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SUPPLEMENTARY INFORMATION

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A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins

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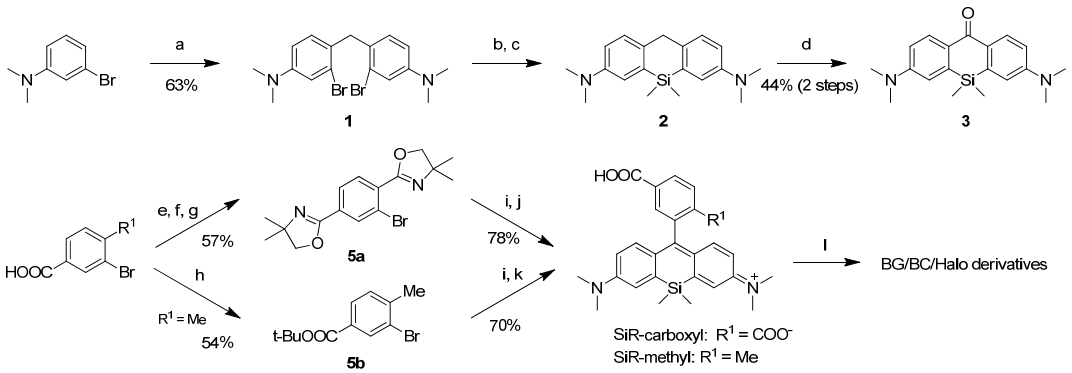
Supplementary figures, table and movies inventory

Supplementary methods.

Chemical synthesis

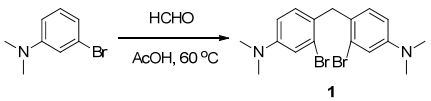
General consideration:

All chemical reagents and dry 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). Thin layer chromatography (TLC) was performed on TLC-aluminum sheets (Silica gel 60 F_{254}). Flash column chromatography was performed with Merck silica gel (230-400 mesh). ^{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 Av-500 (500 MHz for ^{1}H, 125 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 $CDCl_3$ (7.16 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), acetone-d_6 (2.05 ppm for ^{1}H, 29.84 ppm for ^{13}C), and coupling constants reported in Hz. High resolution mass spectra (HRMS) were measured on a Micromass Q-TOf Ultima spectrometer with electron spray ionization (ESI). Analytical reversed phase RP-HPLC was run on a Dionex system equipped with an UltiMate 3000 diode array detector for product visualization on a Waters Symmetry C18 column (5 µm, 3.9 x 150 mm). Buffer A: 0.1% w/v TFA in H_2O Buffer B: acetonitrile. Typical gradient was from 0% to 100% B within 30 min with 1 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% w/v TFA in H_2O Buffer B: acetonitrile. Typical gradient was from 0% to 100% B within 30 min with 4 ml/min flow.



Supplementary Scheme S1. Synthetic route of silicon-rhodamine dyes: (a) HCHO, AcOH, 60 °C, 30 min.; (b) *sec*-BuLi, THF, -78 °C; (c) Me_2SiCl_2, -78 °C to rt.; (d) $KMnO_4$, acetone, -15 °C; (e) $SOCl_2$, cat. DMF, reflux, 3 h.; (f) 2-amino-2-methylpropan-1-ol, DIEA, CH_2Cl_2, 0 °C to r.t., overnight; (g) $SOCl_2$, rt., 1.5 h.; (h) $(Boc)_2O$, DMAP, THF, reflux, overnight; (i) *tert*-BuLi, THF, -78 °C, then **3**, -78 °C to r.t.; (j) 6 N HCl aq. 80 °C, overnight; (k) 6 N HCl, 40 °C., 1 h.; (l) BG- or BC- or Halo-NH_2, PyBOP or HBTU, DIEA, DMSO, r.t.

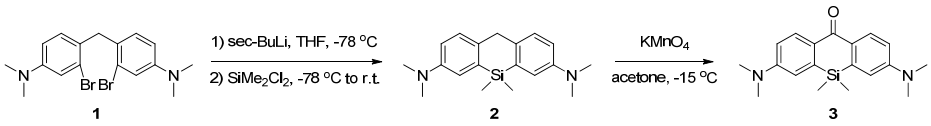
4,4'-Methylenebis(3-bromo-*N,N*-dimethylaniline) **1**



