72 g (91%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (d, *J* = 8.8 Hz, 2H), 6.85 (d, *J* = 3.2 Hz, 2H), 6.60 (dd, *J* = 8.8, 3.2 Hz, 2H), 2.88 (s, 12H), 0.76 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 149.0, 138.9, 133.1, 121.9, 115.4, 40.7, -0.8.

ESI-MS, positive mode: *m/z* (rel. int., %) = 457.2 (100) [M+H, ^{79}Br+^{81}Br]$^+$.

HR-MS (ESI, positive mode): 455.0155 [M+H, 2×^{79}Br]$^+$ (found), 455.0148 (calculated for $C_{18}H_{25}Br_2N_2Si$, [M+H, 2×^{79}Br]$^+$).

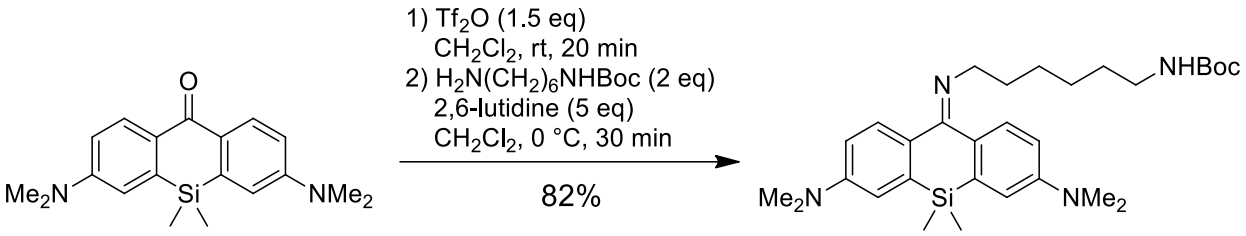
Compound SI-7

tert-Butyllithium (12.8 mL of 2.5 M in hexanes, 21.76 mmol, 4 eq) was added quickly dropwise to a cold (-78 °C) solution of **SI-6** (2.48 g, 5.44 mmol) in anhydrous THF (100 mL). The resulting dark yellow solution was stirred at -78 °C for 1.5 h. Neat *N*,*N*-dimethylcarbamoyl chloride (0.55 mL, 5.98 mmol, 1.1 eq) was then injected dropwise (the color of the reaction mixture changed to light yellow). The resulting mixture was stirred at -78 °C for 30 min, then allowed to warm up to rt and left stirring overnight. It was then quenched with sat. aq. NH_4Cl (50 mL), water was added to dissolve solids, and the mixture was extracted with ethyl acetate (3×40 mL). The combined extracts were washed with brine, dried over Na_2SO_4, filtered, and the product was isolated by flash column chromatography (80 g Redisep Rf; gradient 0% to 10% ethyl acetate – CH_2Cl_2) to give 1.46 g (83%) of the ketone **SI-7** as bright yellow crystalline solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.40 (dt, *J* = 9.1, 0.5 Hz, 1H), 6.84 (ddd, *J* = 9.1, 2.8, 0.5 Hz, 1H), 6.80 (d, *J* = 2.8 Hz, 1H), 3.09 (s, 3H), 0.47 (d, *J* = 0.5 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 185.4, 151.6, 140.6, 131.7, 129.8, 114.4, 113.3, 40.2, -0.9.

***tert*-Butyl 6-(3,7-bis(dimethylamino)-5,5-dimethyldibenzo[*b*,*e*]silin-10(5*H*)-ylideneamino)hexylcarbamate** (SiX) (**3**)



SI-7 *SiX* (**3**)

Trifluoromethanesulfonic anhydride (42 µL, 0.25 mmol, 1.5 eq) was added dropwise to a solution of ketone **SI-7** (prepared as described above, or according to reported procedure[3]; 53 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (2 mL). The resulting intense blue reaction mixture was stirred at rt for 20 min and then added dropwise to a solution of *N*-Boc-1,6-diaminohexane (71 mg, 0.33 mmol, 2 eq) and 2,6-lutidine (97 µL, 0.83 mmol, 5 eq) in anhydrous CH_2Cl_2 (2 mL), cooled in ice-water bath. The resulting solution was stirred for 30 min in ice-water bath, sat. aq. $NaHCO_3$ (20 mL) was added, the mixture was extracted with ethyl acetate (3×20 mL), and the combined extracts were dried over Na_2SO_4. The filtrate was evaporated on Celite and the product was isolated by flash column chromatography (Büchi Sepacore Silica HP 12 g; gradient 0% to 100% A:B, A = 85:15:2 CH_2Cl_2 – ethanol – 25% aq. NH_3, B = CH_2Cl_2) and freeze-dried from 1,4-dioxane to give fluffy yellow solid, yield 71 mg (82%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, *J* = 8.6 Hz, 1H), 7.34 (d, *J* = 8.7 Hz, 1H), 6.94 (d, *J* = 2.8 Hz, 1H), 6.82 (d, *J* = 2.7 Hz, 1H), 6.79 (dd, *J* = 8.7, 2.8 Hz, 1H), 6.71 (dd, *J* = 8.7, 2.8 Hz, 1H), 4.52 (br.s, 1H), 3.79 (t, *J* = 7.1 Hz, 2H), 3.08 – 3.04 (m, 2H), 3.04 (s, 6H), 2.99 (s, 6H), 1.76 (p, *J* = 7.1 Hz, 2H), 1.44 – 1.23 (m, 6H), 1.43 (s, 9H), 0.45 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 156.1, 150.2, 149.8, 140.1, 136.3, 130.0, 128.3, 116.0, 115.0, 114.1, 111.4, 53.4, 40.6, 40.3, 31.5, 30.1, 28.6, 27.2, 26.7, -2.3.

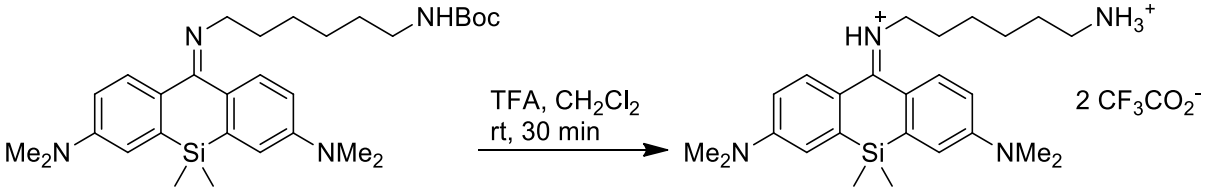
ESI-MS, positive mode: *m/z* (rel. int., %) = 523.3 $[M+H]^+$.

HR-MS (ESI, positive mode): 523.3463 $[M+H]^+$ (found), 523.3463 (calculated for $C_{30}H_{47}N_4O_2Si$, $[M+H]^+$).

UV-Vis (10% methanol – PBS, pH 7.4): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 458 (17000); emission, λmax, nm (Φ): 623 (0.28), excitation at 470 nm.

UV-Vis (acetonitrile): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 317 (21000), 462 (450); emission, λmax, nm (Φ): 607 (0.58), excitation at 470 nm.

6-(3,7-bis(dimethylamino)-5,5-dimethyldibenzo[*b*,*e*]silin-10(5*H*)-ylideneamino)hexylamine bis(trifluoroacetate) (SI-8)



SiX (**3**) **SI-8**

Trifluoroacetic acid (200 µL) was added to a solution of the dye SiX (**3**) (5 mg, 9.06 µmol) in CH_2Cl_2 (2 mL). The resulting yellow-orange solution was evaporated to dryness, re-evaporated with toluene (2×5 mL) and freeze-dried from aqueous dioxane. The resulting free amine (trifluoroacetate salt, yellow hygroscopic solid) was dissolved in DMSO (0.4 mL) and used directly in the next step.

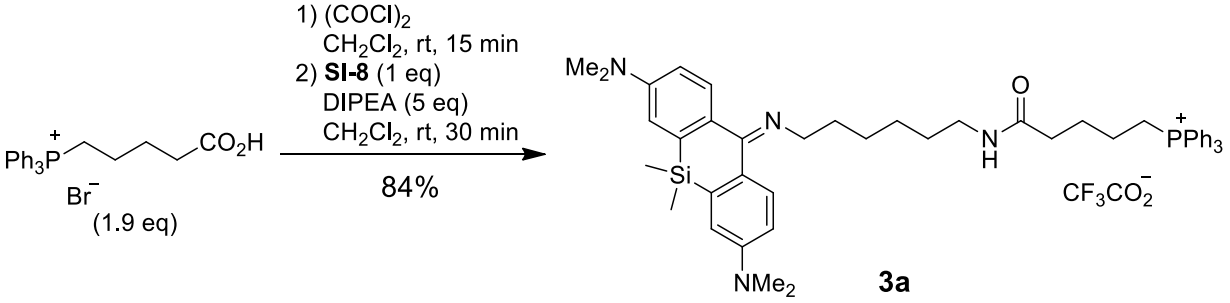
^{1}H NMR (400 MHz, $CDCl_3$): δ 10.80 (br.s, 1H, NH^+), 7.95 (br.s, 4H, NH_3^+), 7.84 (br.d, *J* = 8.9 Hz, 1H), 7.44 (br.d, *J* = 9.0 Hz, 1H), 6.86 (d, *J* = 2.7 Hz, 1H), 6.76 (d, *J* = 2.5 Hz, 1H), 6.69 (dd, *J* = 8.9, 2.9 Hz, 2H), 3.85 (br.s, 2H), 3.06 (s, 6H), 3.01 (s, 6H), 2.84 (br.s, 2H), 1.78 (br.s, 2H), 1.59 (br.s, 2H), 1.34 – 1.16 (m, 4H), 0.41 (s, 6H); signals of the aliphatic chain show significant broadening.

^{19}F NMR (376 MHz, $CDCl_3$): δ -75.75.

ESI-MS, positive mode: *m/z* (rel. int., %) = 423.3 $[M+H]^+$.

