Supplementary information

Fluorogenic probes for multicolor imaging in living cells

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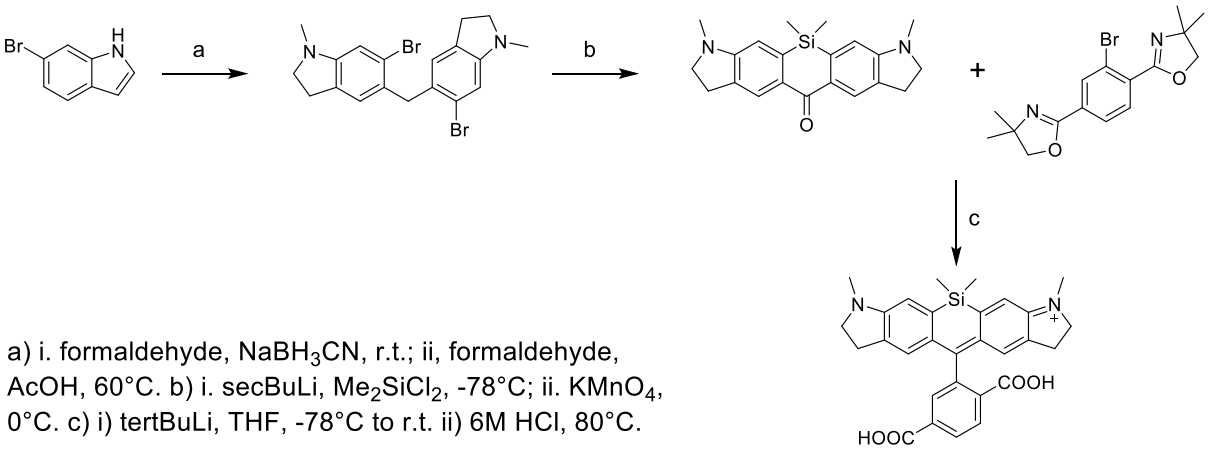
# These authors contributed equally to the present work.

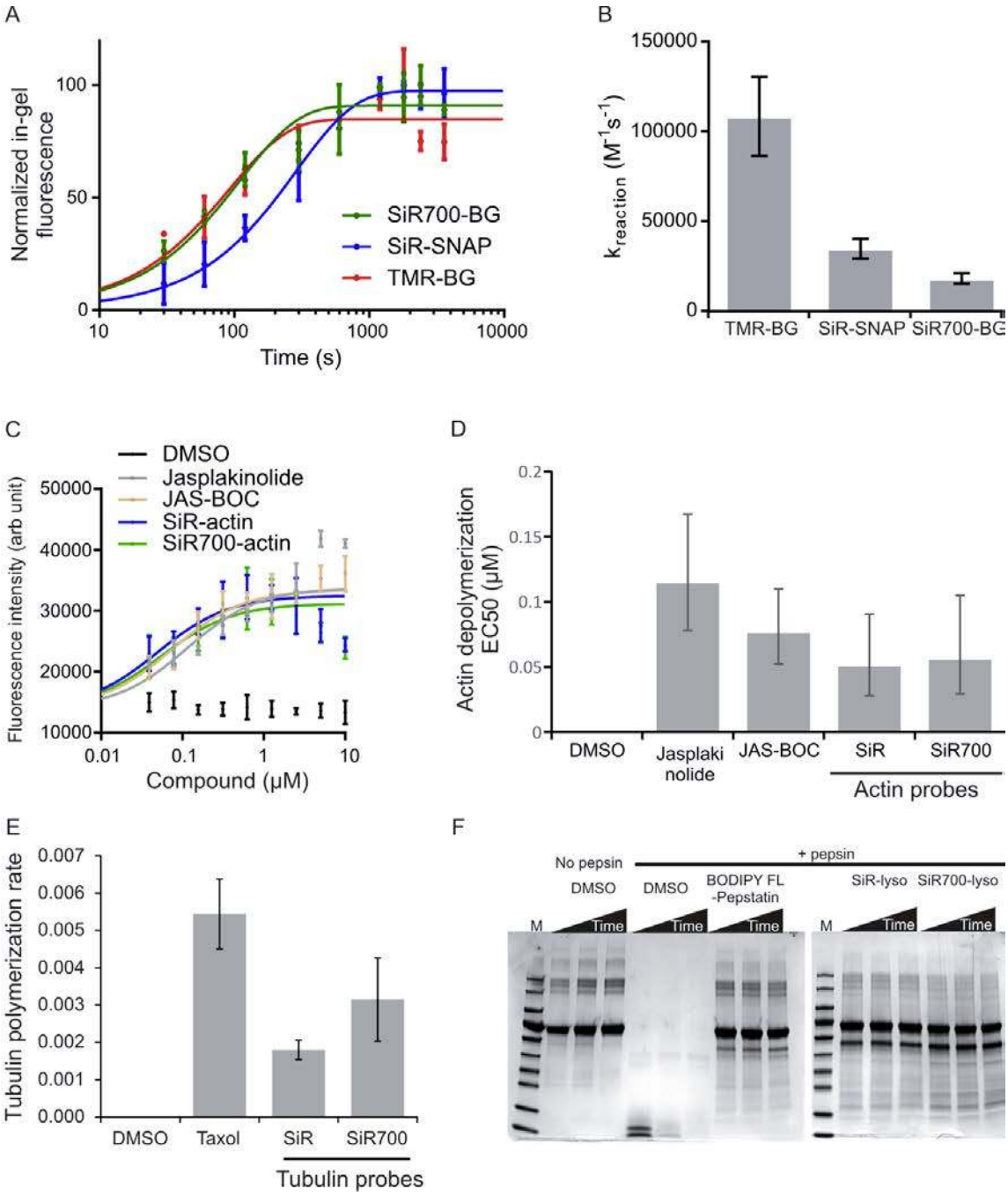
Supplementary inventory

Supplementary Figures

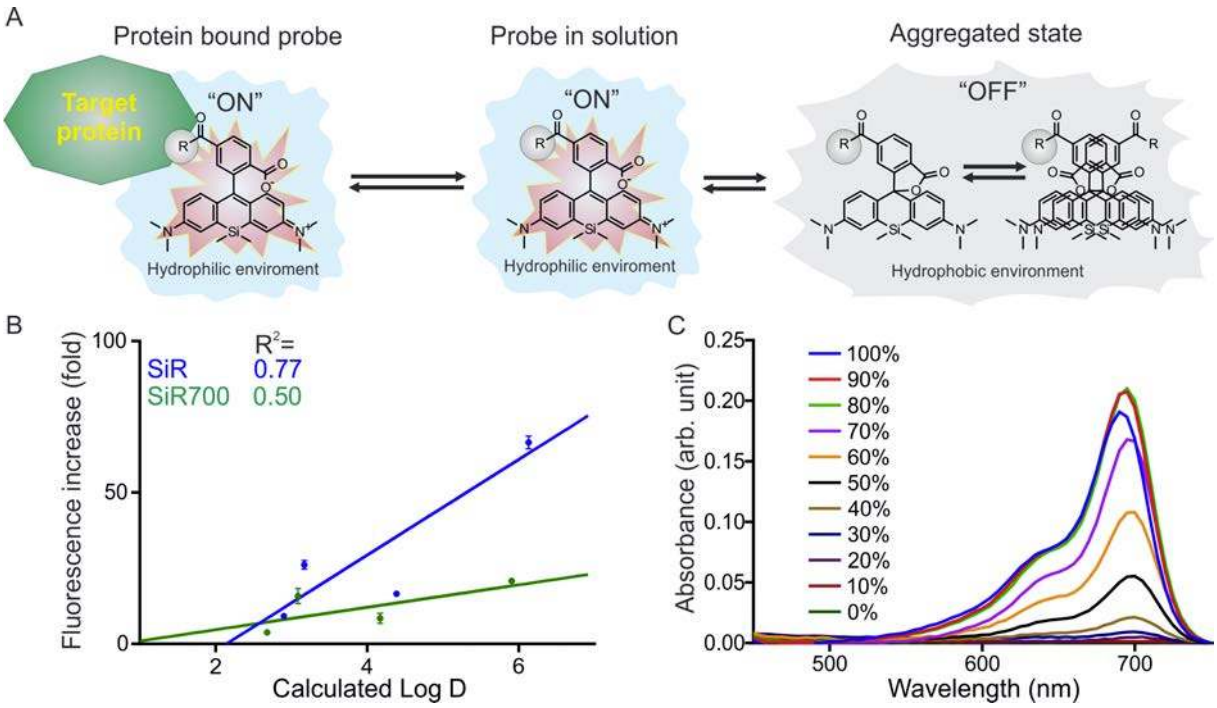


a) i. formaldehyde, $NaBH_3CN$, r.t.; ii, formaldehyde, AcOH, 60°C. b) i. secBuLi, Me_2SiCl_2, -78°C; ii. $KMnO_4$, 0°C. c) i) tertBuLi, THF, -78°C to r.t. ii) 6M HCl, 80°C.

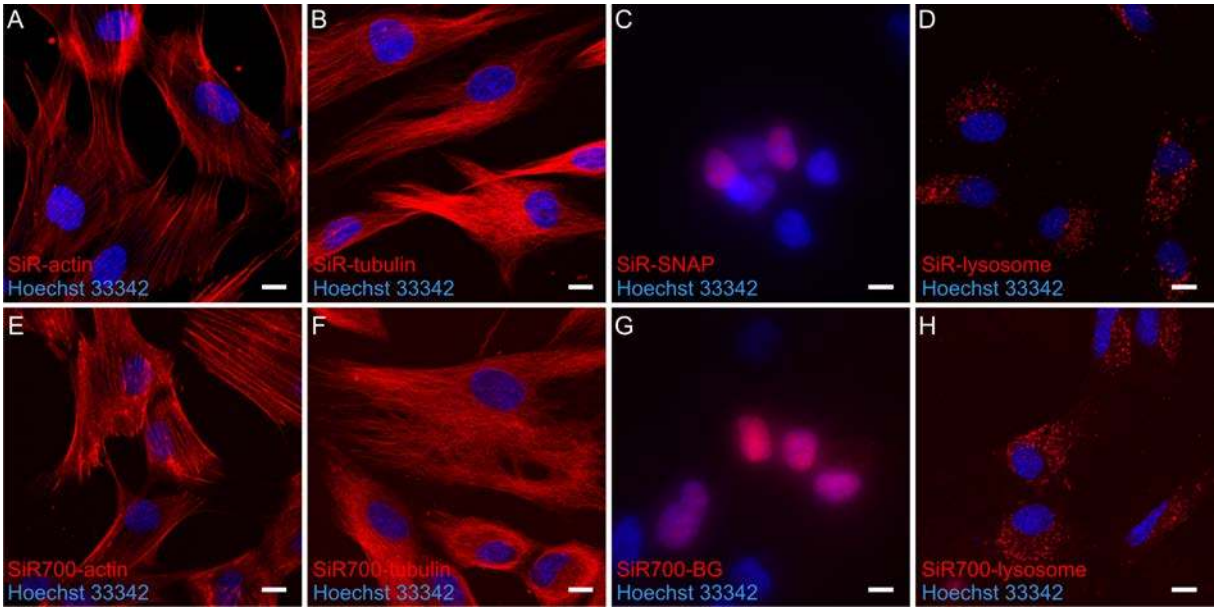
Supplementary Figure 1. Chemical synthesis of carboxy-SiR700.