3-Bromo-*N,N*-dimethylaniline (5.00 g, 25 mmol, 2 equiv) was dissolved in 37% formaldehyde solution (5 ml) and acetic acid (40 ml), and stirred at 60 °C for 30 min. After cooling, acetic acid was evaporated, then saturated $NaHCO_3$ aqueous solution was added carefully. The aqueous phase was extracted with ethyl acetate (3 x), and the combined organic phase was washed with water and brine, dried over Na_2SO_4, then filtered and evaporated. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 90/10) to obtain 4,4'-methylenebis(3-bromo-*N,N*-dimethylaniline) **1** as a white solid. (3.24 g, 63%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (d, 2 H, *J* = 2.7 Hz), 6.85 (d, 2 H, *J* = 8.6 Hz), 6.59 (dd, 2 H, *J* = 8.6, 2.6 Hz), 4.00 (s, 2 H), 2.92 (s, 12 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.2, 130.9, 127.2, 125.7, 116.4, 112.0, 40.7, 40.0. HRMS (ESI): *m/z* calc. for $C_{17}H_{20}Br_2N_2$ 411.0071, 413.0052; found 411.0092 (5.21 ppm), 413.0056 (0.97 ppm) $[M+H]^+$

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

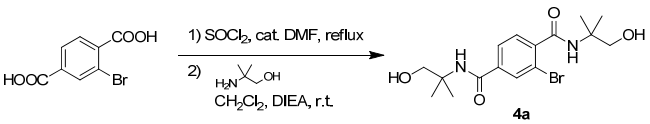


4,4'-Methylenebis(3-bromo-*N*,*N*-dimethylaniline) **1** (2.00 g, 4.85 mmol, 1 equiv) was dissolved in dry THF (200 ml) and stirred at -78 °C. *sec*-BuLi (1.4 M solution in *n*-hexane, 10 ml, 14.0 mmol, 3 equiv) was slowly added for 30 min to the solution and stirred for further 2 h at the same temperature. $SiMe_2Cl_2$ (1 ml, 8.22 mmol, 1.8 equiv) was added to the reaction mixture and stirred at room temperature for 2 h. 1N HCl aqueous solution was added carefully to neutralize the solution, and THF was evaporated. The resulting aqueous solution was extracted with EtOAc, and the organic phase was washed with saturated $NaHCO_3$ aqueous solution, water and brine, dried over Na_2SO_4, filtered and evaporated to obtain the crude including N^3,N^3,N^7,N^7,5,5-hexamethyl-5,10-dihydrodibenzo[*b*,*e*]siline-3,7-diamine **2**, which was used for the next reaction immediately due to its high sensitivity towards oxygen.

The residue was dissolved in acetone (30 ml) and stirred at -15 °C. $KMnO_4$ powder was added portionwise (6 x 300 mg) for 30 min, and stirring was continued for further 2 h at the same temperature. The purple suspension was filtered through a Celite pad, and the yellow filtrate was evaporated. The residue was purified by silica gel column chromatography (*n*-hexane/CH_2Cl_2 = 20/80) to obtain **3** as a yellow solid. (689 mg, 44%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (d, 2 H, *J* = 8.9 Hz), 6.87 (dd, 2 H, *J* = 9.0 Hz, 2.4 Hz), 6.83 (d, 2 H, *J* =2.4 Hz), 3.12 (s, 12 H), 0.51 (s, 6 H).^{13}C NMR (100 MHz, $CDCl_3$) δ 185.4, 151.5, 140.6, 131.7, 129.7, 114.4, 113.2, 40.2, -0.8. HRMS (ESI): *m/z* calc. for $C_{19}H_{24}N_2OSi$ 325.1736; found 325.1730 (-1.85 ppm), $[M+H]^+$

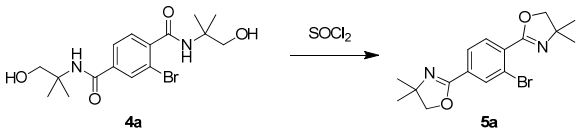
2-Bromo-N^1,N^4-bis(1-hydroxy-2-methylpropan-2-yl)terephthalamide **4a**