HR-MS (ESI, positive mode): 423.2939 $[M+H]^+$ (found), 423.2939 (calculated for $C_{25}H_{39}N_4Si$, $[M+H]^+$).

SiX-**TPP** (**3a**)



3a

Oxalyl chloride (85 µL, 1 mmol) was added to the mixture of (4-carboxybutyl)triphenylphosphonium bromide (44 mg, 0.1 mmol) in CH_2Cl_2 (1 mL), cooled in ice-water bath. The reaction mixture was stirred at rt for 15 min, the

resulting clear yellowish solution was evaporated to dryness, the residue was redissolved in anhydrous CH_2Cl_2 (0.6 mL), and the solution of the resulting acyl chloride was used in the following acylation.

Trifluoroacetate salt **SI-8**, prepared from 10 mg of SiX (~20 µmol), was dissolved in anhydrous CH_2Cl_2 (1 mL) and cooled in ice-water bath. DIPEA (20 µL, 110 µmol, ~5 eq) was added, followed immediately by the solution of acyl chloride (220 µL, 37 µmol, ~1.9 eq) dropwise. The cold bath was removed and the mixture was stirred at rt for 30 min, evaporated to dryness and the product was isolated by prep. HPLC (column Kinetex 5 µm C18 100, diam. 21 mm × 250 mm, gradient 40/60 – 100/0 A:B over 20 min, A = acetonitrile, B = 0.05% TFA in water, t = 7.3 min) to give 14 mg (84%) of the product as viscous orange oil.

^{1}H NMR (400 MHz, CD_3CN): δ 10.43 (br.s, 1H), 7.89 (d, *J* = 8.9 Hz, 1H), 7.87 – 7.81 (m, 3H), 7.76 – 7.65 (m, 12H), 7.61 (d, *J* = 9.0 Hz, 1H), 7.08 (d, *J* = 2.3 Hz, 1H), 7.00 (d, *J* = 2.3 Hz, 1H), 6.90 (br.t, *J* = 6.0 Hz, 1H), 6.83 (dd, *J* = 9.0 Hz, 2.3 Hz, 1H), 6.76 (dd, *J* = 9.0 Hz, 2.3 Hz, 1H), 3.89 (q, *J* = 6.7 Hz, 2H), 3.34 – 3.22 (m, 2H), 3.11 (s, 6H), 3.06 (s, 6H), 3.01 (q, *J* = 6.5 Hz, 2H), 2.16 (t, *J* = 7.0 Hz, 2H), 1.82 (p, *J* = 7.1 Hz, 2H), 1.74 (p, *J* = 7.1 Hz, 2H), 1.68 – 1.53 (m, 2H), 1.34 (p, *J* = 6.8 Hz, 2H), 1.30 – 1.17 (m, 4H), 0.49 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 174.1, 173.9, 160.4 (q, $^2J_{C–F}$ = 37.5 Hz, $CF_3CO_2^-$), 151.9, 151.7, 142.2, 138.7, 135.5 (d, $^4J_{C–P}$ = 2.9 Hz), 133.6 (d, $^3J_{C–P}$ = 9.9 Hz), 131.3, 130.7 (d, $^2J_{C–P}$ = 12.6 Hz), 130.0, 122.9 (q, $^1J_{C–F}$ = 515.8 Hz, $CF_3CO_2^-$), 118.0 (d, $^1J_{C–P}$ = 86.2 Hz), 116.3, 115.3, 113.3, 111.6, 50.1, 40.1, 39.4, 34.0, 28.9, 28.5, 26.5 (d, $^2J_{C–P}$ = 17.0 Hz), 25.9, 22.2 (d, $^1J_{C–P}$ = 51.8 Hz), 21.4 (d, $^3J_{C–P}$ = 4.2 Hz), -2.1.

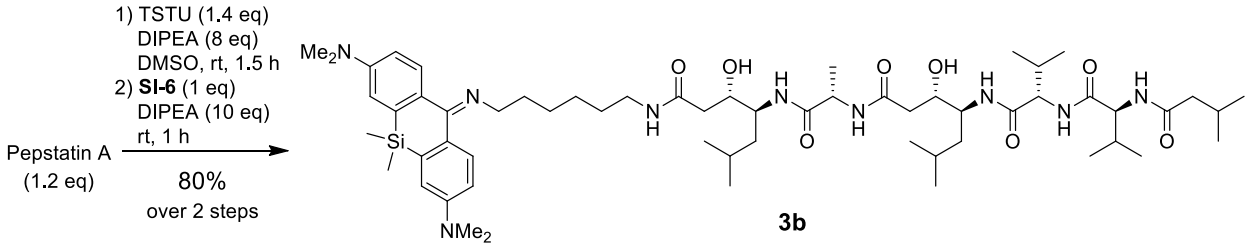
^{19}F NMR (376 MHz, CD_3CN): δ -76.54.

^{31}P NMR (162 MHz, CD_3CN): δ 23.7.

ESI-MS, positive mode: *m/z* (rel. int., %) = 767.6 (63) $[M]^+$, 384.3 (100) $[M+H]^{2+}$.

HR-MS (ESI, positive mode): 767.4261 $[M]^+$ (found), 767.4269 (calculated for $C_{48}H_{60}N_4OPSi$, $[M]^+$).

SiX-**lyso (3b)**



TSTU (*O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; 20 µL of 20 mg/100 µL stock solution in DMSO, 13.2 µmol, 1.4 eq) was added to a mixture of Pepstatin A (7.5 mg, 11 µmol, 1.2 eq) and DIPEA (16 µL, 88 µmol, 8 eq) in

anhydrous DMSO (0.8 mL). TLC control (silica, 10% methanol – CH_2Cl_2) showed complete conversion: R_f = 0.06 (starting material), 0.32 (product), both stained light-brown with vanillin stain. The resulting solution of the NHS ester was added dropwise to a solution of **SI-8** and DIPEA (20 µL, 110 µmol, 10 eq) in DMSO (0.4 mL), and the reaction mixture was stirred at rt for 1 h. TLC control (silica, 90:10:2.5 CH_2Cl_2 – methanol – 25% aq. NH_3): R_f = 0.19 (starting material), 0.35 (product). The reaction mixture was evaporated with CH_2Cl_2, the remaining DMSO solution was freeze-dried, and the product was isolated by preparative HPLC (column Kinetex 5 µm C18 100, diam. 21 mm × 250 mm, isocratic 65/35 A:B over 10 min, A = acetonitrile, B = 0.05% TFA in water, t = 6.8 min). Yield 9 mg (80% over 2 steps) of fluffy orange-yellow solid.

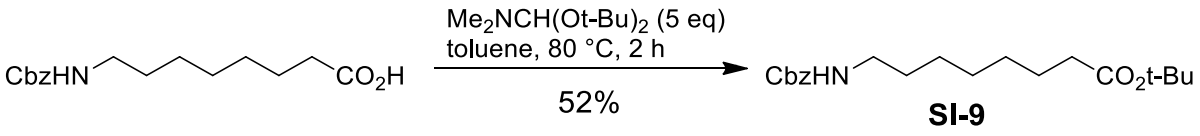
^{1}H NMR (400 MHz, DMSO-d_6): δ 10.93 (br.t, *J* = 5.9 Hz, 1H), 7.93 (d, *J* = 7.3 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 1H), 7.77 (dd, *J* = 9.0, 2.9 Hz, 2H), 7.68 – 7.62 (m, 2H), 7.46 (d, *J* = 8.8 Hz, 1H), 7.30 (d, *J* = 9.1 Hz, 1H), 7.10 (d, *J* = 2.8 Hz, 1H), 7.03 (d, *J* = 2.8 Hz, 1H), 6.90 (dd, *J* = 8.8, 2.6 Hz, 1H), 6.87 (dd, *J* = 8.9, 2.6 Hz, 1H), 4.79 (br. s, 2H), 4.28 – 4.08 (m, 3H), 3.88 (q, *J* = 6.6 Hz, 2H), 3.85 – 3.69 (m, 2H), 3.10 (s, 6H), 3.08 (s, 6H), 3.04 – 2.86 (m, 2H), 2.11 (d, *J* = 6.1 Hz, 2H), 2.05 – 2.00 (m, 3H), 2.00 – 1.87 (m, 4H), 1.76 (q, *J* = 7.4 Hz, 2H), 1.60 – 1.45 (m, 2H), 1.40 – 1.12 (m, 14H), 0.90 – 0.73 (m, 30H), 0.49 (s, 6H).

^{13}C NMR (101 MHz, DMSO-d_6): δ 131.3, 129.1, 116.2, 115.3, 112.1, 111.0, 68.9, 68.8, 57.7, 57.6, 50.5, 50.1, 49.4, 48.2, 44.2, 39.8, 39.4, 39.3, 39.0, 38.4, 38.0, 29.9, 28.7, 27.9, 25.6, 25.5, 23.9, 23.1, 22.0, 21.5, 20.6, 19.0, 18.1, 18.0, -2.8 (indirect detection from the gHSQC experiment, only H-coupled carbons are resolved).

ESI-MS, positive mode: *m/z* (rel. int., %) = 1090.8 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 1090.7437 $[M+H]^+$ (found), 1090.7459 (calculated for $C_{59}H_{100}N_9O_8Si$, $[M+H]^+$).

tert-Butyl 8-(benzyloxycarboxamido)octanoate (SI-9)



SI-9

8-(Benzyloxycarboxamido)octanoic acid[4] (452 mg, 1.54 mmol) was dissolved in toluene (5 mL), dimethylformamide di(*tert*-butyl)acetal (1.8 mL, 7.71 mmol, 5 eq) was added and the reaction mixture was stirred for 2 h at 80 °C (bath temperature). Upon cooling down to rt, sat. aq. $NaHCO_3$ (20 mL) was added, the resulting mixture was extracted with ethyl acetate (3×20 mL), the combined extracts were washed with water (40 mL), brine and dried over Na_2SO_4. The filtrate was evaporated to yellowish oil, from which the product was isolated by flash column chromatography (Teledyne Isco RediSep Rf 24 g; gradient 0% to 40% ethyl acetate – hexane) as viscous colorless oil (280 mg) along with the recovered starting material (120 mg). Yield 52% (or 71% considering starting material recovery).