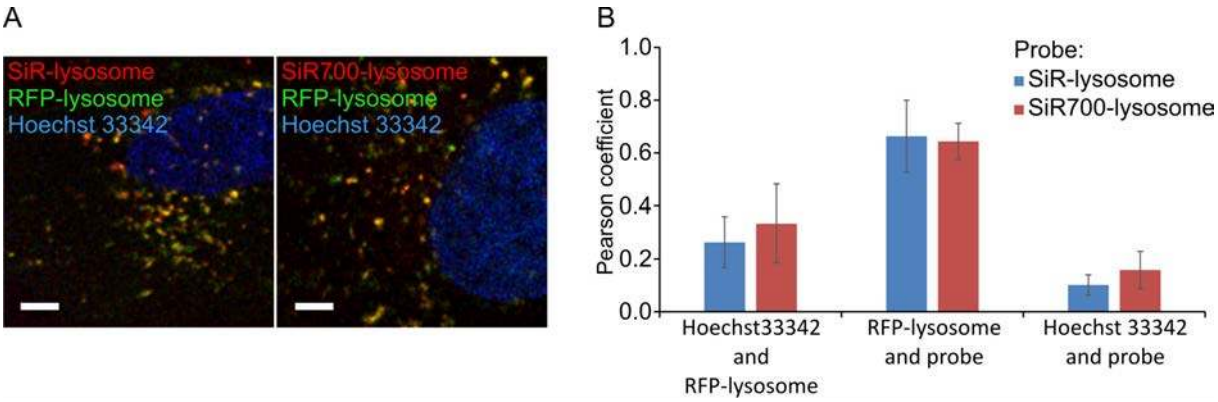
Supplementary Figure 2. Properties of SiR and SiR700 probes. A. Quantification of the SDS-PAGE of SNAP-tag labelling with fluorescent probes. Data presented as mean ± SD, N ≥ 2. **B.** Comparison of the reaction rates of different substrates. Data presented as mean ± SD, N ≥ 2. **C.** Actin depolymerization end-point assay in the presence of actin probes or Jasplakinolide. Data presented as mean ± SD, N = 4. **D.** EC50 of actin depolymerisation assay shown in A. Data presented as mean ± CI 95%. **E.** Tubulin polymerization rate in the presence of tubulin probes or taxol. Data presented as mean ± SD, N = 2. **F**. Inhibition of BSA hydrolysis by pepsin. SDS-PAGE gel showing degradation of BSA (1mg/ml) by 1 μM pepsin. Addition of lysosomal probes 3 μM effectively inhibits degradation. 10, 30 and 60 minutes time points of the reaction are shown.



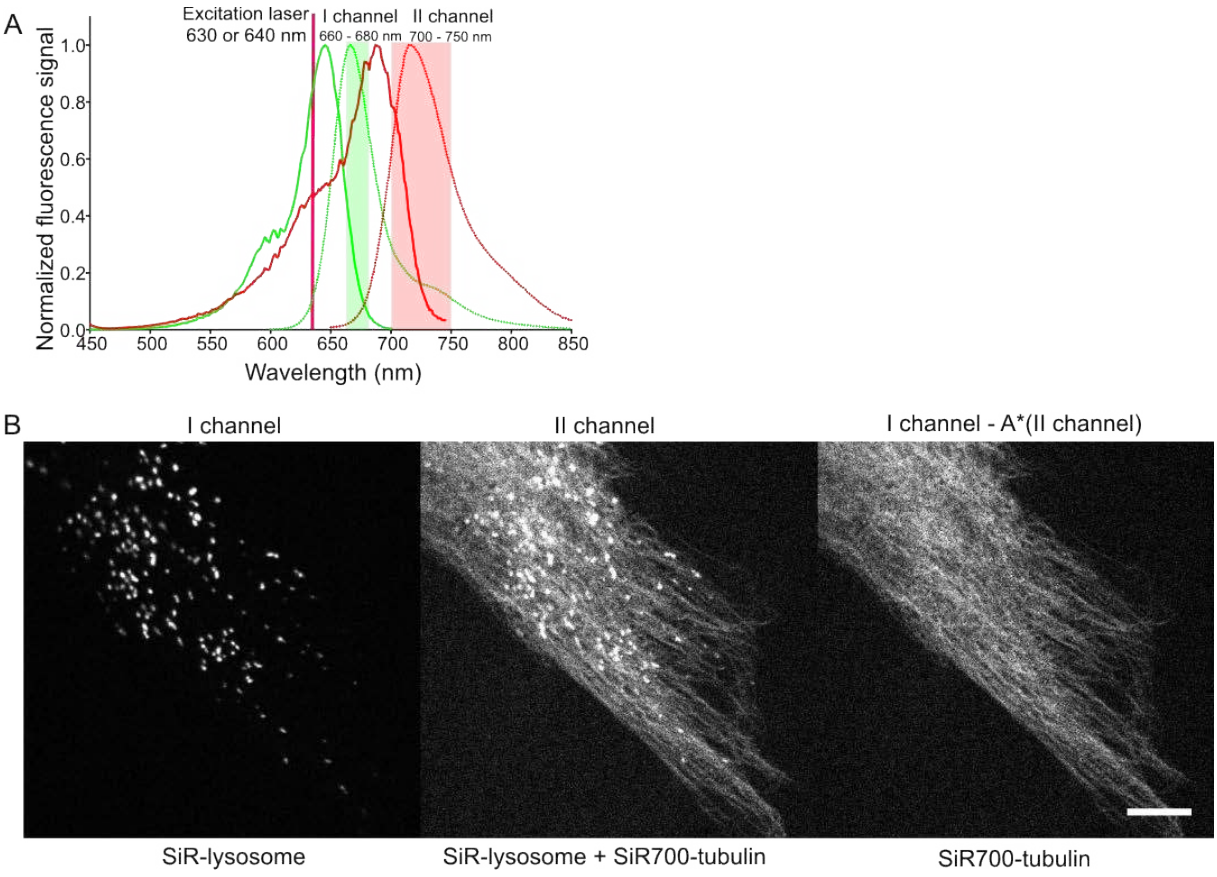
Supplementary Figure 3. Properties of silicon-rhodamine fluorophores. A: Proposed fluorogenicity mechanism of SiR-based probes. B. Correlation between observed fluorogenicity (taken from Supplementary Table 3) and calculated log D (taken from Supplementary Table 5). C. Absorbance spectra of carboxy-SiR700 in different water dioxane mixtures. The percentage of water in the mixture in given in the legend.



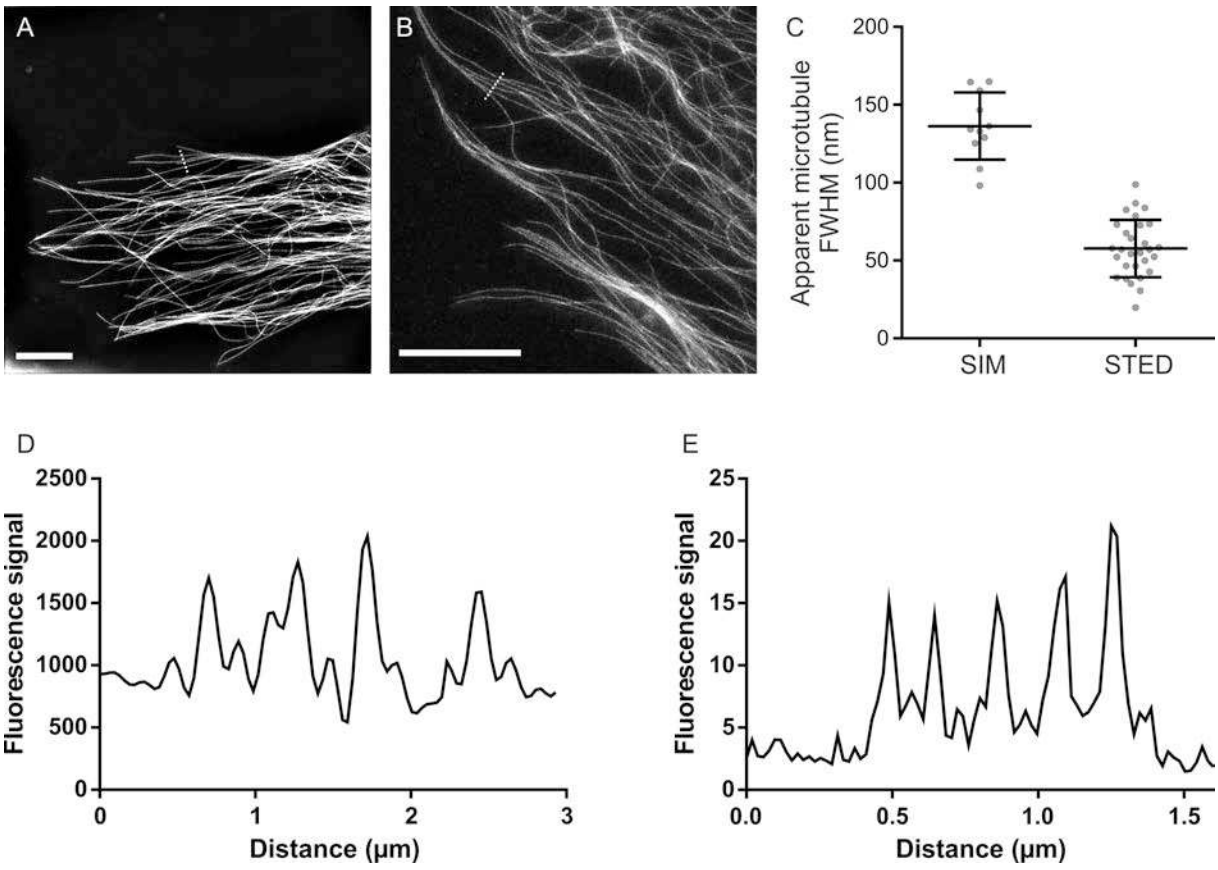
Supplementary Figure 4. Wide field and confocal imaging of SiR and SiR 700 probes. A. and **E.** Confocal images of human primary fibroblasts stained with corresponding SiR- and SiR700-actin probes respectively. **B.** and **F.** Confocal images of human primary fibroblasts stained with corresponding SiR- and SiR700-tubulin probes respectively. **C.** and **G.** Wide field fluorescence microscopy image of U2OS cells expressing nuclear localized SNAP-tag labelled with 5 μM O^6-benzylguanine (BG) SiR and SiR700 respectively. **D.** and **H.** Confocal images of human primary fibroblasts stained with corresponding SiR- and SiR700-lysosome probes, respectively. Probes images in red overlaid with Hoechst 33342 (0.1 μg / ml) nuclear staining. Cells stained with SNAP-tag substrates were washed twice with growth medium and incubated for 1 h at 37°C in the growth medium before imaging. No washing before imaging applied for the cells stained with 2 μM tubulin, actin and lysosome probes in growth medium for 1 h at 37°C. Scale bar 10 μm.



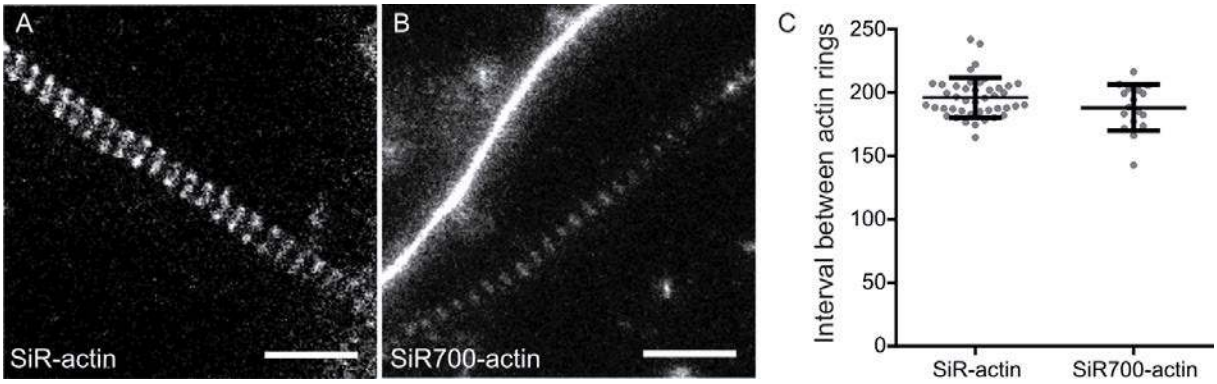
Supplementary Figure 5. Colocalization of lysosomal probes with lysosomal marker Lamp1-tagRFP. A. Images showing co-localization of lysosomal probes (red) with CellLight® Lysosomes-RFP (green, Life technologies) marker. Nuclear Hoechst 33342 (0.1 μg / ml) staining is shown in blue. Scale bar 5 μm. **B.** Estimation of colocalization of lysosomal probes (red), CellLight® Lysosomes-RFP and Hoechst 33342. Pearson coefficient is calculated from confocal images. Data presented as mean ± SD. N = 15 cells for SiR-lysosomesome, N = 12 cells for SiR700-lysosome.