2-Bromoterephthalic acid (750 mg, 3.06 mmol, 1 equiv) was suspended into $SOCl_2$ (5 ml) in the presence of DMF (1 drop), and the solution was refluxed for 3 h. After cooling to room temperature, evaporation of the solvent and drying under vacuum, the residue was dissolved in CH_2Cl_2 (10 ml), and added dropwise to a solution of 2-amino-2-methylpropan-1-ol (750 mg, 8.41 mmol, 2.7 equiv) and DIEA (1.5 ml) in CH_2Cl_2 (10 ml), the solution was stirred at room temperature overnight. Saturated $NaHCO_3$ aqueous solution was added to the reaction mixture which was extracted with EtOAc (3 x). Combined organic phases were washed with water and brine, dried over Na_2SO_4, filtered and evaporated to obtain 2-bromo-N^1,N^4-bis(1-hydroxy-2- methylpropan-2-yl)terephthalamide **4a** as a white solid. (922 mg, 78%)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (d, 1 H, *J* = 1.5 Hz), 7.86 (br s, 1 H), 7.82 (dd, 1 H, *J* = 7.9, 1.7 Hz), 7.72 (br s, 1 H), 7.43 (d, 1 H, *J* = 7.9 Hz), 4.87 (t, 1 H, *J* = 6.1 Hz), 4.82 (t, 1 H, *J* = 6.1 Hz), 3.51 (d, 2 H, *J* = 6.2 Hz), 3.50 (d, 2 H, *J* = 6.2 Hz), 1.31 (s, 6 H), 1.30 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.2, 164.9, 142.3, 137.7, 131.6, 128.8, 126.9, 119.1, 67.8, 67.5, 55.8, 24.0, 23.9. HRMS (ESI): *m/z* calc. for $C_{16}H_{23}BrN_2O_4$ 389.0919; found 389.0915 (-1.03 ppm), $[M+H]^+$

2,2'-(2-Bromo-1,4-phenylene)bis(4,4-dimethyl-4,5-dihydrooxazole) **5a**

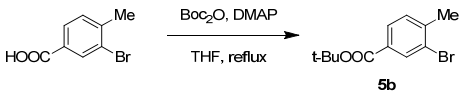


4a **5a**

2-Bromo-N^1,N^4-bis(1-hydroxy-2-methylpropan-2-yl)terephthalamide **4a** (880 mg, 2.27 mmol) was dissolved in $SOCl_2$ (4 ml) and stirred at room temperature for 90 min. After evaporation of the $SOCl_2$, saturated $NaHCO_3$ aqueous solution was added carefully to neutralize the solution. The resulting water phase was extracted with EtOAc (3 x), and the combined organic phases were washed with water and brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified by silica gel column chromatography (EtOAc/CH_2Cl_2 = 50/50 to 100/0) to obtain 2,2'-(2-bromo-1,4-phenylene)bis(4,4-dimethyl-4,5-dihydrooxazole) **5a** as a white solid. (578 mg, 73%)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.08 (d, 1 H, *J* = 1.5 Hz), 7.91 (dd, 1 H, *J* = 8.1, 1.7 Hz), 7.77 (d, 1 H, *J* = 8.0 Hz), 4.16 (s, 2 H), 4.13 (s, 2 H), 1.32 (s, 6 H), 1.30 (s, 6 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.0, 159.3, 132.7, 132.6, 132.1, 131.3, 127.3, 121.4, 79.3, 79.1, 68.7, 68.3, 28.6, 28.4. HRMS (ESI): *m/z* calc. for $C_{16}H_{19}BrN_2O_2$ 351.0708; found 351.0719 (3.13 ppm), $[M+H]^+$

tert-Butyl 3-bromo-4-methylbenzoate **5b**

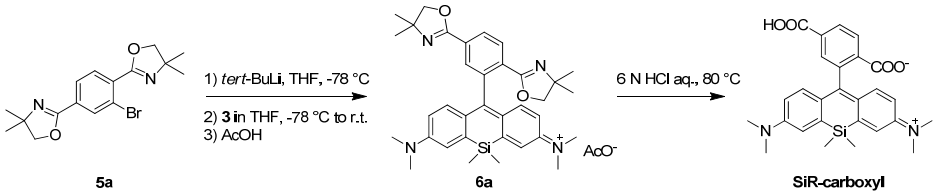


5b

3-Bromo-4-methylbenzoic acid (1.40 g, 6.51 mmol, 1 equiv), $(Boc)_2O$ (3.62 g, 16.6 mmol, 2.5 equiv) and DMAP (180 mg, 1.47 mmol, 0.2 equiv) were dissolved in dry THF (20 ml) and refluxed overnight. After cooling to room temperature and evaporation of the solvent, the residue was dissolved in Et_2O, washed with saturated $NaHCO_3$ aqueous solution, water and brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 95/5) to obtain *tert*-butyl 3-bromo-4-methylbenzoate **5b** as a colorless liquid. (951 mg, 54%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, 1 H, *J* = 1.6 Hz), 7.83 (dd, 1 H, *J* = 7.9, 1.6 Hz), 7.29 (d, 1 H, *J* = 7.9 Hz), 2.46 (s, 3 H), 1.61 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.5, 142.7, 133.3, 131.4, 130.5, 128.2, 124.6, 81.4, 28.2, 23.2.