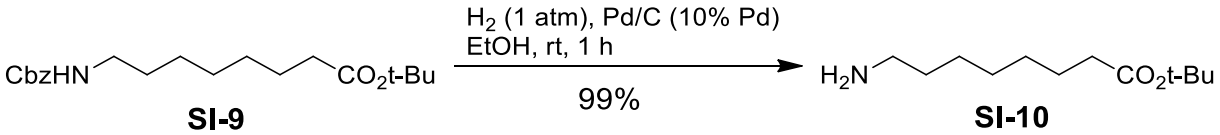
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 – 7.27 (m, 5H), 5.09 (s, 2H), 4.78 (br.s, 1H), 3.17 (q, *J* = 6.7 Hz, 2H), 2.19 (t, *J* = 7.5 Hz, 2H), 1.61 – 1.45 (m, 4H), 1.43 (s, 9H), 1.30 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 173.3, 156.5, 136.8, 128.6, 128.2, 128.2, 80.1, 66.7, 41.2, 35.6, 30.0, 29.1, 29.0, 28.2, 26.7, 25.1.

ESI-MS, positive mode: *m/z* (rel. int., %) = 372.2 $[M+Na]^+$.

HR-MS (ESI, positive mode): 372.2145 $[M+Na]^+$ (found), 372.2145 (calculated for $C_{20}H_{31}NO_4Na$, $[M+Na]^+$).

tert-Butyl 8-aminooctanoate (SI-10)



SI-9 **SI-10**

A degassed solution of **SI-9** (263 mg, 0.75 mmol) in absolute ethanol (15 mL) was stirred vigorously with 100 mg 10% Pd/C under hydrogen atmosphere (balloon). After 1 h, TLC (silica/20% ethyl acetate – hexane, stained with vanillin) showed complete consumption of the starting material. The reaction mixture was diluted with CH_2Cl_2 (20 mL), filtered through a plug of Celite (washed with ethanol), the filtrate was evaporated and dried *in vacuo*, providing 160 mg of the product as light brown oil (99% yield, traces of colloidal Pd).

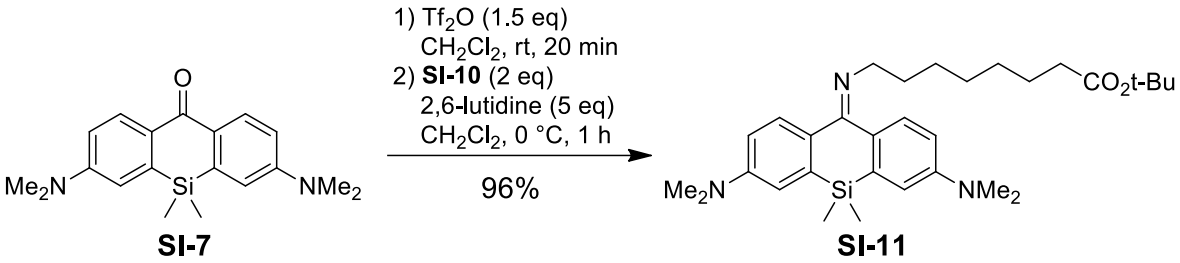
^{1}H NMR (400 MHz, $CDCl_3$): δ 3.23 (br.s, 2H), 2.72 (app.t, *J* = 7.2 Hz, 2H), 2.18 (t, *J* = 7.5 Hz, 2H), 1.62 – 1.45 (m, 4H), 1.42 (s, 9H), 1.36 – 1.22 (m, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 173.4, 80.1, 41.7, 35.7, 32.4, 29.2, 29.1, 28.3, 26.8, 25.1.

ESI-MS, positive mode: *m/z* (rel. int., %) = 216.2 $[M+H]^+$.

HR-MS (ESI, positive mode): 216.1963 $[M+H]^+$ (found), 216.1958 (calculated for $C_{12}H_{26}NO_2$, $[M+H]^+$).

tert-Butyl 8-(3,7-bis(dimethylamino)-5,5-dimethyldibenzo[_b_,_e_]silin-10(5_H_)-ylideneamino)octanoate (SI-11)



SI-7 **SI-11**

Trifluoromethanesulfonic anhydride (120 µL of 1 M solution in CH_2Cl_2, 0.12 mmol, ~1.5 eq) was added dropwise to a solution of ketone **SI-7** (prepared as described above, or according to reported procedure[3]; 25 mg, 0.077 mmol) in anhydrous CH_2Cl_2 (1 mL). The resulting intense blue reaction mixture was stirred at rt for 20 min and added dropwise (rinsing with 0.5 mL of anhydrous CH_2Cl_2) to a solution of **SI-10** (33 mg, 0.154 mmol, 2 eq) and 2,6-lutidine (45 µL, 0.386 mmol, 5 eq) in anhydrous CH_2Cl_2 (2 mL), cooled in ice-water bath. The resulting solution was stirred (ice-water bath) for 1 h, gradually turning brown-orange. Sat. aq. $NaHCO_3$ (20 mL) was added, and the resulting mixture was extracted with ethyl acetate (3×25 mL), the combined extracts were washed with brine and dried over Na_2SO_4. The filtrate was evaporated on Celite and the product (yellow-orange) was isolated by flash column chromatography (Büchi Sepacore Silica HP 12 g; gradient 0% to 100% A:B, A = 85:15:2 CH_2Cl_2 – ethanol – 25% aq. NH_3, B = CH_2Cl_2) and freeze-dried from dioxane to give viscous orange oil, yield 41 mg (96%, containing 0.33 eq dioxane).

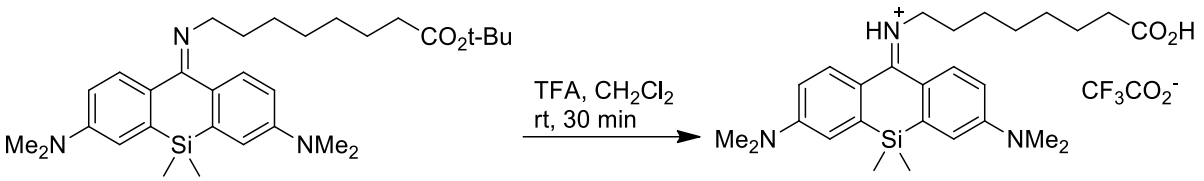
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, *J* = 8.6 Hz, 1H), 7.32 (d, *J* = 8.7 Hz, 1H), 6.95 (d, *J* = 2.8 Hz, 1H), 6.83 (d, *J* = 2.7 Hz, 1H), 6.78 (dd, *J* = 8.6, 2.7 Hz, 1H), 6.70 (dd, *J* = 8.7, 2.8 Hz, 1H), 3.76 (t, *J* = 7.1 Hz, 2H), 3.02 (s, 6H), 2.97 (s, 6H), 2.17 (t, *J* = 7.5 Hz, 2H), 1.73 (p, *J* = 7.2 Hz, 2H), 1.54 (dq, *J* = 12.1, 7.3 Hz, 2H), 1.43 (s, 9H), 1.39 – 1.23 (m, 4H), 0.45 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 173.4, 149.9, 149.4, 139.7, 135.9, 129.7, 128.0, 116.0, 115.0, 114.1, 111.4, 79.9, 54.1, 40.7, 40.4, 35.7, 32.1, 29.3, 29.2, 28.2, 27.6, 25.2, -2.4.

ESI-MS, positive mode: *m/z* (rel. int., %) = 522.4 $[M+H]^+$.

HR-MS (ESI, positive mode): 522.3508 $[M+H]^+$ (found), 522.3510 (calculated for $C_{31}H_{48}N_3O_2Si$, $[M+H]^+$).

8-(3,7-Bis(dimethylamino)-5,5-dimethyldibenzo[*b*,*e*]silin-10(5*H*)-ylideneamino)octanoic acid (SiX-C_8 acid)



SI-11 *SiX*-C_8 acid

Trifluoroacetic acid (1 mL) was added to a solution of **SI-11** (42 mg, 80.6 µmol) in CH_2Cl_2 (3 mL), cooled in ice-water bath. The cooling bath was removed, and the mixture was allowed to stir at rt for 30 min. The mixture was then evaporated to dryness, re-evaporated with toluene (2×5 mL) and freeze-dried from aqueous dioxane, giving 46 mg of SiX-C_8 acid as dark orange fluffy solid (trifluoroacetate salt, 100% yield; remainder dioxane).

^{1}H NMR (400 MHz, $CDCl_3$): δ 10.85 (s, 1H, NH), 10.55 (br.s, 1H, CO_2H), 7.96 (d, *J* = 8.8 Hz, 1H), 7.51 (d, *J* = 9.0 Hz, 1H), 6.94 (d, *J* = 2.7 Hz, 1H), 6.82 (d, *J* = 2.6 Hz, 1H), 6.78 (dd, *J* = 8.8, 2.8 Hz, 1H), 6.75 (dd, *J* = 9.0, 2.7 Hz, 1H), 3.94 (q, *J* = 6.6 Hz, 2H), 3.13 (s, 6H), 3.07 (s, 6H), 2.25 (t, *J* = 7.4 Hz, 2H), 1.82 (p, *J* = 7.0 Hz, 2H), 1.51 (p, *J* = 7.1 Hz, 2H), 1.28 – 1.21 (m, 6H), 0.48 (s, 6H).