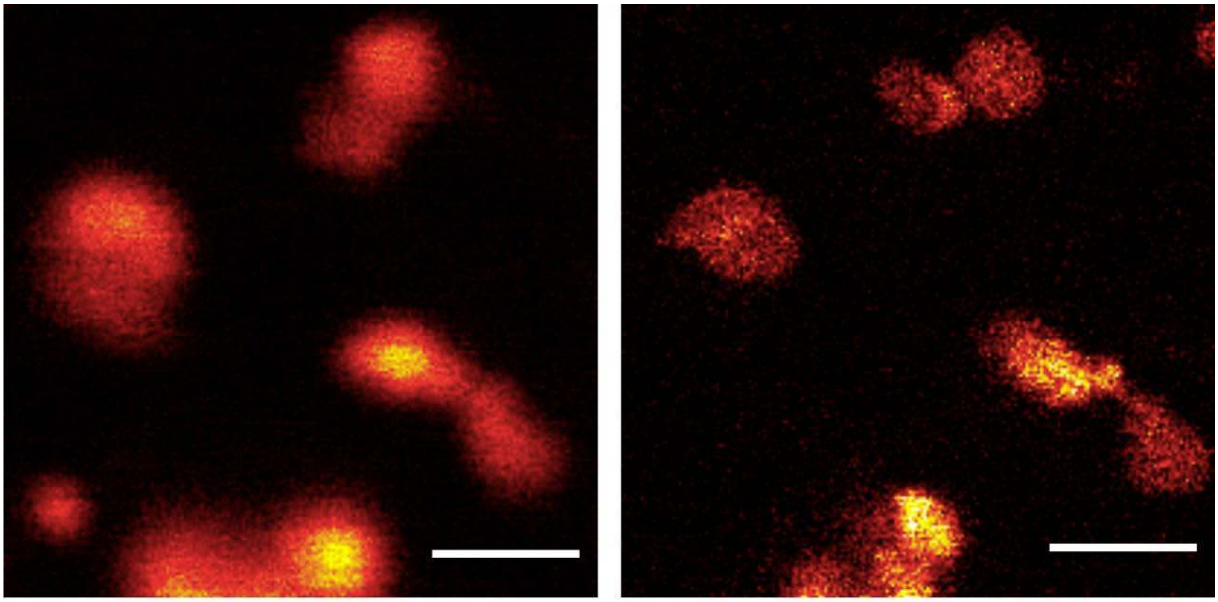
Supplementary Figure 6. Two-colour confocal microscopy using SiR and SiR700 probes. A. Microscopy setup used for imaging SiR and SiR700 simultaneously with single laser excitation. **B.** Example of two colour microscopy of primary human fibroblasts stained with 1 μM SiR-lysosome, 0.5 μM SiR700-tubulin probes at 37°C for 2 h before imaging. No washing was applied to cells. Two raw data channels are shown. Crosstalk between SiR and SiR700 channels could be eliminated by simple channel subtraction. Coefficient A is selected empirically. No washing was applied to cells. Scale bar 10 μm.



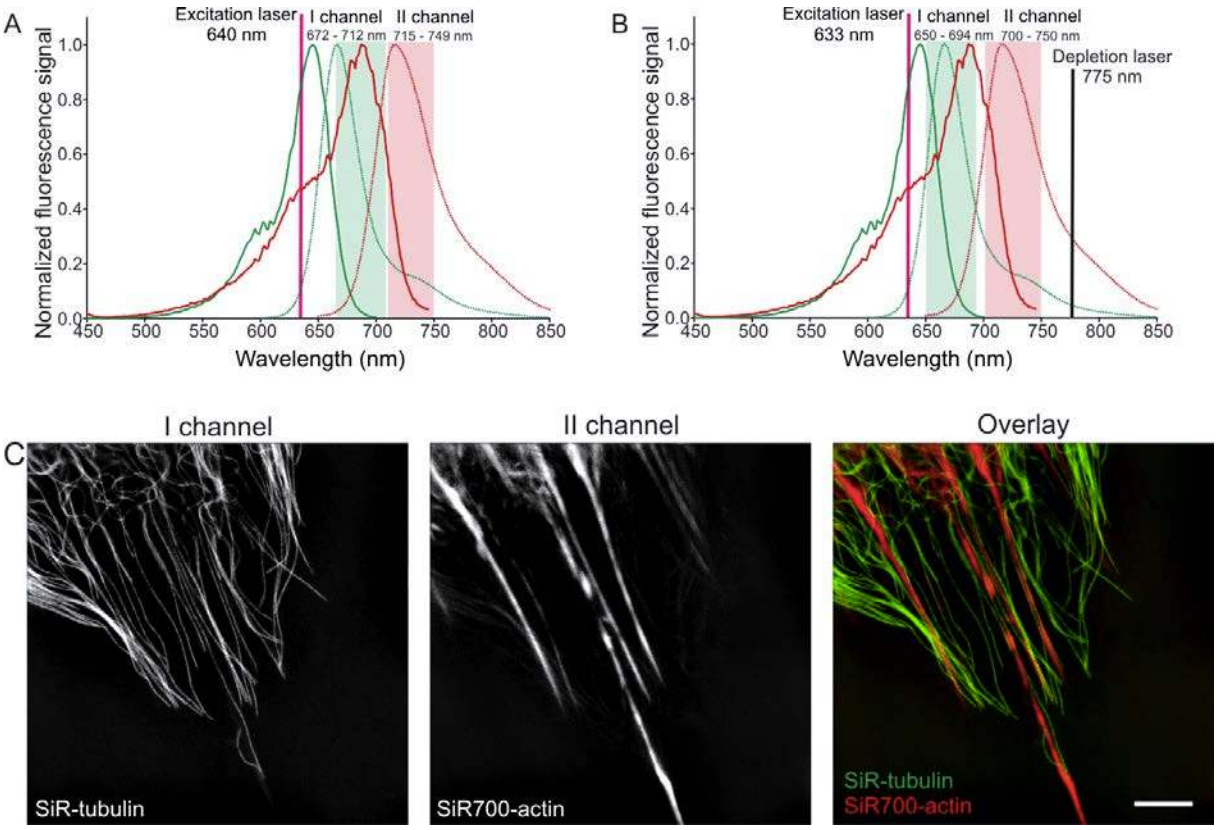
Supplementary Figure 7. Performance of SiR700-tubulin probe in super-resolution microscopy. A. SIM microscopy images of living human primary fibroblasts stained with 2 µM SiR700-tubulin for 1 h at 37°C in growth medium, media replaced to fresh and imaged of NIKON N-SIM microscope. **B.** STED microscopy images of human primary fibroblasts stained with 2 µM SiR700-tubulin probe for 1 h at 37°C in the growth medium used. Medium changed to no probe containing medium just before imaging. **C.** Apparent microtubule diameter in living human primary fibroblasts imaged with SIM or STED microscopes. Data presented as mean ± standard deviation. Scale bars 5 µm. **D.** Intensity line profile across microtubules on image A. **E.** Intensity line profile across microtubules on image B. Dashed line in the images indicates line profile location.



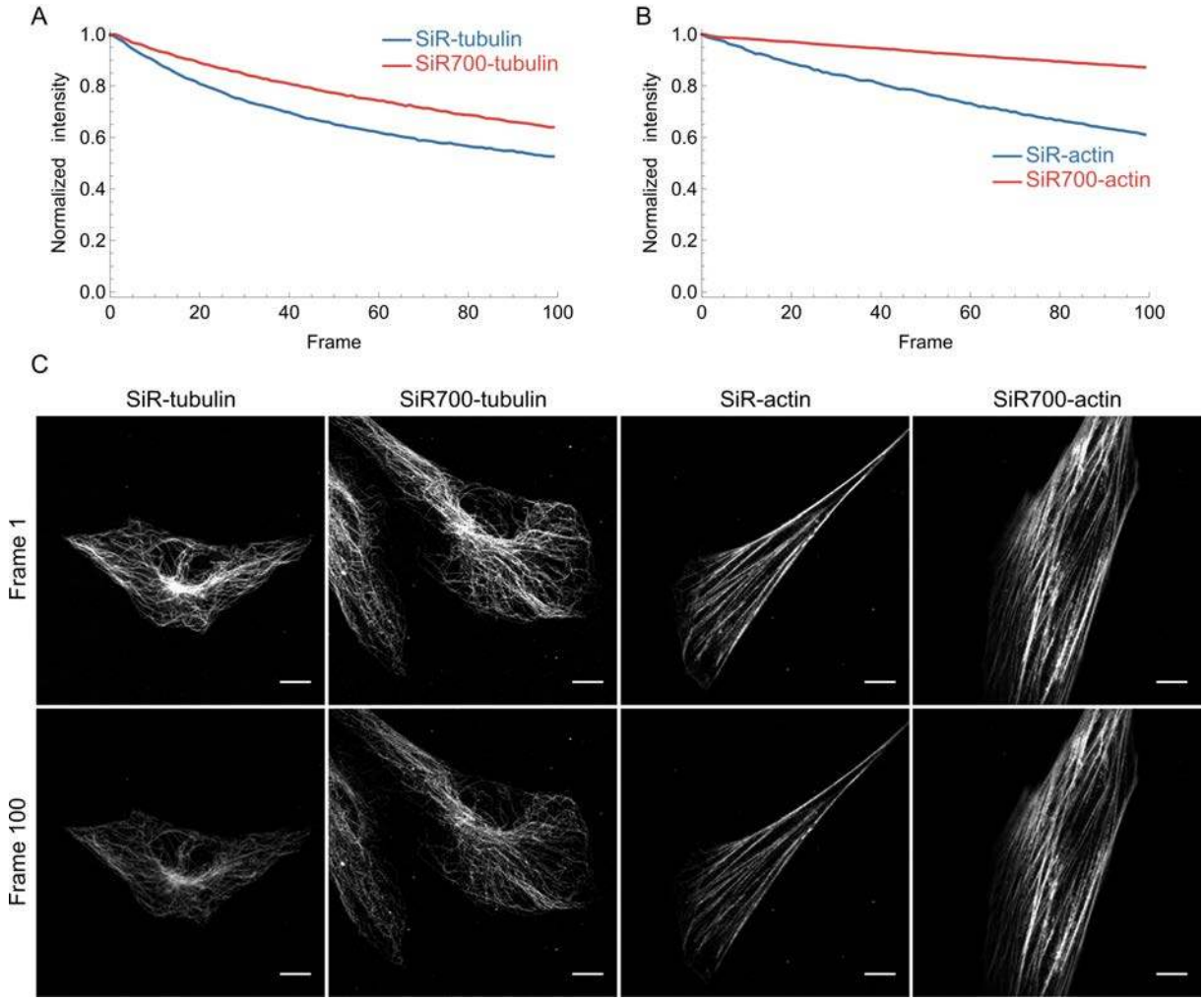
Supplementary Figure 8. Performance of actin probes in stimulated emission depletion (STED) microscopy. A. and **B.** STED microscopy images of primary rat hippocampal neurons stained with 100 nM actin probes for 1 h at 37°C in the growth medium. Medium changed to no probe containing medium (ACSF) just before imaging. 775 nm STED laser was used for SiR and 810 nm STED laser was used for SiR700. Scale bars 1 µm. **C.** Measured interval between actin rings in the axons of primary rat hippocampal neurons. Data presented as mean ± standard deviation.