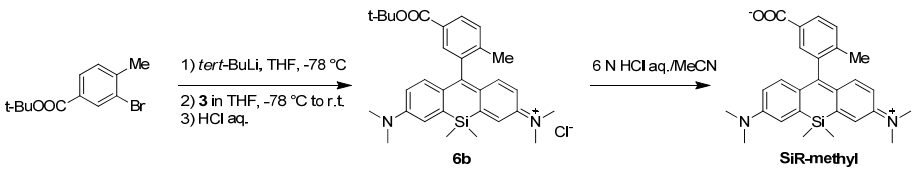
SiR-carboxyl



In an argon-flushed flask fitted with a septum cap, **5a** (150 mg, 0.427 mmol, 2 equiv) was dissolved in dry THF (5 ml) and cooled at -78 °C. *tert*-BuLi (300 µl, 0.481 mmol, 2 equiv) was slowly added dropwise and the solution was stirred at the same temperature for 1 h. Compound **3** (69.0 mg, 0.214 mmol, 1 equiv) in dry THF (5 ml) was added dropwise via a syringe at -78 °C. and the solution was warmed to room temperature and stirred for 2 h. Acetic acid (1 ml) was added to the cooled reaction mixture (ice bath), the resulting intense blue solution was evaporated and lyophilized to obtain compound **6a** as a blue solid, which was used for the next reaction without further purification. Compound **6a** was dissolved in 6 N HCl aq. (12 ml) and stirred at 80 °C overnight. After cooling to room temperature, the solution was added to saturated $NaHCO_3$ aqueous solution (50 ml) to adjust the pH (1-2), and extracted with CH_2Cl_2 (3 x). The combined organic phase was washed with 0.1 N HCl (3 x) and brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified with silica gel column chromatography (CH_2Cl_2/MeOH = 90/10) to obtain **SiR-carboxyl** as a blue solid. (79.2 mg, 78%)

^{1}H NMR (400 MHz, CD_3OD) δ 8.25 (dd, 1 H, *J* = 8.0, 1.1 Hz), 8.05 (d, 1 H, *J* = 8.0 Hz), 7.86 (d, 1 H, *J* = 1.0 Hz), 7.08 (d, 2 H, *J* = 2.8 Hz), 6.77 (d, 2 H, *J* = 9.2 Hz), 6.68 (dd, 2 H, *J* = 9.0, 2.9 Hz), 3.00 (s, 12 H), 0.68 (s, 3 H), 0.58 (s, 3 H). ^{13}C NMR (125 MHz, CD_3OD) δ 172.0, 171.6, 154.6, 149.8, 144.1, 136.7, 131.4, 129.6, 127.8, 127.3, 124.9, 124.4, 116.6, 113.5, 39.2, -0.9, -2.7. HRMS (ESI): *m/z* calc. for $C_{27}H_{29}N_2O_4Si$ 473.1897; found 473.1898 (0.21 ppm), $[M+H]^+$

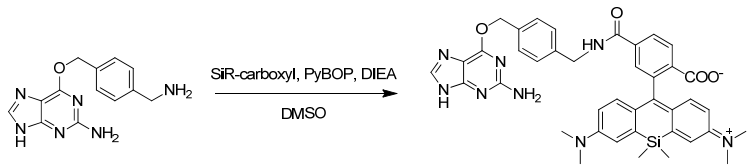
SiR-methyl



In an argon-flushed flask fitted with a septum cap, **5b** (100 mg, 0.369 mmol, 4 equiv) was dissolved in dry THF (3 ml) and cooled at -78 °C. *tert*-BuLi (250 µl, 0.401 mmol, 4 equiv) was slowly added dropwise and the solution was stirred at the same temperature for 1 h. Compound **3** (24.0 mg, 0.074 mmol, 1 equiv) in dry THF (2 ml) was added dropwise via a syringe at -78 °C, the solution was warmed to room temperature and stirred for 2 h. 0.1 N HCl aq. was added to the reaction mixture, and the resulting intense blue solution was basified with saturated $NaHCO_3$ aqueous solution, and extracted with CH_2Cl_2 (3 x). The combined organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated to obtain compound **6b** as a blue solid which was used for the next reaction without further purification. Compound **6b** was dissolved in 6 N HCl aq. (8 ml) and MeCN (2 ml) and stirred at 40 ^oC for 1 h. After cooling to room temperature, the solution was added to 0.1 N NaOH aq. (45 ml) to adjust the pH to 2-3, and extracted with CH_2Cl_2 (2 x), and the combined organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified with silica gel column chromatography (CH_2Cl_2/MeOH = 90/10) to obtain **SiR-methyl** as a blue solid. (23 mg, 70%)