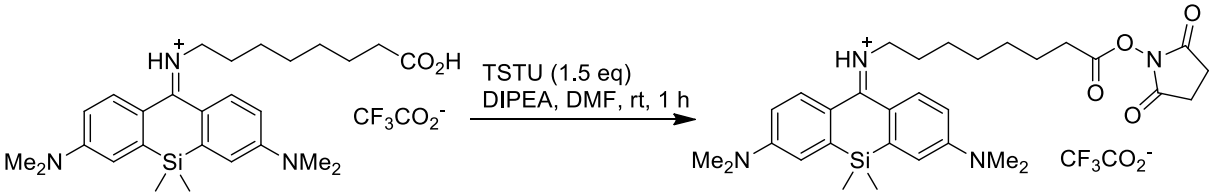
^{19}F NMR (376 MHz, $CDCl_3$): δ -75.72.

^{13}C NMR (101 MHz, $CDCl_3$): δ 173.9, 151.8, 151.6, 142.3, 138.6, 131.4, 129.6, 125.5, 120.2, 116.3, 115.3, 113.3, 111.4, 50.5, 40.04, 40.02, 34.1, 28.9, 28.6, 28.4, 26.3, 24.6, -2.2.

ESI-MS, positive mode: *m/z* (rel. int., %) = 466.3 $[M+H]^+$.

HR-MS (ESI, positive mode): 466.2886 $[M+H]^+$ (found), 466.2884 (calculated for $C_{27}H_{40}N_3O_2Si$, $[M+H]^+$).

8-(3,7-Bis(dimethylamino)-5,5-dimethyldibenzo[*b*,*e*]silin-10(5*H*)-ylideneamino)octanoic acid *N*-hydroxysuccinimide ester (SiX-C_8 acid NHS ester)



SiX-C_8 acid *SiX*-C_8 acid NHS ester

A solution of TSTU (*O*-(*N*-Succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; 15.5 mg, 51.6 µmol, 1.5 eq) in DMF (100 µL) was added to the mixture of SiX-C_8 acid (19.5 mg, 34.4 µmol) and DIPEA (30 µL, 172 µmol, 5 eq) in DMF (500 µL), and the reaction mixture was stirred at rt for 1 h. TLC control of a sample, quenched in acetonitrile – aq. NH_3 mixture (silica, 90:10:2 CH_2Cl_2 –

methanol – 25% aq. NH_3): R_f = 0.08 (starting material), 0.20 (amide). The reaction mixture was diluted with anhydrous CH_2Cl_2 (4 mL), the volatiles were removed on a rotary evaporated and the remaining DMF was evaporated at rt under high vacuum. The product was isolated by preparative HPLC (gradient A:B 50/50 → 20/80, A – 0.1% TFA in water, B – acetonitrile) and freeze-dried from dioxane. Orange solid (18 mg, trifluoroacetate salt, 77% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 11.00 (br.s, 1H), 9.85 (br.s, 1H), 8.02 (br.d, *J* = 8.4 Hz, 1H), 7.52 (d, *J* = 8.9 Hz, 1H), 6.94 (d, *J* = 2.6 Hz, 1H), 6.82 (br.s, 2H), 6.76 (dd, *J* = 8.9, 2.7 Hz, 1H), 3.97 (br.s, 2H), 3.14 (s, 6H), 3.09 (s, 6H), 2.82 (s, 4H), 2.54 (t, *J* = 7.4 Hz, 2H), 1.87 (br.s, 2H), 1.67 (p, *J* = 7.4 Hz, 2H), 1.41 – 1.21 (m, 6H), 0.49 (s, 6H).

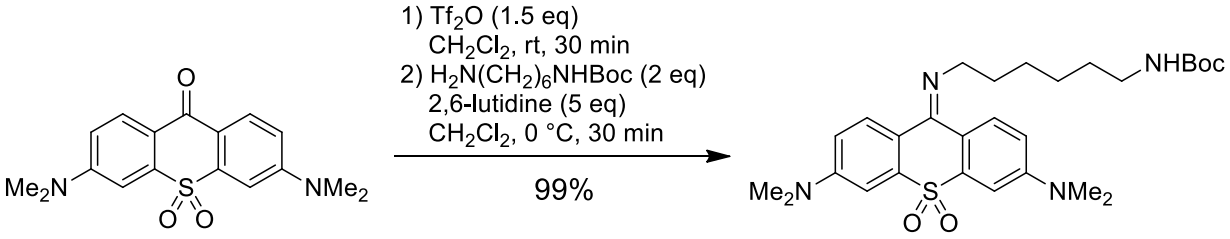
^{19}F NMR (376 MHz, $CDCl_3$): δ -75.80.

^{13}C NMR (101 MHz, $CDCl_3$): δ 131.3, 129.7, 116.2, 115.1, 113.4, 111.2, 50.3, 39.98, 39.95, 30.8, 28.8, 28.4, 28.3, 26.3, 25.6, 24.4, -2.2 (indirect detection from the gHSQC experiment, only H-coupled carbons are resolved).

ESI-MS, positive mode: *m/z* (rel. int., %) = 563.3 $[M+H]^+$.

HR-MS (ESI, positive mode): 563.3058 $[M+H]^+$ (found), 563.3048 (calculated for $C_{31}H_{43}N_4O_4Si$, $[M+H]^+$).

***tert*-Butyl 6-(3,6-bis(dimethylamino)-10,10-dioxido-9*H*-thioxanthen-9-ylideneamino)hexylcarbamate** (SO2X) (**4**)



SI-12 *SO2X* (**4**)

Trifluoromethanesulfonic anhydride (51 µL, 0.3 mmol, 1.5 eq) was added dropwise to a solution of ketone **SI-12** (prepared according to reported procedure[5]; 70 mg, 0.2 mmol) in anhydrous CH_2Cl_2 (3 mL). The resulting dark-green reaction mixture was stirred at rt for 30 min and then added dropwise to a solution of *N*-Boc-1,6-diaminohexane (86 mg, 0.4 mmol, 2 eq) and 2,6-lutidine (116 µL, 1 mmol, 5 eq) in anhydrous CH_2Cl_2 (3 mL), cooled in ice-water bath. The resulting solution was stirred for 30 min in ice-water bath, sat. aq. $NaHCO_3$ (20 mL) was added, the mixture was extracted with ethyl acetate (3×20 mL), and the combined extracts were dried over Na_2SO_4. The filtrate was evaporated on Celite and the product was isolated by flash column chromatography (Büchi Sepacore Silica HP 12 g; gradient 0% to 100% A:B, A = 85:15:5 CH_2Cl_2 – ethanol – acetic acid, B = CH_2Cl_2) and freeze-dried from 1,4-dioxane to give red solid, yield 105 mg (99%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.84 (d, *J* = 8.8 Hz, 1H), 7.48 (d, *J* = 8.8 Hz, 1H), 7.39 (d, *J* = 2.8 Hz, 1H), 7.22 (d, *J* = 2.7 Hz, 1H), 6.82 (dd, *J* = 8.8, 2.7 Hz, 1H), 6.76 (dd, *J* = 8.8, 2.8 Hz, 1H), 4.55 (br.s, 1H), 3.90 (t, *J* = 7.0 Hz, 2H), 3.10 (s, 6H), 3.06 (s, 6H), 1.81 (p, *J* = 7.2 Hz, 2H), 1.52 – 1.24 (m, 6H), 1.43 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 150.8, 150.6, 140.8, 138.2, 130.6, 128.9, 115.5, 113.2, 106.8, 104.9, 54.2, 40.4, 40.3, 31.6, 30.1, 28.6, 27.1, 26.6.

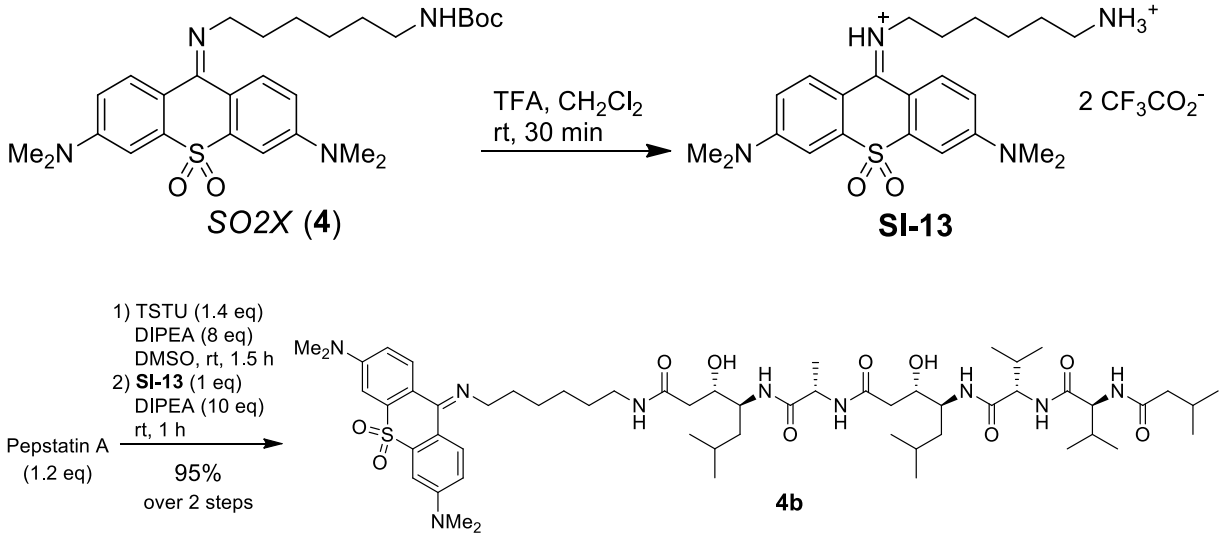
ESI-MS, positive mode: *m/z* (rel. int., %) = 529.3 $[M+H]^+$.

HR-MS (ESI, positive mode): 529.2846 $[M+H]^+$ (found), 529.2843 (calculated for $C_{28}H_{41}N_4O_4S$, $[M+H]^+$).