Supplementary Figure 9. Comparison of confocal and STED images of lysosomes stained with SiR-lysosome probe. Human primary fibroblasts stained with 1 μM SiR-lysosome probe for 1 h at 37°C in the growth medium. Medium changed to no probe containing medium just before imaging. Scale bars 1 μm.



Supplementary Figure 10. Two color structured illumination (SIM) and stimulated emission depletion (STED) microscopy using SiR and SiR700 probes. A. SIM setup used for imaging SiR and SiR700 simultaneously excited with 633 nm single laser excitation. **B.** STED microscopy setup used for imaging SiR and SiR700 simultaneously excited with 633 nm single laser excitation and 775 nm single laser depletion. **C.** Example of SIM image of human primary fibroblasts stained with 2 µM SiR-tubulin and 2 µM SiR700-actin probes. Scale bar 5 µm.



Supplementary Figure 11. Comparison of SiR and SiR700 photostability by confocal microscopy. A. Relative Intensity profile as a function of frame number of live NIH-3T3 cells stained with 0.5 μm SiR-tubulin (blue line) or SiR700-tubulin (red line) for 1.5 h. The cells were washed 3 times with fresh medium before imaging. **B.** Relative Intensity as a function of frame number of live NIH-3T3 cells stained with 0.5 μm SiR-actin (blue line) or SiR700-actin (red line) for 1.5 h. The cells were washed 3 times with fresh medium before imaging. **C.** Confocal images showing the intensity of the SiR-probe signal after the 1st frame and 100th frame for the probes SiR-tubulin, SiR700-tubulin, SiR-actin and SiR700 actin. Scale bar 10 μm.

Supplementary Tables.

Supplementary Table 1. Physicochemical properties of unconjugated fluorophores.

Fluorophore	$\lambda_{abs\ (max)}$ (nm)	ε_{max} ($M^{-1} \cdot cm^{-1}$)	$\lambda_{ex\ (max)}$ (nm)	$\lambda_{em\ (max)}$ (nm)	Sensitivity to dielectric constant (D_{50})	QY
TMR	550	85,000	551	574	6.4	0.7
Carboxy-SiR	652	100,000	645	667	59.0	0.4
Carboxy-SiR700	689	100,000	687	716	43.1	0.13

Supplementary Table 2. Probe fluorescence increase upon addition of 0.1% SDS.

	Probe target			
Fluorophore	SNAP-tag	Lysosome	Tubulin	Actin
TMR	3.2	n.d.	2.2	8.5
SiR	15.0	13.6	18.4	86.2
SiR700	3.4	14.9	7.7	13.7

Supplementary Table 3. Probe fluorescence increase upon binding to the target.

	Probe target			
Fluorophore	SNAP-tag	Lysosome	Tubulin	Actin
TMR	2.4 ± 0.1	n.d.	1.3 ± 0.1	6.4 ± 0.3
SiR	9.2 ± 0.4	26.1 ± 1.5	16.6 ± 0.8	66.6 ± 2.1
SiR700	3.8 ± 0.1	15.8 ± 2.5	8.5 ± 1.6	20.8 ± 0.9

Supplementary Table 4. Reactivity of SNAP-tag substrates.

Substrate	[SNAP-tag] nM	[Substrate] nM	Rate	Number of replicates, N
BG-TMR	100	20	108300 ± 22040	2
SiR-SNAP	100	20	34730 ± 5502	3
SiR700-BG	500	100	18098 ± 2920	3

Supplementary Table 5. Calculated LogD of synthesized probes.

	Probe target			
Fluorophore	SNAP-tag	Lysosome	Tubulin	Actin
SiR	2.9	3.2	4.4	6.1
SiR700	2.7	3.1	4.2	5.9

Supplementary Table 6. Fluorescence lifetime of SiR probes.

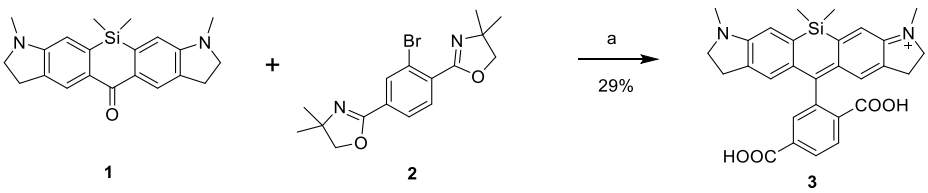
Name	Free probe	Target bound probe
Carboxy-SiR650	2.7 ns	n.a.
Carboxy-SiR700	1.4 ns	n.a.
SiR-SNAP	3.2 ns	3.3 ns
SiR700-BG	2.2 ns	2.8 ns
SiR650-lysosome	3.2 ns*	3.0 ns*
SiR700-lysosome	2.5 ns*	2.1 ns*

*-measurements done in $HCOONH_4$, pH 4.0, n.d. – not determined, n.a. – not available.

Supplementary Methods

Chemical synthesis of probes.

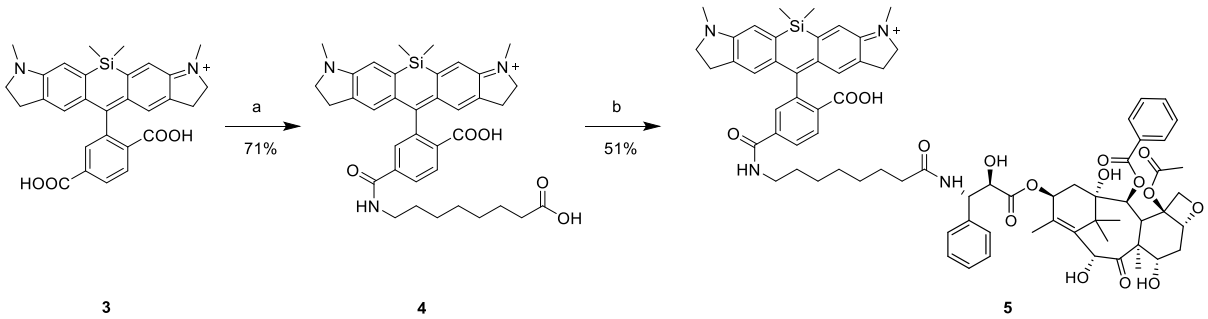
All chemical reagents and anhydrous solvents for synthesis were purchased from commercial suppliers (Sigma-Aldrich, Fluka, Acros) and were used without further purification or distillation. The composition of mixed solvents is given by the volume ratio (v/v). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX 400 (400 MHz for ^{1}H, 100 MHz for ^{13}C, respectively), Bruker AVANCE III 400 Nanobay (400 MHz for ^{1}H, 100 MHz for ^{13}C, respectively), Bruker AVANCE 500 (500 MHz for ^{1}H, 126 MHz for ^{13}C, respectively), or Bruker DRX-600 (600 MHz for ^{1}H, 151 MHz for ^{13}C, respectively), with chemical shifts (δ) reported in ppm relative to the solvent residual signals of $CDCl_3$ (7.26 ppm for ^{1}H, 77.16 ppm for ^{13}C), CD_3OD (3.31 ppm for ^{1}H, 49.00 ppm for ^{13}C), DMSO-d_6 (2.50 ppm for ^{1}H, 39.52 ppm for ^{13}C). Coupling constants are reported in Hz. High resolution mass spectra (HRMS) were measured on a Micromass Q-TOF Ultima spectrometer with electrospray ionization (ESI) or Bruker MicroTOF with ESI-TOF (time-of-flight). LC-MS was performed on a Shimadzu MS2020 connected to a Nexerra UHPLC system equipped with a Waters ACQUITY UPLC BEH C18 1.7µm 2.1x50mm column. Buffer A: 0.05% HCOOH in H_2O Buffer B: 0.05% HCOOH in acetonitrile. Analytical gradient was from 5% to 95% B within 5.5 min with 0.5 ml/min flow. Preparative RP-HPLC was performed on a Dionex system equipped with an UVD 170U UV-Vis detector for product visualization on a Waters SunFire™ Prep C18 OBD™ 5 µm 10×150 mm Column (Buffer A: 0.1% TFA in H_2O Buffer B: acetonitrile. Typical gradient was from 0% to 100% B within 30 min with 4 ml/min flow.), or a JASCO PU-1587 system with equipped a GL Sciences Inertsil ODS-3 10 mm X 250 mm column (Buffer A: H_2O, including 1% acetonitrile and 0.1% TFA, Buffer B: acetonitrile, including 1% H_2O. Typical gradient was from 10% to 90% B within 30 min with 20 ml/min flow.), respectively. After lyophilization of HPLC purified compounds, the solid residue was generally dissolved in dry DMSO and the concentration of the SiR-derivatives was measured by UV-Vis spectroscopy in PBS containing 0.1% SDS, using a molar extinction coefficient of 100'000 $M^{-1}cm^{-1}$ at 652 nm for SiR of 100'000 $M^{-1}cm^{-1}$ at 689 nm for SiR700.



Supplementary Scheme 1. Carboxy-SiR700 **3**: (a) i) **2**, tBuLi, THF -78°C. ii) **1**, THF -78°C to r.t. iii) 6M HCl, 80°C.

Carboxy-SiR700 **3**

In an argon-flushed flask fitted with a septum cap, compound **2** [1] (250 mg, 0.68 mmol, 5 eq.) was dissolved in dry THF (5 ml) and cooled at -78 °C. A 1.6 M solution of *tert*-BuLi (350 µl, 0.55 mmol, 4 eq.) was slowly added dropwise and the solution was stirred at the same temperature for 30 min. Compound **1** [2] (48 mg, 0.14 mmol, 1 eq.) in dry THF (5 ml) was added dropwise via a syringe at -78 °C. The solution was allowed to warm to room temperature and stirred for 3 h. 0.1 N HCl was added to the cooled reaction mixture (ice bath), then saturated $NaHCO_3$ aqueous solution was added. The aqueous phase was extracted with CH_2Cl_2 (3 x), and the combined organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated to afford a blue-green sticky solid. it was dissolved in 6 N HCl aq. (2 ml) and MeCN (1 ml), and stirred at 80 °C overnight. After cooling to room temperature, the solution was added to saturated $NaHCO_3$ aqueous solution (50 ml) and 0.1 N HCl aqueous solution to adjust the pH (1-2), and extracted with CH_2Cl_2 (3 x). The combined organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified with HPLC using a JASCO PU-1587 system to obtain **3** as a blue-green solid. (19 mg, 29%). ^{1}H NMR (400 MHz, CD_3OD) δ 0.54 (s, 3H), 0.61 (s, 3H), 2.90 (t, 4H, *J* = 7.9), 3.19 (s, 6H), 3.77 (t, 4H, *J* = 7.9), 6.56 (s, 2H), 7.10 (s, 2H), 7.78 (d, 2H, *J* = 1.2), 8.26 (dd, 1H, *J* = 1.2, 8,2), 8.30 (d, 1H, *J* = 8.2). ^{13}C NMR (100 MHz, CD_3OD) δ -1.9, -1.0, 27.3, 33.7, 55.6, 115.2, 130.3, 130.8, 132.2, 132.3, 133.0, 134.4, 135.4, 135.8, 143.8, 151.2, 158.2, 167.8, 168.1. HRMS (ESI) calcd for $C_{29}H_{29}N_2O_4Si^+$ $[M]^+$ 497.1891; found 497.1877.



Supplementary Scheme 2. SiR700-tubulin **5**: (a) 8-aminooctanoic acid TSTU, DIEA, DMSO, r.t.. (b) 3'-aminodocetaxel, HBTU, DIEA, DMSO, r.t.