^{1}H NMR (400 MHz, CD_3OD) δ 8.12 (d, 1 H, *J* = 7.3 Hz), 7.77 (s, 1 H), 7.49 (d, 1 H, *J* = 7.6 Hz), 7.39 (s, 2 H), 7.07 (d, 2 H, *J* = 9.5 Hz), 6.78 (d, 2 H, *J* = 9.6 Hz), 3.37 (s, 12 H), 2.10 (s, 3 H), 0.65 (s, 3 H), 0.63 (s, 3 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.3, 158.4, 158.1, 154.2, 147.7, 140.4, 138.6, 130.4, 130.0, 126.8, 122.0, 119.3, 116.3, 115.1, 41.0, 19.4, -0.6, -0.9. HRMS (ESI): *m/z* calcd for $C_{27}H_{31}N_2O_2Si$ 443.2155; found 443.2162 (1.58 ppm), $[M+H]^+$

SiR-SNAP

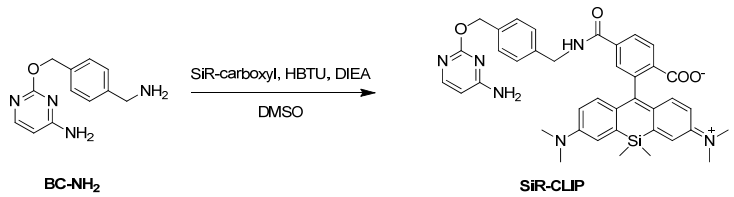


SiR-carboxyl (5.0 mg, 1 equiv), BG-NH_2 (6.5 mg 2.4 equiv)[1], PyBOP (12.0 mg, 2.4 equiv) were dissolved in dry DMSO (500 µl) in the presence of DIEA (10 µl), and the solution was stirred at room temperature for 3 h. The reaction mixture was purified by RP-HPLC to obtain **SiR-SNAP** as a blue solid. (7.0 mg, 91%)

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.36 (t, 1 H, *J* = 5.0 Hz), 8.50 (s, 1 H), 8.14 (d, 1 H, *J* = 7.6 Hz), 8.07 (d, 1 H, *J* = 7.8 Hz), 7.71 (s, 1 H), 7.49 (d, 2 H, *J* = 7.9 Hz), 7.34 (d, 2 H, *J* = 7.8 Hz), 7.05 (s, 2 H), 6.66 (m, 4 H), 5.52 (s, 2 H), 4.45 (d, 2 H, *J* = 5.2 Hz), 2.95 (s, 12 H), 0.63 (m, 3 H), 0.53 (s, 3 H). ^{13}C NMR (100 MHz, acetone-d_6) δ 169.2, 165.2, 159.9, 157.7, 155.5, 149.6, 139.6, 136.3, 131.4, 128.9, 128.8, 128.4, 128.0, 125.2, 123.1, 116.6, 113.7, 68.5,

65.2, 43.1, 39.4, 14.7, -0.6, -1.9. HRMS (ESI): *m/z* calc. for $C_{40}H_{41}N_8O_4Si$ 725.3020; found 725.3032 (1.65 ppm), $[M+H]^+$

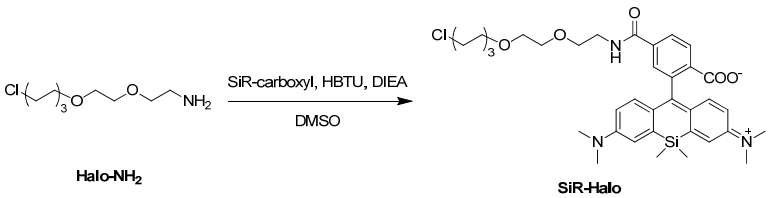
SiR-CLIP



To 0.1 ml of a 15 mM solution of SiR-carboxyl in DMSO were successively added 15 µl 0.1 M HBTU in DMSO and 10 µl DIEA. After 1 min, 15 ul of 0.1 M BC-NH_2 [2] in DMSO were added. The reaction was let 15 min at r.t. Then 50 µl H_2O were added. The reaction was purified by RP-HPLC and lyophilized. The residue blue was dissolved in 0.45 ml DMSO to obtain a 2.2 mM solution of **SiR-CLIP** (66% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.35 (t, 1 H, *J* = 5.9 Hz), 8.69 (br, s, 2 H), 8.13 (d, 1 H, *J* = 8.1 Hz), 8.06 (d, 1 H, *J* = 8.0 Hz), 7.97 (d, 1 H, *J* = 6.9 Hz), 7.70 (s, 1 H), 7.44 (d, 2 H, *J* = 8.0 Hz), 7.34 (d, 2 H, *J* = 8.0 Hz), 7.03 (s, 2 H), 6.65 (m, 4 H), 6.33 (d, 1 H, *J* = 6.9 Hz), 5.41 (s, 2 H), 4.45 (d, 2 H, *J* = 5.7 Hz), 2.93 (s, 12 H), 1.24 (s, 1 H), 0.64 (s, 3 H), 0.53 (s, 3 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.4, 165.3, 149.7, 140.2, 140.1, 136.3, 130.9, 129.3, 128.7, 128.1, 128.0, 126.0, 123.3, 116.9, 114.3, 100.3, 70.0, 69.8, 66.0, 63.1, 43.1, 40.5, 34.0, 31.8, 29.5, 29.4, 29.2, 28.9, 24.9, 22.6, 0.5, -0.8. HRMS (ESI): *m/z* calc. for $C_{39}H_{41}N_6O_4Si$ 685.2958; found 685.2963 (0.73 ppm), $[M+H]^+$