UV-Vis (10% acetonitrile – PBS, pH 7.4): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 349 (7600), 408 (11000), 509 (13000); emission, λmax, nm (Φ): 647 (0.15), excitation at 500 nm.

UV-Vis (acetonitrile): absorption, λmax, nm (ε, $M^{-1}cm^{-1}$): 328 (17000); no long Stokes shift emission observed in this solvent.

SO2X-**lyso** (**4b**)



Trifluoroacetic acid (200 µL) was added to a solution of the dye SO2X (**4**) (6 mg, 11.4 µmol) in CH_2Cl_2 (2 mL). The resulting orange solution was evaporated to dryness, re-evaporated with toluene (2×5 mL) and the residue of **SI-13** (trifluoroacetate salt) was dissolved in anhydrous DMSO (0.2 mL) to be used directly in the next step.
TSTU (*O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; 25 µL of 20 mg/100 µL stock solution in DMSO, 16.4 µmol, 1.4 eq) was added to a mixture of Pepstatin A (9.4 mg, 13.7 µmol, 1.2 eq) and DIPEA (20 µL, 115 µmol, 8 eq) in anhydrous DMSO (1 mL). TLC control (silica, 10% methanol – CH_2Cl_2) showed complete conversion: R_f = 0.06 (starting material), 0.32 (product), both stained light-brown with vanillin stain. DIPEA (25 µL, 143 µmol, 10 eq), followed immediately by the resulting solution of the NHS ester, were added dropwise to a solution of **SI-13** in DMSO (0.2 mL), and the reaction mixture was stirred at rt for 1.5 h. TLC control

(silica, 90:10:2 CH_2Cl_2 – methanol – 25% aq. NH_3): R_f = 0.80 (SO2X), 0.50 (product). The reaction mixture was evaporated with CH_2Cl_2, the remaining DMSO solution was freeze-dried, and the product was isolated by preparative HPLC (gradient 30/70 – 100/0 A:B over 20 min, A = acetonitrile, B = 0.1% TFA in water, t = 14.3 min). Yield 8 mg (56% over 2 steps) of fluffy red solid.

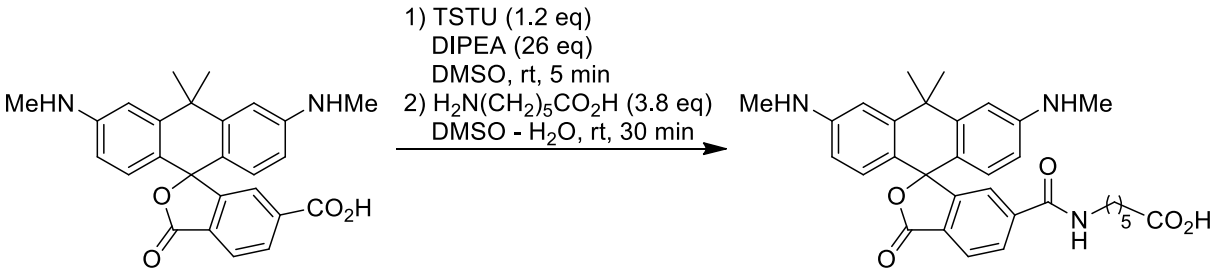
^{1}H NMR (400 MHz, DMSO-d_6): δ 11.7 (br.s, 1H), 7.95 (d, *J* = 4.2 Hz, 1H), 7.93 (d, *J* = 2.4 Hz, 1H), 7.86 (d, *J* = 9.2 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 1H), 7.78 (d, *J* = 9.0 Hz, 1H), 7.67 (t, *J* = 5.6 Hz, 1H), 7.46 (d, *J* = 8.7 Hz, 1H), 7.36 (d, *J* = 2.7 Hz, 1H), 7.31 (d, *J* = 9.1 Hz, 1H), 7.26 (d, *J* = 2.7 Hz, 1H), 7.09 (dd, *J* = 9.1, 2.7 Hz, 1H), 7.05 (dd, *J* = 9.2, 2.8 Hz, 1H), 4.26 – 4.09 (m, 3H), 4.00 (br.t, *J* = 7.0 Hz, 2H), 3.88 – 3.68 (m, 2H), 3.20 (s, 6H), 3.17 (s, 6H), 2.99 (tdd, *J* = 19.7, 12.5, 6.0 Hz, 2H), 2.12 (d, *J* = 6.1 Hz, 2H), 2.08 – 2.00 (m, 3H), 2.00 – 1.89 (m, 3H), 1.82 (t, *J* = 7.2 Hz, 2H), 1.52 (s, 2H), 1.42 – 1.14 (m, 10H), 0.90 – 0.74 (m, 30H).

^{13}C NMR (101 MHz, DMSO-d_6): δ 132.4, 130.6, 114.0, 113.3, 107.2, 105.7, 68.9, 66.2, 57.9, 57.8, 50.6, 50.3, 48.3, 44.3, 39.9, 39.8, 39.1, 38.2, 30.1, 28.9, 28.1, 25.7, 24.1, 23.2, 22.2, 21.6, 19.2, 18.2, 18.1 (indirect detection from the gHSQC experiment, only H-coupled carbons are resolved).

ESI-MS, positive mode: *m/z* (rel. int., %) = 1096.7 (100) $[M+H]^+$.

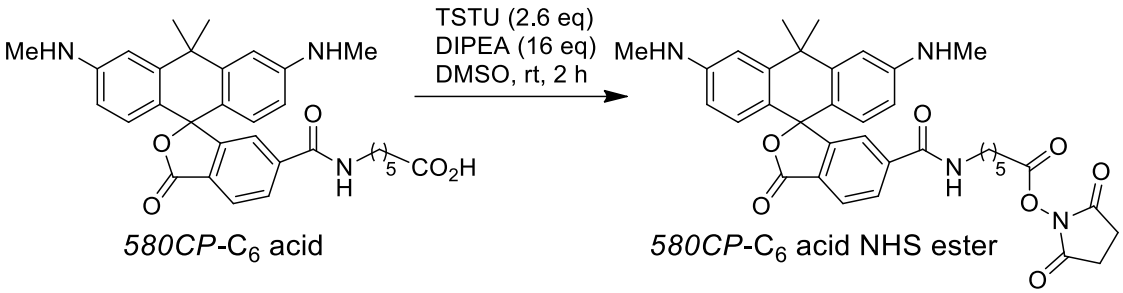
HR-MS (ESI, positive mode): 1096.6832 $[M+H]^+$ (found), 1096.6839 (calculated for $C_{57}H_{94}N_9O_{10}S$, $[M+H]^+$).

580CP-actin

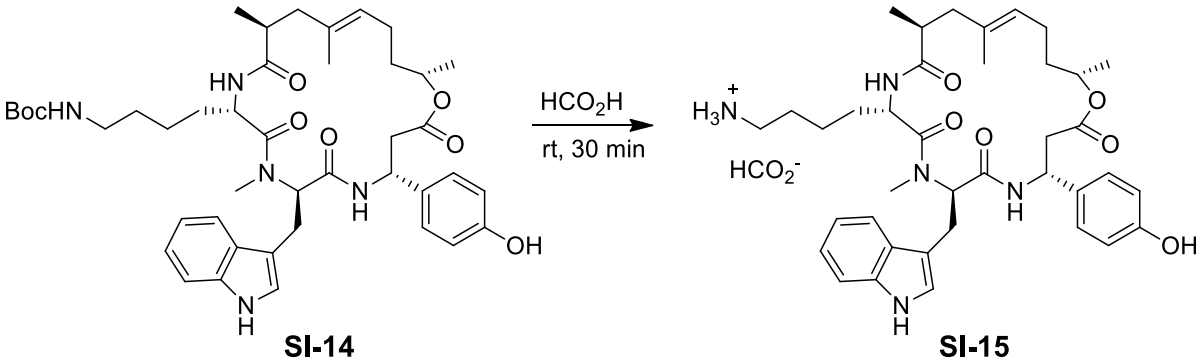


580CP *580CP*-C_6 acid

Dye *580CP* (2 mg, 4.6 µmol) [1] was dissolved in dry DMSO (0.4 mL). *N*-Ethyldiisopropylamine (DIPEA; 21 µL, 15.5 mg, 120 µmol, 26 eq) and TSTU (*O*-(*N*-Succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; 1.7 mg, 5.52 µmol, 1.2 eq) were then added, and the reaction mixture was stirred for 5 min at rt. 6-Aminohexanoic acid (2.3 mg, 17.48 µmol, 3.8 eq) was then introduced, and the resulting suspension was sonicated for 15 min. Water (20 µL) was added, and the mixture was stirred at rt for further 15 min. The reaction progress was checked by HPLC; after 30 min, acetic acid (8.3 µL, 30 eq) was added and the solvents were removed on a lyophilizer. The product was isolated by preparative HPLC (gradient 20/80 – 80/20 A:B over 15 min, A = acetonitrile, B = 0.05% TFA in water, t = 5.2 min), and the intermediate was used directly in the next step.