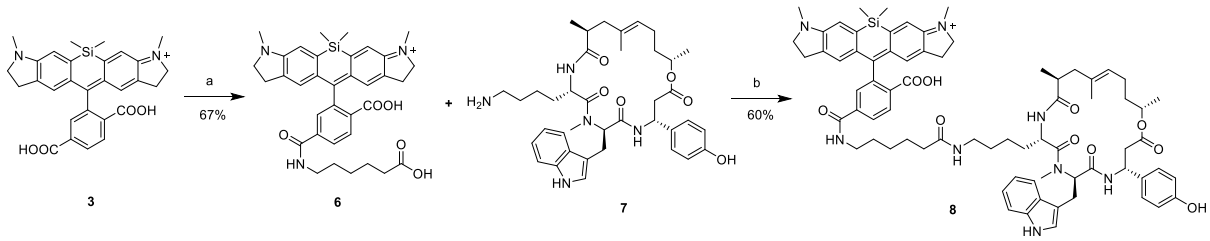
SiR700-C8-COOH **4**

SiR700-COOH **3** (150 µl of a 22.3 mM solution in DMSO, 3.3 µmol, 1 eq.) was successively treated with DIEA (15 ul, 87 µmol, 26 eq.) and TSTU (1.2 mg, 4.1 µmol, 1.2 eq.). After 5 min, 8-aminooctanoic acid (2.0 mg, 12.6 µmol, 3.8 eq.) was added. The reaction was sonicated 15 min at r.t. Then H_2O (20 µl) was added and the mixture was incubated for 15 min at r.t. AcOH was added until the pH was neutral and the reaction was purified by RP-HPLC and lyophilized and dissolved in dry DMSO. 400 µl of 5.9 mM solution of **4** were obtained (71% yield) as a green solution ^{1}H NMR all signals were broadened by a slow exchange between forms (400 MHz, DMSO-d6) δ 0.50 (3H, s), 0.60 (3H, s), 1.26 (7H, s), 1.48-1.45 (4H, m), 2.17 (2H, t, J=7.4), 2.81-2.79 (6H, m), 3.21-3.20 (4H, m), 6.50 (2, s), 6.83 (1, s), 7.27 (1H, s), 7.52 (1, s), 8.04 (2, s), 8.71 (1, t, J=5.5). ^{13}C NMR (151 MHz, MeOD) δ 177.57, 168.13, 167.69, 158.21, 151.35, 143.80, 139.08, 134.49, 134.35, 133.26, 132.39, 130.28, 130.19, 128.45, 115.24, 55.56, 49.85, 41.21, 34.87, 33.72, 30.77, 30.26, 30.10, 30.04, 27.89, 27.25, 25.96. HRMS (ESI) calcd for $C_{36}H_{44}N_3O_5^+$ $[M]^+$ 598.3275; found 598.3279.

SiR700-Tubulin **5**

SiR700-C8-COOH **4** (170 µl of a 5.9 mM solution in DMSO, 1.0 µmol, 1 eq.) was treated with DIEA (20 ul, 116 µmol) and HBTU (15 µl of a 100 mM solution in DMSO, 1.5 µmol, 1.5 eq.). After 5 min, 3'-aminodocetaxel [3] (30 µl of a 50 mM solution in DMSO, 1.5 µmol, 1.5 eq.) was added. The mixture was incubated 2h at r.t. The product was purified by RP-HPLC and lyophilized. 300 µl of 1.7 mM solution of **5**

were obtained (51% yield) as a green solution. ^{1}H NMR (400 MHz, MeOD) δ 0.56 (3H, s), 0.62 (3H, s), 1.12 (3H, s), 1.19 (3H, s), 1.26 (3H, dd, J=1.1,6.8), 1.35 (6H, d), 1.63-1.57 (4H, m), 1.70 (3H, s), 1.87-1.81 (1H, m), 1.91 (3H, d), 2.07-2.04 (1H, m), 2.32-2.24 (3H, m), 2.37 (3H, s), 2.90 (4H, m), 3.22 (6H, s), 3.80 (4H, t, J=7.4), 3.88 (1H, d, J=7.2), 4.25-4.20 (3H, m), 4.60 (1H, d, J=4.4), 5.02-4.99 (1H, m, J=1.3,9.3), 5.27 (1H, s), 5.48 (1H, d, J=4.3), 5.65 (1H, d, J=7.2), 6.17 (1, t, J=8.9), 6.57 (2H, s), 7.13 (2H, s), 7.29-7.25 (1H, m), 7.44-7.37 (4H, m), 7.56 (2H, m), 7.67-7.64 (2H, m), 8.12-8.08 (3H, m), 8.32 (1H, d, J=8.2). ^{13}C NMR (151 MHz, MeOD) δ 174.58, 173.03, 170.43, 166.74, 166.27, 156.77, 151.16, 138.82, 137.84, 137.76, 136.60, 133.13, 132.98, 131.66, 130.92, 130.05, 129.80, 128.97, 128.77, 128.29, 128.23, 127.43, 126.99, 113.80, 84.52, 80.99, 77.69, 76.22, 75.08, 74.27, 73.44, 71.30, 71.27, 69.78, 65.09, 62.70, 57.51, 55.38, 54.19, 48.18, 46.52, 43.16, 39.80, 36.13, 35.51, 33.56, 32.38, 31.69, 29.38, 29.21, 29.09, 29.03, 28.89, 28.82, 28.63, 26.44, 25.91, 25.78, 25.53, 24.62, 21.82, 20.30, 13.05, 9.09. HRMS (ESI) calcd for $C_{73}H_{89}N_8O_{10}Si^+$ $[M]^+$ 1265.6465; found 1265.6465.



Supplementary Scheme 3. SiR700-actin **8**: (a) i) 6-aminohexaoic acid methyl ester, TSTU, DIEA, DMSO, r.t.; ii) aq. NaOH. (b) **7,** TSTU, DIEA, DMSO, r.t.

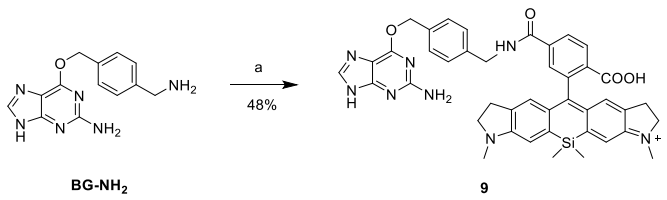
SiR700-C6-COOH **6**

SiR700-COOH **3** (100 µl of 12 mM solution in DMSO, 1.2 µmol, 1 eq.) was treated with DIEA (10 ul, 58 µmol, 48 eq.) and TSTU (14 µl of 100 mM solution in DMSO, 1.4 µmol, 1.2 eq.) were successively added. After 5 min, 6-aminohexanoic acid methyl ester (20 µl of 100 mM solution in DMSO, 2 µmol, 2 eq.) was added. The mixture was incubated for 15 min at r.t. Then 2 M NaOH (75 µl) was added and the mixture was incubated for 15 min at r.t.. AcOH was added until the pH was neutral and the reaction was purified by RP-HPLC and lyophilized. 200 µl of 4.0 mM solution of **6**

were obtained (67% yield) as a green solution.[1]H NMR (400 MHz, MeOD) δ 0.56 (3H, s), 0.61 (3H, s), 1.48-1.40 (2H, m), 1.70-1.62 (3H, m), 2.32 (2H, t, J=7.2), 2.93 (4H, m), 3.22 (6H, s), 3.41 (2H, t, J=7.1), 3.80 (4H, t, J=8.0), 6.59-6.58 (2H, m), 7.13 (2H, s), 7.66 (1H, s), 8.10 (1H, m), 8.34-8.28 (1H, m). ^{13}C NMR (101 MHz, MeOD) δ 176.09, 166.76, 166.30, 161.16, 160.81, 156.85, 150.02, 142.39, 137.65, 133.15, 132.98, 131.91, 131.05, 128.88, 127.09, 113.89, 54.18, 39.69, 33.40, 32.34, 28.69, 26.22, 25.86, 24.36, -2.40, -3.22. HRMS (ESI) calcd for $C_{35}H_{40}N_3O_5Si^+$ $[M]^+$ 610.2732; found 610.2732.

SiR700-actin **8**

SiR700-C6-COOH **6** (250 µl of 18.6 mM solution in DMSO, 4.6 µmol, 1 eq.) was treated with DIEA (6.1 ul, 37.2 µmol, 8 eq.) and TSTU (1.8 mg, 6.0 µmol, 1.3 eq.). After 5 min, lysine modified depsipeptide **7** [4] (50 µl of a 140 mM DMSO solution, 7.0 µmol, 1.5 eq.) was added. The mixture was incubated for 2 h at r.t. The product was purified by RP-HPLC, lyophilized and dissolved in dry DMSO. 200 µl of 13.9 mM solution of **8** were obtained (60% yield) as a green solution. ^{1}H NMR HRMS (400 MHz, MeOD) δ 0.55 (3H, s), 0.60 (3H, s), 1.03-0.95 (3H, m), 1.08 (3H, d, J=6.8), 1.25-1.16 (5H, m), 1.44-1.38 (3H, m), 1.53 (3H, s), 1.70-1.58 (5H, m), 1.91-1.87 (3H, m), 2.18 (2H, t, J=7.3), 2.27 (1H, m, J=12.3,14.1), 2.78-2.60 (3H, m), 3.04-2.86 (6H, m), 3.11 (3H, s), 3.17 (2H, d, J=8.2), 3.20 (6H, s), 3.42 (2H, t, J=6.9), 3.76 (4H, t, J=8.2), 4.77-4.75 (1H, m), 4.84 (1H, m), 5.06-5.02 (1H, m), 5.25 (1H, m, J=3.9,9.0,9.0), 5.64 (1H, m), 6.56 (2H, m), 6.74 (2H, m), 6.99 (1H, m), 7.09-7.05 (4H, m), 7.11 (2H, s), 7.31 (1H, d, J=8.1), 7.60 (1H, d, J=7.9), 7.69 (1H, d, J=1.7), 8.10 (1H, dd, J=8.2,1.8), 8.32 (1H, d, J=8.2), 8.42 (1H, d, J=8.5). ^{13}C NMR (151 MHz, MeOD) δ 176.49, 174.60, 173.12, 170.88, 170.27, 170.19, 166.73, 166.22, 156.86, 156.43, 150.24, 142.16, 137.61, 136.60, 133.34, 133.25, 132.95, 132.20, 132.03, 131.13, 129.03, 128.81, 127.13, 127.02, 126.90, 124.54, 122.99, 121.05, 118.40, 118.07, 114.96, 113.92, 110.96, 109.41, 70.44, 56.17, 54.12, 49.44, 49.34, 48.88, 43.16, 40.44, 39.67, 39.00, 38.66, 35.57, 35.25, 32.28, 31.29, 30.05, 29.39, 28.72, 28.62, 26.19, 25.80, 25.32, 24.34, 23.15, 21.68, 21.54, 18.95, 18.67, 15.21. (ESI) calcd for $C_{73}H_{89}N_8O_{10}Si^+$ $[M]^+$ 1265.6465; found 1265.6465.

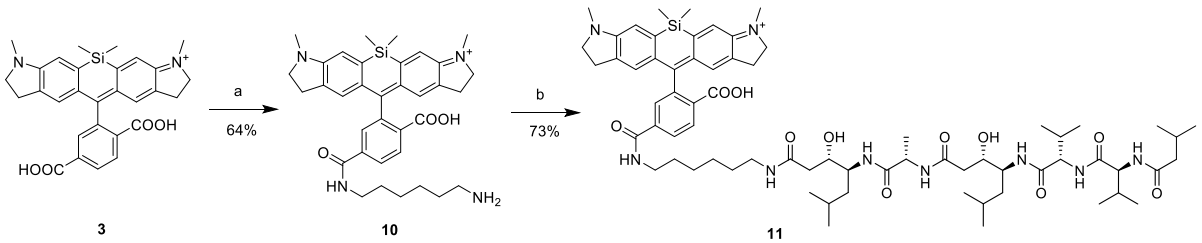


Supplementary Scheme 4. SiR700-BG **9**: (a) **3,** TSTU, DIEA, DMSO, r.t..