SiR-Halo

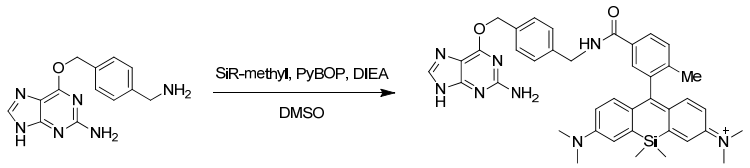


To 0.1 ml of a 15 mM solution of SiR-carboxyl in DMSO were successively added 15 µl 0.1 M HBTU in DMSO and 10 µl DIEA. After 1 min, 15 µl of 0.1 M HaloTag amine (O2) ligand (Halo-NH_2 [3]) in DMSO were added. The reaction was let 15 min at r.t. Then 50 µl H_2O were added. The reaction was purified by RP-HPLC and lyophilized. The blue residue was dissolved in 0.45 ml DMSO to obtain a 1.5 mM solution of **SiR-Halo** (45% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (t, 1 H, *J* = 5.5 Hz), 8.09 (d, 1 H, *J* = 8.0 Hz), 8.03 (d, 1 H, J = 8.1 Hz), 7.68 (s, 1 H), 7.05 (s, 2 H), 6.65 (m, 4 H), 3.58 (t, 2 H, *J* = 6.7 Hz), 3.50 (m, 4

H), 3.43 (m, 2 H), 3.38 (m, 2 H), 3.30 (t, 2 H, *J* = 6.5 Hz), 2.94 (s, 12 H), 1.65 (m, 2 H), 1.41 (m, 2 H), 1.18-1.37 (m, 4 H), 0.65 (s, 3 H), 0.53 (s, 3 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.3, 155.3, 149.7, 140.3, 136.4, 131.0, 128.7, 128.2, 127.9, 125.8, 123.2, 117.0, 114.3, 70.6, 70.6, 70.0, 70.0, 69.9, 69.9, 69.1, 69.1, 66.0, 63.1, 45.8, 45.8, 40.5, 32.5, 29.5, 29.5, 26.6, 25.4, 0.5, -0.8. HRMS (ESI): *m/z* calc. for $C_{37}H_{49}ClN_3O_5Si$ 678.3130; found 678.3134 (0.59 ppm), $[M+H]^+$

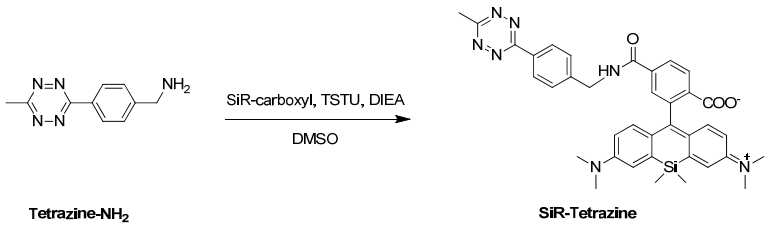
SiR(methyl)-SNAP



SiR-methyl (5.0 mg, 1 equiv), BG-NH_2 (6.5 mg 2.4 equiv), PyBOP (12.0 mg, 2.4 equiv) were dissolved in dry DMSO (500 µl) in the presence of DIEA (10 µl), and the solution was stirred at room temperature for 3 h. The reaction mixture was purified by RP-HPLC to obtain **SiR(methyl)-SNAP** as a blue solid. (3.9 mg, 50%)