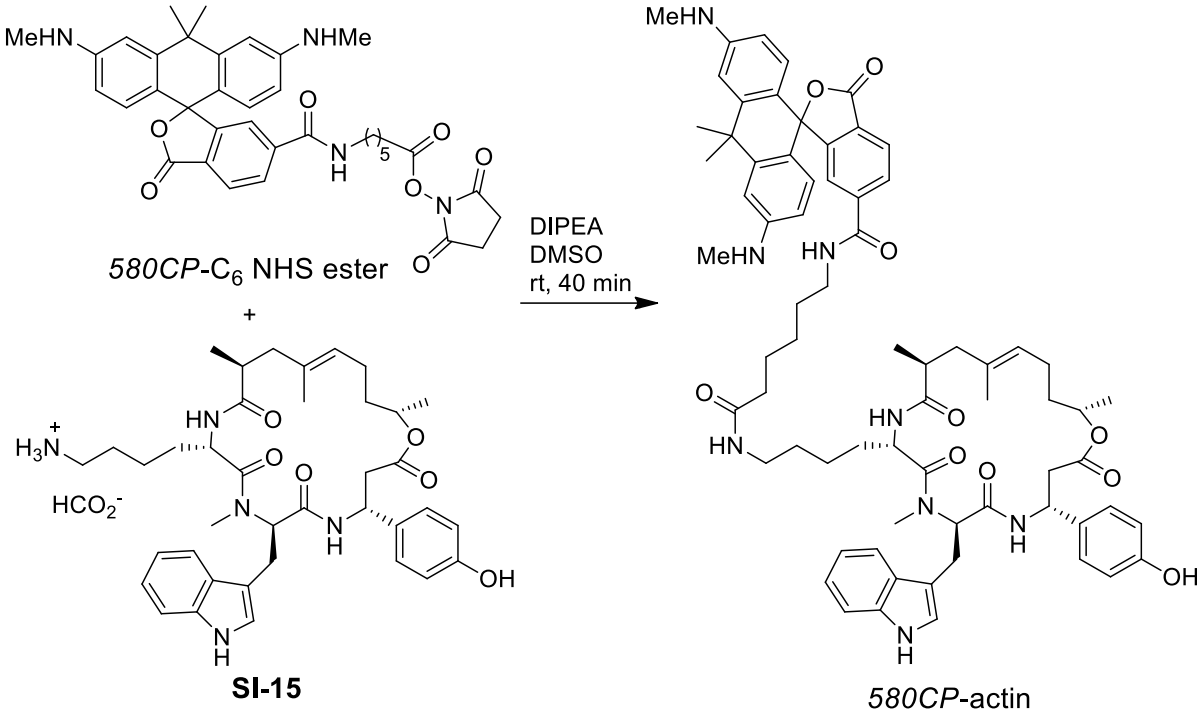


A portion of the resulting *580CP*-C_6 acid (0.2 mg, 0.37 µmol) was dissolved in dry DMSO (100 µL), and DIPEA (5 µL of 10% v/v solution in DMSO, 2.94 µmol, 8 eq) and TSTU (10 µL of 7 mg/50 µL DMSO stock solution, 0.48 µmol, 1.3 eq) were then added. The reaction mixture was stirred at rt for 1 h, and the progress was controlled by HPLC. The second addition of DIPEA (5 µL of 10% v/v solution in DMSO, 2.94 µmol, 8 eq) and TSTU (10 µL of 7 mg/50 µL DMSO stock solution, 0.48 µmol, 1.3 eq) was made, and the reaction was stirred at rt for another 1 h period. The solvents were then removed on a lyophilizer, and the product was isolated by preparative HPLC (gradient 20/80 – 80/20 A:B over 15 min, A = acetonitrile, B = 0.05% TFA in water, t = 5.2 min), and the intermediate was used directly in the next step HPLC (gradient 20/80 – 80/20 A:B over 15 min, A = acetonitrile, B = 0.05% TFA in water, t = 6.3 min).



SI-14 **SI-15**

Desbromo-desmethyljasplakinolide analogue **SI-14** (0.2 mg, 0.26 µmol) [compound 12 in ref [6]] was dissolved in formic acid (21 µL) and stirred for 30 min at rt. The mixture was the diluted with water (17 µL) and lyophilized; the residue was dissolved in water (17 µL) and lyophilized. HPLC control (gradient 20/80 – 100/0 A:B over 20 min, A = acetonitrile, B = 0.05% TFA in water): starting material t = 11.8 min, product t = 6.3 min. The entire amount of deprotected amine **SI-15** (as formate salt) was used for labeling with *580CP* dye.

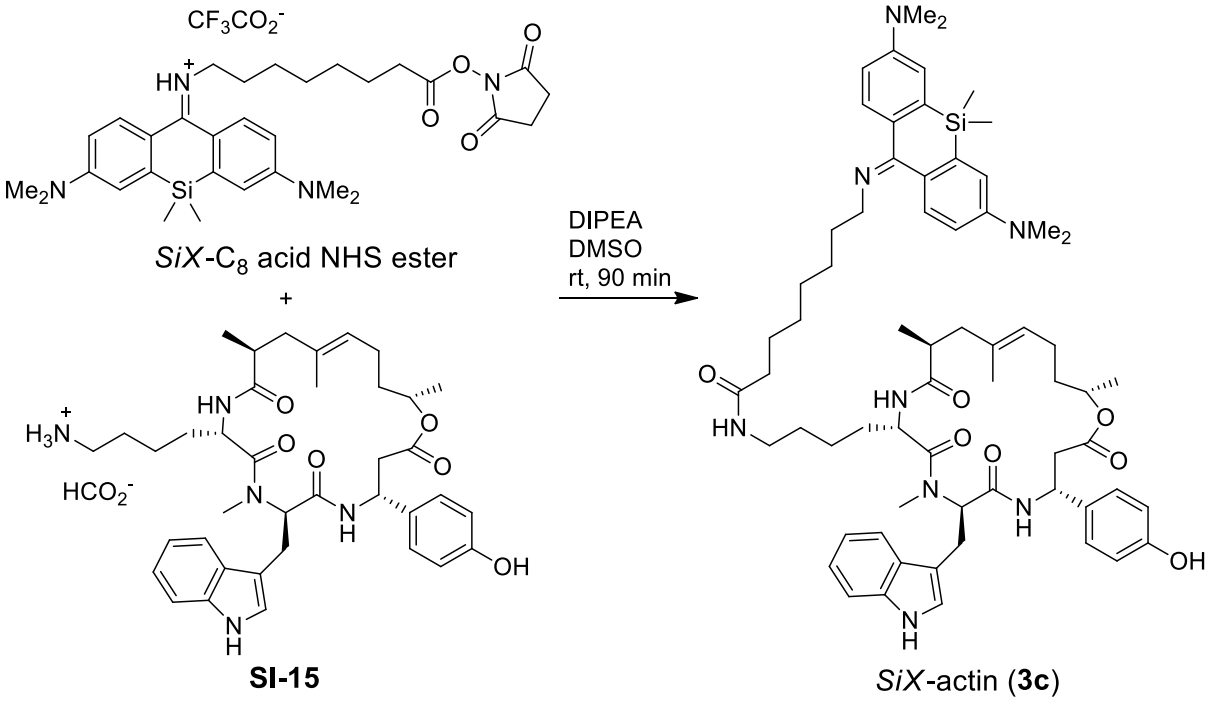


580CP-C_6 acid NHS ester (0.2 mg, 0.31 µmol) was dissolved in dry DMSO (100 µL) under argon, and DIPEA (8.5 µL of 10% v/v solution in DMSO, 2.94 µmol, 8 eq) followed by the solution of the formate salt of deprotected desbromo-desmethyljasplakinolide analogue (**SI-15**) from the previous step (~0.2 mg, ~0.26 µmol) in DMSO (20 µL, rinsed with 10 µL DMSO) were added. The reaction mixture was stirred at rt (under argon) for 40 min, the solvents were removed on a lyophilizer, and the product was isolated by preparative HPLC (column: Kinetex 5µm 100A 250×4.6 mm, gradient 20/80 – 100/0 A:B over 20 min, A = acetonitrile, B = 0.05% TFA in water; 580CP-C_6 acid NHS ester t = 9.0 min, 580CP-actin t = 11.3 min).

ESI-MS, positive mode: *m/z* (rel. int., %) = 599.3 $[M+2H]^{2+}$.

HR-MS (ESI, positive mode): 599.3217 $[M+2H]^{2+}$ (found), 599.3228 (calculated for $C_{70}H_{86}N_8O_{10}$, $[M+2H]^{2+}$).

SiX-**actin** (**3c**)

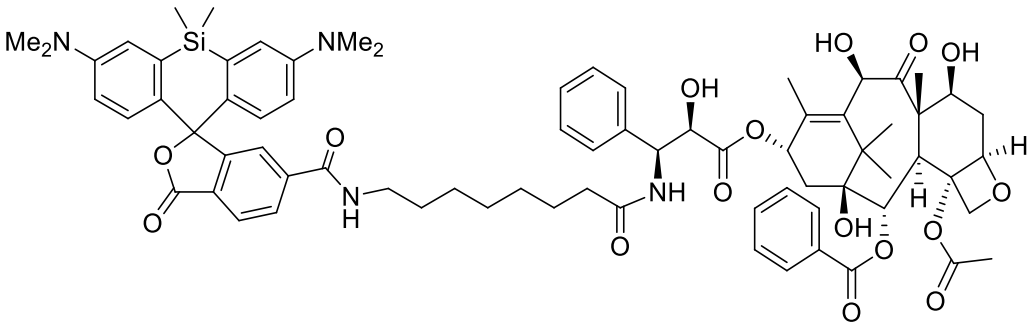


SiX-C_8 acid NHS ester (87.3 µg, 0.155 µmol) was dissolved in dry DMSO (50 µL) under argon, and DIPEA (4.3 µL of 10% v/v solution in DMSO, 2.48 µmol, 16 eq) followed by the solution of the formate salt of deprotected desbromo-desmethyljasplakinolide analogue prepared as described above (94.8 µg, 0.129 µmol) in DMSO (10 µL, rinsed with 10 µL DMSO) were added. The reaction mixture was stirred at rt (under argon) for 90 min, the solvents were removed on a lyophilizer, and the product was isolated by preparative HPLC (column: Kinetex 5µm 100A 250×10 mm, gradient 30/70 – 100/0 A:B over 20 min, A = acetonitrile, B = 0.05% TFA in water; SiX -C_8 acid NHS ester t = 12.8 min, SiX -actin t = 13.9 min).

ESI-MS, positive mode: *m/z* (rel. int., %) = 561.3 $[M+2H]^{2+}$.