SiR700-BG **9**

SiR700-COOH **3** (80 µl of 13.0 mM solution in DMSO, 1.0 µmol, 1 eq.) was treated with DIEA (10 ul, 58 µmol, 58 eq.) and TSTU (12 µl of 130 mM solution in DMSO, 1.7 µmol, 1.7 eq.). After 5 min, BG-NH_2 [5] (2.0 mg, 7.4 µmol, 7.4 eq.) was added. The mixture was incubated for 1 h at r.t. The product was purified by RP-HPLC, lyophilized and dissolved in dry DMSO. 250 µl of 2.0 mM solution of **9** were obtained (48% yield) as a green solution.

^{1}H NMR (600 MHz, DMSO-d6) δ 0.57 (3H, s), 0.61 (3H, s), 2.92 (4H, t, J=7.8), 3.22 (6H, s), 3.80 (4H, t, J=8.1) 4.62 (2H, s), 5.65 (2H, s), 6.52 (2H, br, s), 6.59 (2H, s), 7.13 (2H, s), 7.44 (2H, d, J=8.1), 7.55 (2H, d, J=8.1), 7.71 (1H, d, J=1.7), 8.13 (1H, dd, J=1.8, 8.2), 8.31 (1H, s), 8.33 (2H, d, J=8.2). ^{13}C NMR (101 MHz, MeOD) δ 172.03, 166.80, 166.27, 161.02, 160.66, 159.76, 156.82, 152.60, 149.93, 142.55, 141.76, 139.25, 137.39, 134.29, 133.35, 132.97, 131.82, 131.06, 128.95, 128.88, 128.80, 127.60, 127.08, 113.88, 69.22, 54.18, 43.10, 32.34, 29.40, 25.87, -2.43, -3.21. HRMS (ESI) calcd for $C_{42}H_{41}N_8O_4Si^+$ $[M]^+$ 749.3015; found 749.3008.



Supplementary Scheme 5. SiR700-lysosome **11**: (a) 1,6-diaminohexane, TSTU, DIEA, DMSO, r.t. (b) Pepstatin A**,** TSTU, DIEA, DMSO, r.t.

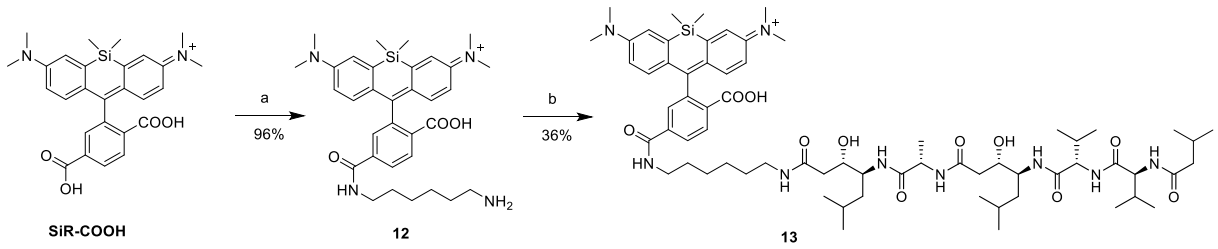
SiR700-C6-NH2 **10**

SiR700-COOH **3** (52 µl of 42.0 mM solution in DMSO, 2.1 µmol, 1 eq.) was treated with DIEA 10 ul, 58 µmol, 26 eq.) and TSTU (26 µl of a 100 mM solution in DMSO, 2.6 µmol, 1.2 eq.). After 5 min, 1,6-diaminohexane (210 µl of a 100 mM DMSO

solution, 21 µmol, 10 eq.) was added. The mixture was incubated for 1 h at r.t. The product was purified by RP-HPLC, lyophilized and dissolved in dry DMSO. 200 µl of 6.7 mM solution of **10** were obtained (64% yield) as a green solution.
^{1}H NMR (400 MHz, MeOD) δ 0.57 (3H, s), 0.61 (3H, s), 1.47 (5H, m), 1.71-1.66 (4H, m), 2.93 (6H, m), 3.21 (6H, s), 3.41 (2H, m), 3.79 (4H, t, J=8.0), 6.58 (2H, s), 7.12 (2H, s), 7.66 (1H, d, J=1.6), 8.09 (1H, dd, J=1.7,8.2), 8.32 (1H, d, J=8.2). ^{13}C NMR (101 MHz, MeOD) δ 166.84, 166.37, 161.34, 160.99, 156.78, 149.74, 142.70, 137.64, 133.14, 132.97, 131.71, 130.94, 128.93, 128.70, 127.03, 113.84, 54.19, 39.59, 39.27, 32.38, 28.85, 27.14, 26.12, 25.90, 25.66, -2.43, -3.16. HRMS (ESI) calcd for $C_{35}H_{43}N_4O_3Si$ $[M]^+$ 595.3104; found 595.3107.

SiR700-lysosome **11**

Pepstatin A (100 µl of 2.2 mM solution in DMSO, 0.22 µmol, 1.5 eq.) was treated with DIEA (5 ul, 29 µmol) and TSTU (100 µl of a 3 mM solution in DMSO, 0.3 µmol, 2.0 eq.). After 5 min, SiR700-C6-NH2 **10** (22 µl of 6.7 mM solution in DMSO, 0.15 µmol, 1.0 eq.) was added. The mixture was incubated for 1 h at r.t. The product was purified by RP-HPLC, lyophilized and dissolved in dry DMSO. 100 µl of 1.1 mM solution of **11** were obtained (73% yield) as a green solution. ^{1}H NMR (400 MHz, MeOD) δ 0.56 (3H, s), 0.61 (3H, s), 1.01-0.88 (31H, m), 1.43-1.31 (10H, m), 1.67-1.53 (8H, m), 2.17-2.01 (5H, m), 2.27 (2H, d, J=6.7), 2.43-2.33 (2H, m), 2.93 (4H, t, J=7.8), 3.25-3.11 (9H, m), 3.42-3.39 (2H, m), 3.81 (4H, t, J=8.0), 4.06-3.93 (4H, m), 4.17-4.13 (2H, m), 4.26 (1H, q, J=7.1), 6.59 (2H, s), 7.13 (2H, s), 7.46 (1H, d, J=9.4), 7.67 (1H, d, J=1.6), 7.71 (1H, d, J=9.1), 7.99 (1H, d, J=8.3), 8.11 (1H, dd, J=1.7,8.2), 8.33 (1H, d, J=8.2). ^{13}C NMR (126 MHz, MeOD) δ 174.4, 174.0, 172.8, 172.7, 172.4, 172.2, 166.7, 166.2, 156.9, 150.2, 137.6, 133.2, 133.0, 132.0, 131.1, 128.9, 128.8, 127.1, 113.9, 70.1, 69.8, 69.6, 65.1, 62.7, 59.5, 59.4, 54.2, 51.3, 50.9, 50.0, 48.2, 44.6, 40.7, 40.3, 40.0, 39.8, 39.7, 38.9, 32.3, 30.1, 30.0, 29.4, 28.9, 26.3, 26.2, 26.1, 25.9, 24.5, 24.5, 22.5, 22.4, 21.4, 21.4, 21.0, 18.7, 18.6, 17.8, 17.6, 16.7, -2.4, -3.2.HRMS (ESI) calcd for $C_{69}H_{105}N_9O_{11}Si^{+2}$ $[M^+ + H]^{+2}$ 631.8846; found 631.8840.



Supplementary Scheme 6. SiR-lysosome **13**: (a) i. TSTU, Boc-1,6-diaminohexane, DIEA, DMSO/MeCN, r.t. ii. TFA. r.t.(b) Pepstatin A**,** TSTU, DIEA, DMSO, r.t.

SiR-C6-NH2 **12**

SiR-COOH (Spirochrome Ltd. SC004) (10.4 mg, 22 µmol, 1 eq.) was dissolved in DMSO/MeCN 1:2 (0.3ml). DIEA (10 ul, 58 µmol, 2.6 eq.) and TSTU (7.9 mg, 26 µmol, 1.2 eq.) were added. After 5 min, N-Boc-1,6-diaminohexane (7.1 mg, 33 µmol, 1.5 eq.) was added. The mixture was incubated for 30 min at r.t.. The mixture was diluted with 5 ml H_2O and extracted with CH_2Cl_2 (2 x 5ml). The combined extracts were concentrated and the residue was dissolved in TFA (0.3 ml). After 10 min, the solvent was evaporated, the residue was taken up in MeCN/H_2O 1:1 (1 ml), purified by RP-HPLC and lyophilized. 12.1 mg of 17 were obtained (96% yield) as a dark blue solid. 1H NMR (400 MHz, DMSO) δ 8.75 (t, 1 H, J = 5.5 Hz), 8.07 (m, 2 H), 7.66 (s, 4 H), 7.09 (s, 2 H), 6.69 (m, 4 H), 3.23 (q, 2 H, J = 6.6 Hz), 2.97 (s, 12 H), 2.77 (m, 2 H), 1.50 (m, 4 H), 1.29 (dd, 4 H, J = 3.4 Hz), 0.65 (s, 3 H), 0.54 (s, 3 H); (LC: tR = 3.11 min); ^{13}C NMR (101 MHz, DMSO) δ 170.30, 165.90, 150.70, 141.11, 131.64, 129.34, 127.26, 124.33, 121.37, 118.46, 118.23, 115.56, 115.31, 112.66, 40.05, 30.05, 28.22, 27.27, 26.76, 1.21, 0.04; HRMS (ESI) calcd for $C_{33}H_{44}N_4O_3Si^{+2}$ $[M+2H]^{+2}$ 286.1586; found 286.1589.

SiR-pepstatin **13**

Pepstatin A (120 µl of 25 mM solution in DMSO, 3.0 µmol, 1.5 eq.) was treated with DIEA (5 ul, 29 µmol) and TSTU (36 µl of a 100 mM solution in DMSO, 3.6 µmol, 1.8 eq.). After 5 min, SiR-C6-NH2 **12** [3] (80 µl of 25 mM solution in DMSO, 2.0 µmol, 1.0 eq.) was added. The mixture was incubated for 1 h at r.t. The product was purified by RP-HPLC, lyophilized and dissolved in dry DMSO. 450 µl of 1.6 mM solution of **13** were obtained (36% yield) as a blue solution.

^{1}H NMR (400 MHz, DMSO-d6) δ 0.53 (3H, s), 0.65 (3H, s), 0.87-0.78 (30H, m), 1.26-1.18 (10H, m), 1.39-1.33 (4H, m), 1.55-1.47 (3H, m), 2.13-1.93 (9H, m), 3.02-2.96 (13H, m), 3.21 (2H, d, J=6.3), 3.83-3.79 (3H, m), 4.26-4.13 (3H, m), 6.67-6.67 (4H,

m), 7.07-7.06 (1H, m), 7.31 (1H, d, J=8.9), 7.46 (1H, d, J=8.7), 7.66 (2H, s), 7.81 (2H, m), 7.92 (1H, d, J=7.4), 8.06 (2H, m), 8.71 (1H, s). ^{13}C NMR (151 MHz, MeOD) δ 174.40, 174.08, 174.01, 172.75, 172.70, 172.48, 172.40, 172.23, 166.62, 137.98, 127.48, 119.83, 113.79, 70.12, 69.67, 59.65, 59.61, 59.56, 59.52, 59.45, 51.44, 51.36, 50.97, 50.89, 50.06, 50.03, 48.19, 44.61, 40.68, 40.33, 40.00, 39.92, 39.88, 39.71, 39.56, 38.91, 30.14, 30.02, 29.39, 28.89, 28.86, 26.26, 26.20, 26.10, 24.54, 24.47, 22.44, 22.34, 21.45, 21.41, 21.06, 18.68, 18.59, 17.77, 17.54, 16.68. HRMS (ESI) calcd for $C_{67}H_{105}N_9O_{11}Si^{+2}$ $[M^+ + H]^{+2}$ 619.8846; found 619.8826.