^{1}H NMR (400 MHz, CD_3OD) δ 8.29 (s, 1 H), 7.99 (dd, 1 H, *J* = 8.0, 1.9 Hz), 7.67 (d, 1 H, *J* = 1.9 Hz), 7.54 (m, 3 H), 7.42 (d, 2 H, *J* = 8.2 Hz), 7.40 (d, 2 H, *J* = 2.9 Hz), 7.06 (d, 2 H, *J* = 9.6 Hz), 6.79 (dd, 2 H, *J* = 9.7, 2.9 Hz), 5.64 (s, 2 H), 4.59 (s, 2 H), 3.37 (s, 12 H), 2.12 (s, 3 H), 0.63 (s, 6 H). ^{13}C NMR (100 MHz, CD_3OD) δ 167.7, 167.6, 159.8, 154.4, 152.6, 148.1, 141.7, 140.7, 139.9, 139.5, 139.1, 134.2, 131.6, 130.3, 128.8, 127.7, 127.5, 127.3, 126.9, 120.9, 114.0, 69.2, 42.9, 39.5, 18.1, -2.6, -2.6. HRMS (ESI): *m/z* calc. for $C_{40}H_{43}N_8O_2Si$ 695.3278; found 695.3277 (-0.14 ppm), $[M]^+$

SiR-tetrazine



SiR-carboxyl (9 mg, 19 µmol, 1 equiv) was dissolved in DMSO (0.25 ml). TSTU (6.6 mg, 22 µmol, 1.15 equiv) and DIEA (10 ul, 58 µmol, 3 equiv) were successively added. After 5 min, tetrazine-NH_2 [4](0.1 M in DMSO, 0.25 ml, 25 µmol, 1.3 equiv) was added. The reaction was let 15 min at r.t. Then 50 µl H_2O were added. The reaction was purified by RP-HPLC and lyophilized. 10.6 mg (85% yield) of **SiR-tetrazine** were obtained as a blue powder.

^{1}H NMR (400 MHz, Acetone) δ 8.72 (t, 1 H, *J* = 5.8 Hz), 8.49 (d, 2 H, *J* = 8.1 Hz), 8.25 (d, 1 H, *J* = 8.0 Hz), 8.13 (d, 1 H, *J* = 8.0 Hz), 7.89 (s, 1 H), 7.65 (d, 2 H, *J* = 8.1 Hz), 7.30 (s, 2 H), 6.89 (d, 2 H, *J* = 9.0 Hz), 6.77 (d, 2 H, *J* = 9.1 Hz), 4.74 (d, 2 H, *J* = 5.8 Hz), 3.11 (s, 12 H), 3.04 (s, 3 H), 0.70 (s, 3 H), 0.59 (s, 3 H). ^{13}C NMR (101 MHz, Acetone) δ 169.11, 168.36, 166.22, 164.66, 159.33, 151.26, 144.92, 140.45, 132.13, 130.37, 129.44, 128.94, 128.55, 127.73, 125.55, 119.11, 115.19, 44.09, 40.80, 21.21, 0.07, -1.21. HRMS (ESI) calcd for $C_{37}H_{38}N_7O_3Si^+$: 656.2805; found 656.2783 (3.3 ppm), $[M]^+$.

Atto655-Halo

Atto-655 NHS ester (1 mg, 1.4 μmol, 1 equiv) was dissolved in 0.1 ml DMSO. Then Halotag-amine (O2) ligand (25 μl of a 0.1 M DMSO solution, 2.5 μmol, 1.8 equiv) was added followed by DIPEA (2 μl, 8 equiv). After 15 minutes, 10 ul H2O were added and the product was purified by RP-HPLC and lyophilized. 0.9 mg (87% yield). of **Atto655-Halo** were obtained as a blue powder. HRMS (ESI) calcd for $C_{37}H_{54}ClN_4O_7S^+$ 733.3402; found 733.3394 $[M]^+$.

Unnatural amino acids (UAAs)

trans-Cyclooctene lysine (termed as TCO) was prepared according to previously described work [5]. Cyclooctyne lysine (termed as SCO) was purchased from SiChem GmbH (Bremen, Germany).

Plasmid construction

pEBTet SNAP_GW and pEBTet GW_SNAP destination vectors were constructed from pEBTet GFP_GW and pEBTet GW respectively. pEBTet plasmids were described previously [6]. For N-terminal fusion SNAP genes were amplified by two consecutive PCR's (introducing FLAG- and His-tags at the N-terminus of SNAP-tag), digested with HindIII and KpnI and ligated into the appropriate vector. Dual expression plasmid pEBTet SNAP_GW/GFP-Centrin2 was constructed by inserting IRES (internal ribosome entry site) via R.NheI and R.SgsI restriction sites into pEBTet SNAP_GW plasmid. Afterwards myc-tag and GFP encoding sequences was inserted via R.Bpu1102I and R.SdaI restriction sites and Centrin2

gene was added using R.SdaI and R.SgsI sites leading to pEBTet SNAP_GW/GFP-Centrin2 destination vector.