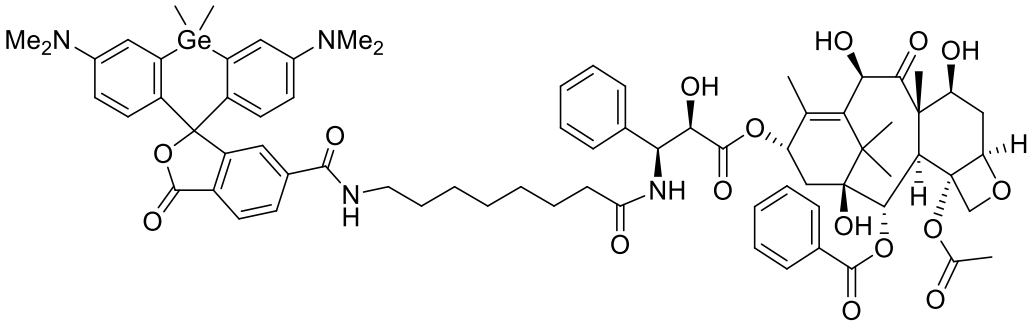
HR-MS (ESI, positive mode): 561.3350 $[M+2H]^{2+}$ (found), 561.3345 (calculated for $C_{65}H_{88}N_8O_7Si$, $[M+2H]^{2+}$).

SiR-tubulin



The *SiR*-tubulin probe was prepared according to the literature procedure [7].

GeR-tubulin



The *GeR*-tubulin probe was prepared according to the literature procedure[2].

DFT Calculations

Density functional theory (DFT and TD-DFT) calculations were carried out with Gaussian 09 program (revision E.01)[8] using Austin-Frisch-Petersson functional with dispersion (APFD) with 6-311++G(2d,p) basis set. The default Polarizable Continuum Model was used as a solvation model. The initial molecular geometries were generated using a built-in molecular mechanics method of ChemBio3D software (ChemBioOffice 12.0, CambridgeSoft) followed by additional refinement with the molecular mechanics method (force field: UFF, 4 steps per update, steepest descent algorithm) of Avogadro 1.1.1 software (http://avogadro.cc/). The computation was performed at the High Performance Computing center for the Georg-August-Universität Göttingen (https://www.gwdg.de/application-services/high-performance-computing).

In vitro characterization of the probes

Estimation of relative photostability of LSS dyes

The stirred solutions of LSS dyes CX (**1**), GeX (**2**), SiX (**3**), Abberior Star470SXP, Atto 490LS and Oregon Green reference in air-saturated 10% methanol – PBS (pH 7.4), matched to absorption A ~ 0.5 at 470 nm, were irradiated at ambient temperature in a stopped quartz cuvette (path length l = 1 cm, volume V = 3 cm³) with a collimated 470 nm light beam (mounted high-power LED M470L3; Thorlabs, USA) with a power density ~30-35 mW/cm². Identical irradiation power and geometries were used for all studied dye solutions. After 1 h irradiation time, the amount of photodegraded dye was estimated spectrophotometrically, assuming the absorption of the photoproducts at 470 nm was negligible.

The reaction rate for the photobleaching of a dye, assuming a single photochemical step, can be expressed according to (ref. 9):

$$-\frac{d[dye]}{dt} = I_0 F \Phi_{bl} \varepsilon_{dye} l [dye]$$

where Φ_{bl} – photobleaching quantum yield, I_0 – incident photonic flux, ε_{dye} – extinction coefficient at the irradiation wavelength, l – optical path length, $F = (1 - 10^{-A})/A$ – photokinetic factor at the irradiation wavelength (A – absorption).
Measuring initial rates (low conversion approximation), the relative photostability of the dyes r with respect to Oregon Green (OG), under 470 nm irradiation, was calculated as follows:

$$r = \frac{(\Phi_{bl} \times \varepsilon^{470nm})_{OG}}{(\Phi_{bl} \times \varepsilon^{470nm})_{dye}} \approx \frac{\frac{\Delta[OG]}{\Delta t}}{\frac{\Delta[dye]}{\Delta t}} \times \frac{I_0 F l [dye]}{I_0 F l [OG]} \approx \frac{\Delta[OG]}{\Delta[dye]} \times \frac{F[dye]}{F[OG]} = \frac{\Delta A_{OG}}{\Delta A_{dye}} \times \frac{\left(1 - 10^{-\bar{A}_{dye}}\right)}{\left(1 - 10^{-\bar{A}_{OG}}\right)}$$

where for the chosen dye and Oregon Green $\Delta A = A - A^0$ is the difference between absorption at t = 1 h (A) and at t = 0 (A^0), and $\bar{A}$ is the average absorption during the irradiation ($[A+A^0]/2$), all expressed at the irradiation wavelength (470 nm). The results are plotted in Supplementary Figure S3.

Inhibition of pepsin activity by lysosomal probes

Bovine serum albumin (BSA) digestion with pepsin was used for the estimation of lysosome probe inhibitory potential. Each reaction mix contained 1 mg/ml BSA, 1 μM pepsin and 3 μM probes in reaction buffer (50 mM $HCOONH_4$, pH 4.0). The reactions were incubated at 37°C for 2 h and stopped by addition of 4x Laemmli sample buffer (Bio-Rad, Cat. No. 161-0747) and 5 μl of 1 M NaOH to each 400 μl of sample to balance the pH. Samples were immediately heated at 95°C for 5 min and loaded on SDS-PAGE gels. Upon completion of electrophoresis, the gels were stained with Coomassie® Brilliant Blue R-250 (Applichem, Cat. No. A1092) to detect the extent of proteolysis of BSA. Images of destained gels were acquired on an Amersham Imager 600 RGB system (GE Healthcare Life Sciences).

Estimation of K_d^{app} for pepstatin probes

Twofold dilution series starting at 100 μM of pepsin was prepared in 50 mM $HCOONH_4$ pH 4.0 buffer. Master solutions containing 0.11 μM probe **1b**, **2b**, **3b** or SiR-lysosome[10] in the same buffer were prepared in separate tubes. Binding assay was started by mixing 100 μl of pepsin solution and 900 μl of probe solution. Afterwards samples were incubated for 2 h at room temperature and fluorescence was measured in a glass bottom 96-well plate (MatTek cat. No. PBK96G-1.5-5-F) on a multiwell plate reader TECAN Spark 20M. Large Stoks shift probes fluorescence emission was recorded at 600 nm with 10 nm bandwidth while exciting at 490 nm with 10 nm bandwidth. SiR-lysosome probe fluorescence emission was recorded at 670 nm with 10 nm bandwidth while exciting at 645 nm with 10 nm bandwidth. All samples were prepared at least in technical triplicates. Obtained fluorescence change was fitted using GraphPad Prism 6 to one site specific binding equation:

$$Y = Fmin + (Fmax - Fmin) * \frac{(p+X+K_d^{app}) - \sqrt{(p+X+K_d^{app})^2 - 4*p*X}}{2*p} \quad (1)$$

where *Fmin* – fluorescence of probe without target, *Fmax* – fluorescence of probe at saturating concentration, *p* – probe concentration, *X* – target protein concentration, K_d^{app} – apparent dissociation constant of the probe.

Labeling of antibodies

Direct labelling of mouse primary mouse anti-acetylated tubulin, clone 6-11B-1 (Sigma cat. No. T7451) was performed using APEX™ antibody labelling kit (Thermo Fisher cat. No. A10468) and SiX-NHS DMSO solution according to manufacturer protocols. Secondary sheep anti-mouse antibody (Dianova, cat. No. 515-005-003) was labelled in 0.1 M $NaHCO_3$ buffer for 1 h at room temperature. Afterwards excess of dye was removed by gel filtration using PD MiniTrap G-25 column (GE Healthcare Life Sciences, cat. No. 28-9180-07).

Photophysical measurements

Unless specified otherwise, all photophysical parameters were measured in PBS (pH 7.4) solutions at room temperature (25 °C). The absorption spectra were recorded on a Varian Cary 4000 UV-Vis spectrophotometer (Agilent). The emission spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent). The fluorescence quantum yields (absolute values) were obtained on a Quantaurus-QY absolute PL quantum yield spectrometer (model C11347-12, Hamamatsu) according to the manufacturer's instructions. Fluorescence lifetimes were measured on a Quantaurus-Tau fluorescence lifetime spectrometer (model C11367-32, Hamamatsu) according to the manufacturer's instructions.

Manipulation of cells

Preparation and maintenance of cells

Human primary dermal fibroblasts, HeLa and U2OS cells were cultured in high-glucose DMEM (Life Technologies, Cat. No. 31053-028) supplemented with GlutaMAX-1 (Life Technologies, Cat. No. 35050-038) and 10% foetal bovine serum (FBS, Life Technologies, Cat. No. 10270-106) in a humidified 5% CO_2 incubator at 37 °C. The cells were split every 3-4 days or at confluence. Cells were seeded in glass bottom 12-well plates (MatTek Corporation, Cat. No. P12G-1.0-14-F).

Transduction with CellLight® Talin-GFP (Life Technologies, Cat. No. C10611) was performed according to manufacturer's recommendations. CellLight® reagent (10 µl per well) was added to 0.5 ml of complete DMEM medium in a 12-well plate. Afterwards, the cells were grown in a humidified 5% CO_2 incubator at 37 °C for 48 h. The staining of Talin-GFP expressing cells with probes was performed as described below.

Cultures of hippocampal neurons were prepared from Wistar rats at postnatal day P0-P1 in accordance with the regulations of the German Animal Welfare Act and

under the approval of local veterinary service. Cells were plated on 100 µg/mL poly-ornithine (Sigma-Aldrich, Cat. No. P3655) and 1 µg/mL Laminin (BD Bioscience, Cat. No. 354232) coated coverslips. Neuronal cultures were maintained in NeuroBasal medium (Gibco, Cat. No. 21103049) supplemented with 2% B27 serum-free supplement (Gibco, Cat. No. 17504044), 2 mM L-Glutamine (Gibco, Cat. No. 25030) and Pen/Strep (100 units/mL and 100 µg/mL respectively, BiochromAG, Cat. No. A2213). The day after plating, 5 µM cytosine β-D-arabinofuranoside (Sigma, Cat. No. C1768) was added to the cultures.