In vitro characterization of the probes

In vitro tubulin polymerization assay

Measurement of the enhancement of the rates of tubulin polymerization by the probes was performed using a commercial tubulin polymerization fluorescence assay kit available from Cytoskeleton, Inc. (cat. BK011P). It is based on DAPI (4',6-Diamidino-2-Phenylindole) fluorescence change upon tubulin polymerization[6]. SiR-tubulin is commercially available at Spirochrome Ltd. (cat. # SC002). All measurements were carried out according to manufacturer's recommendations using standard conditions protocol. Samples were measured in a half area 96-well plate (Greiner bio-one cat. 675076) on an Infinite M1000 spectrofluorometer (TECAN). Fluorescence emission was detected at 430 nm while exciting at 350 nm. The excitation and emission bandwidth for all measurements was set to 20 and 10 nm, respectively. During acquisition the temperature was set to 37 °C. All samples were prepared in duplicates and fluorescence was measured over 90 min (one measurement each minute) until a stable signal was achieved. The lowest value of each data set was normalized to 1. Obtained polymerization curves were analyzed using GraphPad Prism 6.0 version and fitted to a model describing plateau followed by one phase exponential association:

$$y = y_0 + (plateau - y_0) \times (1 - e^{-K \times (x - x_0)})$$

considering that x_0 is the time at which tubulin polymerization begins, y_0 is the average y value up to time x_0, plateau is the y value at infinite times and K is the rate constant.

In vitro actin polymerization and depolymerization assay

The influence of the probes on the rate of actin depolymerization was assessed using a commercial actin polymerization fluorescence assay kit available from Cytoskeleton, Inc. (cat. BK003). It is based on pyrene labeled actin fluorescence change upon depolymerization[7]. SiR-actin is commercially available at Spirochrome Ltd. (cat. # SC003). Measurements were performed in 96-well plates (Greiner bio-one cat. 655900) on an Infinite M1000 spectrofluorometer (TECAN). Fluorescence emission was detected at 420 nm while exciting at 350 nm. The excitation and emission bandwidth for all measurements was set to 20 nm and the temperature was set to 37 °C. All samples were prepared in triplicates and fluorescence was measured over 4 h (one measurement every 2 min). First, G-actin (monomeric actin) was prepared according to manufacturer's recommendation by dissolving lyophilized rabbit pyrene-labeled muscle actin in G-buffer (5 mM Tris-HCl (pH 8.0), 0.2 mM $CaCl_2$ and 0.2 mM ATP) to a final concentration of 0.4 mg/ml. Subsequently, G-actin was mixed with probes (final concentration 3 µM) and pyrene fluorescence was measured until a stable signal was achieved. G-actin polymerization was induced by the addition of actin polymerization buffer (for final buffer composition of 50 mM KCl, 2 mM $MgCl_2$, 5 mM guanidine carbonate and 1 mM ATP) and pyrene fluorescence was measured until completion of polymerization (~1 h). Depolymerization of F-actin (filamentous actin) was induced by a five-fold dilution of the samples with G-buffer and pyrene fluorescence was measured for 3 h until fluorescent signals reached a plateau. For polymerization curves the lowest value of each data set was normalized to 1 and for depolymerization curves the highest value of each data set was normalized to 1.

The compounds were transferred to the assay plates (Black Polystyrene 384-well plate, Clear Flat Bottom, Corning #3655) using an acoustic dispenser (Echo® 550 Liquid Handler, Labcyte Inc., Sunnyvale, CA). The Echo instrument transfers liquid droplets by increments of 2.5 nL to an upside down destination plate through acoustic energy.

For each compound, a range of volumes was selected to obtain the following final compounds concentrations of 50 / 25 / 12.5 / 6.25 / 3.125 / 1.5625 µM in the assay plates. The amount of DMSO in each well was completed to yield a final concentration of 0.5%.

The compounds were diluted with 20 µl of G-Buffer (Cat. # BSA01, Actin Polymerization Biochem Kit, Cytoskeleton Inc., Denver, CO), using a fluidic dispenser (Multidrop Combi, Thermo Fisher Scientific Inc.). A volume of 2 µl of Actin Polymerisation Buffer (Cat. # BSA02, Actin Polymerization Biochem Kit, Cytoskeleton Inc., Denver, CO) was pipetted in the assay plates with a Sciclone liquid handler (Sciclone ALH 3000 Advanced Liquid Handler, Caliper Life Sciences, Hopkinton, MA). Covered assay plates were then incubated for 1 hour at room temperature.

After the reaction, the compounds were further diluted with G-Buffer to a final volume of 80 µl and the assay plates centrifuged at 2000 rpm for 1 min. This was followed by an incubation of 2 hours at room temperature.

The readout was performed by measuring the fluorescence intensity signal using a multimode microplate reader (Saphire2 microplate reader, Tecan Ltd, Männedorf, Switzerland). The excitation and emission wavelengths were set at 355 nm and 415 nm repectively (both with 10 nm bandwidth).

Obtained polymerization and depolymerization curves were fitted using GraphPad Prism 6.0. Polymerization curves were fitted to the model describing "plateau followed by one-phase association": Equation shown below:

$$y = y_0 + (plateau - y_0) \times (1 - e^{-K \times (x - x_0)})$$

considering that x_0 is the time at which actin polymerization begins, y_0 is the average y value up to time x_0, plateau is the y value at infinite times and K is the rate constant. Depolymerization curves were fitted to the model describing "two-phase decay":

$$y = plateau + (y_0 - plateau) \times a \times 0.01 \times e^{-K_{fast} \times x} + (y_0 - plateau) \times (100 - a) \times 0.01 \times e^{-K_{slow} \times x}$$

considering that y_0 is the y value when x (time) is zero, plateau is the y value at infinite times, K_{fast} and K_{slow} are the two rate constants and a is the fraction of the span (from y_0 to plateau) accounted for by the faster of the two components.

Inhibition of pepsin activity by lysosomal probes.

BSA digestions with pepsin was used for the estimation of lysosome probe inhibitory potential. Reaction mix contained 1 mg / ml BSA, 1 µM pepsin and 3 µM probes in reaction buffer (50 mM $HCOONH_4$, pH 4.0). Reaction was incubated for 2h at 37°C

and stopped by adding 4x Laemmli sample buffer (Bio-rad, Cat. # 161-0747) and 5 μl of 1 M NaOH to each 400 μl of sample to balance the pH. Samples were immediately heated 95°C for 5 min and loaded to SDS-PAGE gel. Afterwards gels were stained with PageBlue protein staining solution (Thermo Scientific Cat. # 24620) to detect proteolysis extent of BSA. Experiment was repeated two times with identical outcome.

Measurements of UV absorbance spectra in water-dioxane mixtures

Solutions of 5 μM carboxy-SiR700 in water-dioxane mixtures containing 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% of dioxane (by volume) were prepared. The absorbance spectra were recorded using a SpectraMax Gemini (Molecular devices) plate reader using polypropylene 96-well pates in 5 nm steps. The spectra were integrated from 450 to 750 nm using GraphPad Prism 6.0. Normalized integrals were plotted against dielectric constant of water-dioxane mixture.

Estimation of activity of SNAP-tag substrates

Two separate solutions were prepared in the reaction buffer (80mM Na_2HPO_4, 20mM NaH_2PO_4, 100mM NaCl, 2 mM 2-merkaptoethanol, 0.1 mg/ml BSA, pH7.3): 125 μl of substrate solution and 125 μl of the SNAP-tag protein solution (for concentrations see Supplementary Table 4). Substrates were stocked as DMSO solutions and purified SNAP-tag protein was stored at -20°C in storage buffer (80mM Na_2HPO_4, 20mM NaH_2PO_4, 100mM NaCl, 50% glycerol, 2 mM 2-merkaptoethanol, pH7.3). Final reaction mix contained <1% DMSO and <5% glycerol. After mixing two initial solutions, 20 μl of aliquots were withdrawn at specific time points and supplemented with 10μl of stop buffer composed of protein electrophoresis loading dye 6x containing 5 mM O^6-benzylguanine. These aliquots are incubated for 5 minutes at 95°C before loading a gel. Time points at which reaction was stopped are the following: 60, 120, 300, 600, 1200, 1800, 2400, 3600, 7200 seconds. Finally, 20μl of each aliquot were used to load the SDS-PAGE gel. Obtained fluorescent bands were quantified using Bio-Rad Quantity One 1-D analysis software. Obtained values were plotted vs time and fitted by GraphPad Prism 6.0 using one phase association equation:

$$y = A \times (1 - e^{-K \times (x)})$$

considering that A is the amplitude of fluorescence increase and K is the rate constant. Each time course was repeated at least two times.

Estimation of fluorescence increase upon target binding

Substrate from a 300 µM DMSO stock solution was directly added to the target protein (0.4 mg/ml G-actin or 2 mg/ml monomeric tubulin), to 0.2% SDS or to a BSA solution (0.4 mg/ml or 2 mg/ml) (1:83 dilution). In case of tubulin probes, buffer containing 80 mM piperazine-N,N'-bis[2-ethanesulfonic acid] sequisodium salt, 2 mM $MgCl_2$, 0.5 mM ethylene glycol-bis(b-amino-ethyl ether) N,N,N',N'-tetra-acetic acid (pH 6.9), 1 mM GTP and 15% glycerol was used. In case of actin probes, buffer containing 5 mM Tris-HCl (pH 8.0), 0.2 mM $CaCl_2$ and 0.2 mM ATP was used. This buffer was supplemented with 50 mM KCl, 2 mM $MgCl_2$, 5 mM guanidine carbonate and 1 mM ATP to obtain F-actin. The samples prepared in 1.5 ml tubes (Eppendorf) were incubated for 2 - 3 h at 37 °C and fluorescence was measured in a half area 96-well plate (Greiner bio-one cat. 675076) on an Infinite M1000 spectrofluorometer (TECAN). Fluorescence emission was recorded from 580 nm to 850 nm while exciting at 550 nm. for SiR and from 620 nm to 850 nm while exciting at 590 nm. for SiR700 The excitation and emission bandwidth for all measurements was set to 10 nm. All samples were prepared in triplicates. Ratios $F_{(+SDS)}/F_{(+BSA)}$ or $F_{(+enzyme)}/F_{(+BSA)}$ of fluorescence signals at 667 nm (for SiR) or 716nm (for SiR700) were calculated.

LogD calculation

LogD at pH 7.5 was calculated using MarvinSketch 6.0.5 ([8], http://www.chemaxon.com). Chemical structures of the probes which were used for the calculation are displayed in Supplementary Figure 1.

Fluorescence lifetime measurements

The different free fluorescent compounds were freshly diluted in PBS pH 7.4 (Lonza) for SiR650 and SiR700 or in $HCOONH_4$ pH 4.0 for SiR650-lysosome and SiR700-lysosome to a concentration of 200 nM from concentrated DMSO stocks (final DMSO concentration: ≤ 0.02%). For SNAP-tag labeling, the labeling substrates (SiR-SNAP, SiR700-BG) were diluted to a concentration of 1 µM in PBS pH 7.4 containing 5 µM SNAP-tag protein (5 eq.). After 1 h incubation at room temperature, the eventual unreacted substrates were separated by gel filtration (illustra NAP-5 columns, GE Healthcare). The collected SNAP-tag labeled solutions were further

diluted to a concentration of 200 nM in PBS pH 7.4. For the measurements of the lysosomal probes bound to pepstatin A, the probes were diluted in $HCOONH_4$ buffer pH 4.0 to a concentration of 200 nM and mixed with 20 µM pepstatin A (100-fold excess). The samples were incubated at room temperature for 10 min before the measurement.

The fluorescence lifetime measurements were performed with a laser scanning confocal microscope (Leica TCS SP8 X) equipped with a PicoHarp 300 (PicoQuant) TCSPC module (see description of the microscopy setups) at 22±1°C.