Cell preparation for microscopy

Live-cell staining of hippocampal neurons was achieved by adding the probes to the growth medium. Cells were incubated for 30-60 min in a humidified 5% CO_2 incubator at 37°C. The axon initial segment was stained for 5 min at room temperature with an anti–pan-neurofascin antibody (UC Davis/NIH NeuroMab Facility, Cat. No. 75-172; diluted in artificial cerebrospinal fluid (ACSF buffer) to 10 µg/mL), recognizing the extracellular domain of the protein. After three fast washes with ACSF, the cells were incubated for 30 s with anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen, Cat. No. A11001). After washing, the cells were imaged in ACSF buffer at RT.

Transduction of human fibroblasts with CellLight Lysosomes-RFP, BacMam 2.0 (Thermo Fisher Scientific, cat. C10597) or CellLight Talin-GFP (Thermo Fisher Scientific, cat. C10611) was performed according to manufacturer's recommendations. 10 µl per well of CellLight® reagent was added to 1 ml of complete DMEM medium in a glass bottom 12-well plate (MatTek Corporation, Cat. No. P12G-1.0-14-F). Afterwards, cells were grown for 48 h in a humidified 5% CO2 incubator at 37 °C. Staining of Lysosomes-RFP expressing cells with LSS probes was performed as described below.

Cells were stained with the LSS probes at 37 °C in DMEM (Thermo Fisher Scientific, Cat. No. 31053-028) supplemented with 10% FBS (Thermo Fisher Scientific, Cat. No. 10082139) at 37 °C and 5% CO_2. If needed, MitoTracker™ Orange CMTMRos (Thermo Fisher Scientific, cat. M7510) at 0.5 µg/ml or Hoechst 33342 at 0.1 µg/ml (Sigma Aldrich, cat. B2261) was included in the media. Afterwards, the cells were washed 2 times with HBSS (Hanks' balanced salt solution, Lonza, Cat. No. BE10-527F). Imaging was performed in DMEM with 10% FBS.

For fixed cells staining experiments, human fibroblasts were grown in glass bottom 12-well plates (MatTek Corporation, Cat. No. P12G-1.0-14-F). Afterwards, the samples were fixed for 10 min in -20 ˚C methanol containing 1 mM EGTA, washed in PBS and blocked for 60 min in 1% bovine serum albumin (BSA) in PBS. Next, primary antibodies were diluted in PBS with 1% BSA and added to the samples for overnight incubation at 4°C. Afterwards, the samples were washed 3 times with PBS, mounted in 90% glycerol containing 4% propyl gallate. If secondary antibody staining was required, a secondary antibody, diluted in PBS with 1% BSA, was added for 1 h treatment at room temperature and the samples were imaged after washing 3 times with PBS and mounting in 90% glycerol containing 4% propyl gallate.

Cell cycle analysis by imaging flow cytometry

HeLa cells were grown 6-well plat (250.000 cells per well) for 24h in presence of variable concentration of fluorescent probe. Probes were dissolved in DMSO at 1 mM concentration, thus control samples were prepared by adding only DMSO. We found that HeLa cells do not adhere strongly to the plastic bottom of a 6-well plate and thus the trypsination step could be omitted. The cells were suspended in 2 ml of the growth media by simply washing off with a pipette. Next, the cells were processed according to the NucleoCounter® NC-3000™ two-step cell cycle analysis protocol. In particular, ~500.000 cells harvested cells by centrifuging 5 min at 400 g at room temperature. Afterwards, the cells were resuspended in 250 µl lysis solution (Solution 10, Chemometec Cat. No. 910-3010) supplemented with 10 µg/ml DAPI (Solution 12, Chemometec Cat. No. 910-3012), incubated at 37° C for 5 min, and 250 µl of stabilization solution (Solution 11, Chemometec Cat. No. 910-3011) was added. Cells were counted on a NucleoCounter® NC-3000™ in NC-Slide A2™ slides (Chemometec, Cat. No. 942-0001) loaded with ~30 µl of each of the cell suspensions into the chambers of the slide. Each time, ~10.000 cells in total were measured and the obtained cell cycle histograms were analysed with ChemoMetec NucleoView NC-3000 software, version 2.1.25.8. All experiments were repeated at least three times and the results are presented as mean with standard deviation. The obtained mean values were compared by running multiple t-tests on GraphPad Prism version 6.0 software.

Description of microscopy setups

Confocal microscope

Confocal imaging was performed on a Leica SP8 (Leica Microsystems, Mannheim, Germany) inverted confocal microscope equipped with an HC PL APO CS2 63x/1.40 OIL objective. Images were acquired using a 700 Hz bidirectional scanner, a pixel size of 70 nm × 70 nm, a pinhole of 95.6 µm (1 AU) and frame averaging of 2 - 3. Hoechst 33342 was excited with 405 nm laser and detected with a regular PMT in the 420 – 470 nm range. Large Stokes shift probes were excited with 488 nm laser and detected with Leica HyD detector set within the spectral range of 580–630 nm.

STED microscope

Confocal and STED images were acquired on an Abberior STED 775 QUAD scanning microscope (Abberior Instruments GmbH) equipped with 485 nm, 561 nm and 640 nm 40 MHz pulsed excitation lasers, a pulsed 775 nm STED 40 MHz laser, and UPlanSApo 100x/1.40 Oil objective. The following detection windows were used: GFP/A488 channel 525 / 50 nm, TMR/Cy3 channel 615 / 20 nm and Cy5 channel 685 / 70 nm. Large Stokes shift dyes were excited with 485 nm laser and detected at 615 / 20 nm. Pixel size was 30 – 50 nm for all confocal and STED images acquired on this setup. Laser powers were optimized for each sample. Estimation of STED effect was performed by varying STED laser power from 0 to 100% while measuring

cells stained with mouse anti-tubulin and SiX-labelled sheep anti-mouse antibody. Obtained data were fitted using GraphPad Prism 6 to the following equation:

$$Y = \frac{d_{conf}}{\sqrt{1+I/I_{sat}}} \quad (2)$$

where d_{conf} – confocal resolution, I – STED laser intensity power, I_{sat} – saturating STED laser intensity power.

Processing and visualization of acquired images

All acquired or reconstructed images were processed and visualized using Fiji[11]. Line profiles were measured using the “straight line” tool with the line width set to 3 pixels. All profiles were fitted to Gaussian of Lorentz distributions using appropriate tools available on GraphPad Prism 6.

Supplementary references

(1) Butkevich, A. N.; Mitronova, G. Y.; Sidenstein, S. C.; Klocke, J. L.; Kamin, D.; Meineke, D. N.; D'Este, E.; Kraemer, P. T.; Danzl, J. G.; Belov, V. N.; Hell, S. W., *Angew. Chem., Int. Ed.* **2016,** *55*, 3290-4.
(2) Butkevich, A. N.; Belov, V. N.; Kolmakov, K.; Sokolov, V. V.; Shojaei, H.; Sidenstein, S. C.; Kamin, D.; Matthias, J.; Vlijm, R.; Engelhardt, J.; Hell, S. W., *Chem. Eur. J.* **2017,** DOI: 10.1002/chem.201701216.
(3) Lukinavičius, G.; Umezawa, K.; Olivier, N.; Honigmann, A.; Yang, G.; Plass, T.; Mueller, V.; Reymond, L.; Correa, I. R., Jr.; Luo, Z. G.; Schultz, C.; Lemke, E. A.; Heppenstall, P.; Eggeling, C.; Manley, S.; Johnsson, K., *Nat. Chem.* **2013,** *5*, 132-9.
(4) Sanguinetti, M.; Sanfilippo, S.; Castagnolo, D.; Sanglard, D.; Posteraro, B.; Donzellini, G.; Botta, M., *ACS Med. Chem. Lett.* **2013,** *4*, 852-7.
(5) Liu, J.; Sun, Y. Q.; Zhang, H.; Shi, H.; Shi, Y.; Guo, W., *ACS Appl. Mater. Interfaces* **2016,** *8*, 22953-62.
(6) Milroy, L. G.; Rizzo, S.; Calderon, A.; Ellinger, B.; Erdmann, S.; Mondry, J.; Verveer, P.; Bastiaens, P.; Waldmann, H.; Dehmelt, L.; Arndt, H. D., *J. Am. Chem. Soc.* **2012,** *134*, 8480-6.
(7) Lukinavičius, G.; Reymond, L.; D'Este, E.; Masharina, A.; Göttfert, F.; Ta, H.; Guther, A.; Fournier, M.; Rizzo, S.; Waldmann, H.; Blaukopf, C.; Sommer, C.; Gerlich, D. W.; Arndt, H. D.; Hell, S. W.; Johnsson, K., *Nat. Methods* **2014,** *11*, 731-3.
(8) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J. Wallingford, CT, 2016.
(9) Deniel, M. H.; Lavabre, D.; Micheau, J. C., Crano, J. C.; Guglielmetti, R. J. *Photokinetics under Continuous Irradiation*. In *Organic Photochromic and Thermochromic Compounds*: Volume 2: Physicochemical Studies, Biological Applications, and Thermochromism. Eds. Springer US: Boston, MA, 2002; pp 167-209. DOI: 10.1007/0-306-46912-X_4.
(10) Lukinavičius, G.; Reymond, L.; Umezawa, K.; Sallin, O.; D'Este, E.; Göttfert, F.; Ta, H.; Hell, S. W.; Urano, Y.; Johnsson, K., *J. Am. Chem. Soc.* **2016,** *138*, 9365-8.
(11) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J. Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A., *Nat. Methods* **2012,** *9*, 676-82.