For data acquisition and data analysis, we used the software SymPhoTime 64 (PicoQuant). Typically, the overall decay of the image (sum of the photon counts of all the pixels, N>3x10^6 photon counts) were fitted using an n-exponential reconvolution model,

$$y(t) = \sum_{i=0}^{n-1} IRF \otimes \Big|_{Bkgr_{IRF}|Shift_{IRF}} A[i] \exp\left(\frac{t}{\tau[i]}\right) + Bkgr_{Dec}$$

where the instrument response function (IRF) is calculated from the convolution integral of the model function. $Bkgr_{IRF}$ and $Shift_{IRF}$ correspond to the corrections for the IRF background and displacement and $Bkgr_{Dec}$ correspond to the correction for the decay background. A and τ correspond respectively to the pre-exponential factor and the lifetime. The goodness-of-fit was determined by the reduced chi-square (χ_R^2<1.2) using a nonlinear least-squares analysis and examining the weighted residuals trace. Free SiR650 and SiR700 were fitted with a mono-exponential fitting model, while the functionalized and protein labelled fluorophores were best fitted using a bi-exponential decay model. The intensity weighted average lifetime $\overline{\tau}$ were used to compare the different lifetimes,

$$\overline{\tau} = \frac{a_1\tau_1^2 + a_2\tau_2^2}{a_1\tau_1 + a_2\tau_2} = f_1\tau_1 + f_2\tau_2$$

where a_1, a_2 are the pre-exponential factors (amplitudes), τ_1, τ_2 are the lifetimes and f_1, f_2 are the fractional contribution of each decay time to the steady-state intensity. The determined lifetimes represented as mean of triplicate are shown in Supplementary Table 6.

Photostability measurements

NIH-3T3 cells were stained with SiR-actin, SiR700-actin, SiR-tubulin, or SiR700-tubulin (500 nM, 1,5h) washed 3 times with fresh medium and imaged on a laser scanning confocal microscope (Leica TCS SP8 X) microscope. Excitation was performed at 640nm using high laser power to enhance the bleaching rate (40% laser intensity for tubulin probes and 20% laser intensity for actin). 100 successive frames were recorded on a single plane. After background correction, the normalized intensity of each frame was plotted against the frame number.

Manipulation of cells

Preparation and maintenance of cells

Human primary dermal fibroblasts were cultured in high-glucose DMEM (Life Technologies, cat. 31053-028) supplemented with GlutaMAX-1 (Life Technologies, cat. 35050-038) 10% FBS (Life Technologies, cat. 10270-106) in a humidified 5% CO_2 incubator at 37 °C. Cells were split every 3-4 days or at confluence. Cells were seeded in glass bottom 12-well or 24-well plates (MatTek Corporation, cat. P12GC-1.0-14-F or P24G-1.5-13-F) or glass bottom 35 mm dishes (MatTek Corporation, cat. P35GC-1.5-10-C) one day before imaging.

Transduction with CellLight® Tubulin-GFP (Life Technologies, cat. C10613) and CellLight® Actin-RFP reagents (Life Technologies, cat. C10583) was performed according to manufacturer's recommendations. 5 µl per well of CellLight® reagent was added to 0.5 ml of complete DMEM medium in a 24-well plate, 10 µl per well in a 12-well plate. Afterwards, cells were grown for 24 h in a humidified 5% CO_2 incubator at 37 °C. Staining of GFP-tubulin or RFP-actin expressing cells with SiR-based 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. P3655) and 1 µg/mL Laminin (BD Bioscience, cat. 354232) coated coverslips. Neuronal cultures were maintained in NeuroBasal medium (Gibco, cat. 21103049) supplemented with 2% B27 serum-free supplement (Gibco, cat. 17504044), 2 mM L-Glutamine (Gibco, cat. 25030) and Pen/Strep (100 units/mL and 100 µg/mL respectively, BiochromAG, cat. A2213). The day after

plating 5 µM cytosine β-D-arabinofuranoside (Sigma, cat. C1768) was added to the cultures. Medium was replaced once per week.

Live-cell staining of actin in hippocampal neurons was achieved by adding the SiR-Actin or SiR700-Actin probes to the growth medium. Cells were incubated for 30-60 min in a humidified 5% CO_2 incubator at 37°C. After washing, cells were imaged in artificial cerebrospinal fluid (ACSF buffer) at RT.

Cell preparation for STED microscopy

All the used cell lines were cultured in DMEM (Life Technologies, cat. 61965-059) supplemented with 10% FCS (FCS, PAA, cat. A15-151) and pen/strep (100 units/ml and 100 µg/ml respectively, BiochromAG, cat. A2213) at 37 °C and 5% CO_2. Cells were stained with the probes at 37 °C in HDMEM (phenol red–free DMEM—Invitrogen, cat. 31053-028—buffered with 10 mM HEPES) supplemented with 10% FCS and pen/strep. Imaging was performed in HDMEM buffer with 10% FCS.

Description of microscopy setups

Wide field microscopes

For imaging GFP, RFP and SiR a Leica DMI6000B microscope equipped with a Leica HCX PL APO 100x 1.47 OIL objective and a Hamamatsu-C9100 EM-CCD camera (512 x 512 pixels) was used. The following dichroic mirror and filters were used for Hoechst 33342 signal detection: excitation BP 360/40, emission BP 470/40 and dichroic mirror at 400 nm. The following dichroic mirror and filters were used for SiR and SiR700 signal detection: excitation BP 635/30, emission BP 700/72 and dichroic mirror at 650 nm. Z-stacks with voxel size of 240 x 240 x 692 nm were acquired and images were presented as maximal intensity projections (MIP) or the best focus plane.

Confocal microscopes

Labeled samples were imaged on a Leica SP8 (Leica, Germany) inverted confocal microscope equipped with a HC PL APO CS2 63x/1.20 WATER objective. Images were taken using a 400 - 1000 Hz bidirectional scanner, a voxel size of 60 - 120 x 60 - 120 x 120 - 250 nm, a pinhole of 111.5 µm (1 AU) and frame averaging of 1 – 3. Hoechst 33342 was excited with 405 nm laser light source set to 0.1 – 0.2% and detected with regular PMT at 415 – 480 nm range. Other fluorophores were

excited with a white light laser set to the following values: 550 nm (power at 5 - 10%, RFP excitation), 633 or 640 nm (power at 10 - 20%, dual SiR and SiR700 excitation) and 670 nm (power at 2 - 5%, SiR700 excitation). Emission light was registered with Leica HyD™ detectors set to the following spectral ranges: 560 – 610 nm (RFP channel), 650 – 700 nm (SiR channel), 700 – 750 nm (SiR700 channel) and time-gating switched "on" for the interval 0.3 – 24 ns. Subsequently, deconvolution using a computed PSF was applied and the colocalization of fluorescence signals was measured (Colocalization analyzer, Huygens Essentials package).

The fluorescence lifetime measurements were performed on the same confocal microscope equipped with a PicoHarp 300 (PicoQuant, Germany) TCSPC module and with a HC PL APO CS2 63x/1.40 oil-immersion objective. The images were acquired in glass-bottom 24-well plates (MatTek Corporation, cat. P24G-1.5-13-F) using an image size of 46 x 46 µm with 256 x 256 pixels, scan speed of 400 Hz, pinhole at 1 airy unit, with a laser power adjusted to 10^5 average counts per second to avoid pile-up effects and a target photon counts of 100/pixel. The white-light laser was set respectively to 633 nm and 640 nm for SiR650 and SiR700 matching the notch filters installed on the setup. The HyD SMD detection range was set respectively to 650-750 nm and 660-795 nm for SiR650 and SiR700.

SIM microscope

The images were acquired with a commercially available setup Nikon Eclipse Ti microscope equipped with a Nikon Apo TIRF 100X/1.49 objective and 2.5 x internal magnification. 640 nm laser was used for excitation in combination with Cy5 HC filterset (AHF analysentechnik AG, cat. no.: F36-523) and Sir700 filter set 732/68 BrightLine HC (AHF analysentechnik AG, cat. no.: F39-732). The signal was detected by an EM-CCD camera Andor DU897 (image size 512 x 512 pixels). Structured illumination is produced by projecting the image of a grating block onto the image plane inducing interferences with the sample image. The SIM images are reconstructed from the raw images through the Nikon proprietary software NIS-Element. The field of view is 32 µm x 32 µm with 64 nm / pixel and 32 nm / pixel after reconstruction of SIM images.

STED microscopes

A part of the images was taken using a STED-microscope described previously[9]. The fluorophores were excited with a pulsed laser diode at 640 nm wavelength (PicoQuant, Berlin, Germany), whereas the pulsed STED beam of 775 nm wavelength originating from a frequency-doubled fiber laser (IPG Photonics, Oxford, MA) was optically modified so that the focal intensity distribution exhibited a central intensity zero, i.e. a 'doughnut'. Thus, fluorescence outside the doughnut zero was prevented by stimulated emission at 775 nm, leading to sub-diffraction sized probing areas. Using pulse energies of 3 nJ a resolution down to ~40 nm was achieved. The images were acquired by scanning the sample with a piezo stage (Mad City Labs, Madison, WI) and detecting the fluorescence with an avalanche photo diode (Micro Photon Devices, Bolzano, Italy). The SiR signal was acquired using a 660 – 720 nm detection window. A confocal (i.e. diffraction limited resolution) channel with 470 nm excitation and 500 – 550 nm detection wavelengths was implemented to additionally image GFP. The overlay of the STED and confocal channels was adjusted and verified on 100nm TetraSpeck fluorescent beads (Life Technologies).

A second STED microscope configuration was equipped with an excitation 635 nm pulsed diode laser (LDH-D-C-635 and PDL 800-B, PicoQuant, 100ps pulse width), and a titanium-sapphire mode locked laser for STED, operating at 810 nm (Mira900, Coherent). The repetition rate of the original STED pulse train was 76 MHz; the STED pulses were temporally stretched from their original 150 fs to about 120 ps using a 121 m single mode glass fiber (PMJ-A3HPC, AMS Technologies). The excitation pulses were synchronized to the STED pulses and triggered at every 7th pulse thus reducing the effective repetition rate to ~10 MHz. Detection was accomplished with avalanche photodiodes (SPCMAQR-13-FC, Perkin Elmer Optoelectronics). The focal STED doughnut was created by passing the STED beam to a polymer vortex phase plate (RPC Photonics). Both excitation and STED pulses were fed into a 1.4 NA objective lens (HCX PL APO 100X 1.40-0.70, Leica).

Alternatively, we used a commercial Leica SP8 gSTED 3X (Leica, Germany) inverted microscope equipped with a HC PL APO STED 100x/1.40 OIL objective and a 775 nm STED laser. Images were taken using a 700 – 1400 Hz bidirectional scanner, a pixel size of 10 - 30 nm, a pinhole of 151.7 µm (1 AU) and a line

averaging of 2 – 3 lines. Fluorophores were excited by a white light laser set to 633 nm. If weak fluorescence of SiR700 was observed, the 670 nm laser could be used additionally. In this system, the fluorescence signal is detected using HyD detectors operating in standard mode, with time gating intervals of 0.3 – 6 ns set to detection ranges of 650 – 694 nm and 700 – 750 nm for SiR and SiR700, respectively.

Processing and visualization of acquired images.

All acquired or reconstructed images were processed and visualized using Fiji[10]. For visualization of lysosomes of super-resolution microscopy images the “red hot” lookup table was used for. Deconvolution of STED images was performed using the Richardson-Lucy algorithm and ImSpector (Max-Plank Innovation) software. Acquired SIM images were reconstructed with Nikon Nis-Elements 4.11. 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 OriginPro 9 (http://www.originlab.com/).

General statistical analysis

All measured parameters were measured at least in duplicates and presented as mean with standard deviation or standard error range as well as sample size indicated. *In vitro* replicates are measured on different days using the same setup. Each sample measured at least in technical duplicates. Cellular imaging experiments were performed at least twice on different days. Images at several different places of cell culture were acquired. If applicable, these parameters were indicated in the figure legends of the manuscript or supplementary information. Cell staining with probes was independently reproduced by the groups of S.W.H. and K.J..

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