Commercially available Cep41 entry clones were purchased from Genecopoeia (GC-V1653 and GC-V1653-CF). Expression clones were generated using standard LR recombination procedure as recommended by LR clonase supplier (Life Technologies). All obtained expression vectors and intermediate constructs were verified by sequencing (data available on request).

pSNAPf-H2B, pSNAP-Actin, pCLIPf-H2B and pCLIPf-Cox8A plasmids were obtained from NEB (New England Biolabs). pHalo-Actin was a kind gift from the Wymann laboratory.

CLIP-Halo fusion protein purification

E. coli Rosetta-gami(DE3)pLysS strain was electroporated with plasmid pET-51b CLIP-Halo. Single colony was picked for the expression of fusion protein. Expression has been performed overnight at 16 °C in autoinducing MagicMedia *E.coli* Expression Medium (Life Technologies) according to manufacturer recommendations. CLIP-Halo protein contained C-terminal His-tag and N-terminal Strep-tag. For the purification of the protein, Ni-NTA (Qiagen) was used according to the instructions of the supplier. Eluted protein was further purified by Strep-Tactin superflow (IBA) according to the instructions of the supplier.

Cell Culture and Transfection

U2OS cells were cultured in high-glucose DMEM with GlutaMAX-1 (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum, Life Technologies) in a humidified 5% CO_2 incubator at 37 °C. Cells were split every 3-4 days or at confluence. To generate inducible cell lines, cells were transiently transfected with appropriate expression vector at 80-90% confluence. 48 h after transfection, cells were exposed to selective medium containing 1 µg/ml puromycin (Life Technologies), which led to a substantial death of non-transfected cells over 4-6 days. After amplification of the transfected cell population under selective conditions for 4-6 days, the cells were frozen in 10% DMSO and stored at -80 °C.

Cells were seeded on the glass coverslips or glass bottom 35 mm dishes depending on imaging conditions one day before imaging. Expression of tagged proteins was induced with 0.1 µg/ml doxycycline (Sigma-Aldrich) for 1-2 days.

Transient transfection of HeLa 157 [7] was performed using Lipofectamine™ LTX with PLUS™ reagent (Life Technologies) according to manufacturer recommendations: 2 µg of DNA was mixed with 200 µl of OptiMEM I (Life Technologies) and 1.5 µl of PLUS™ reagent (Life Technologies). Solution was incubated for 5 min at room temeprature. Then 4 µl of Lipofectamine™ LTX was added, mixed and incubated for 15 min at room temperature. Prepared DNA-Lipfectamince complex was added to a glass bottom 35 mm dish (Mattek) with HeLa 157 cells at 50-70% confluence covered with 1ml of OPTI-MEM I. After 6 h incubation in a humidified 5% CO_2 incubator at 37 °C the medium was changed to a fresh high-glucose DMEM with GlutaMAX-1 (Life Technologies, Carlsbad, CA) supplemented with 10% FBS. The cells were incubated for 2 days before imaging.

Fixed cell labeling

SNAP-tagged Cep41 expressing U2OS cells were grown on glass coverslips (0.17 µm thickness, 22 mm diameter). After removal of growth medium, cells were rapidly extracted with a pre-warmed to 37 °C BRB80 buffer (80 mM K-PIPES, pH 6.8, 1 mM $MgCl_2$; 1 mM EGTA, 0.2% NP-40) for 30 s, fixed for 3-10 min in -20˚C methanol, washed in PBS (phosphate buffered saline, Lonza) and blocked for 60 min with 1% BSA (bovine serum albumin, Sigma-Aldrich) in PBS. Afterwards, SNAP-tagged proteins were labeled by incubating with 0.3 µM substrate (SNAP-Surface® 549 (NEB) or SiR-SNAP) in PBS containing 1% BSA for 1h at room temperature. Excess of the dye was removed by washing three times for 5 min with PBS containing 0.05% TX-100 reduced (Sigma-Aldrich).

The samples were incubated with primary antibodies at 4 °C overnight. Then samples were washed three times and incubated with secondary antibodies for 1 h at room temperature.

All antibodies were diluted in PBS with 1% BSA before addition to the samples. Primary antibody used in this study was mouse anti-α-tubulin (dilution 1:500, clone DM1A, Sigma-

Aldrich). Secondary antibodies used in this study (dilution 1:1000 – 1:500) were: goat anti-mouse coupled to Alexa 488 (Life Technologies), Alexa647 (Life Technologies) or Atto647N (Active Motif).

Long-term wide field microscopy of living cells

SNAP-Cep41 expression in U2OS cells was induced for 48 h with 0.1 µg/ml of doxycycline growing in glass bottom 35 mm dish (Mattek). During data acquisition cells were placed in DMEM^{gfp} media (Evrogen) containing 10% FBS supplemented with 0.1 µg/ml of doxycycline, 1 µg/ml of puromycin and 2.5 µM SiR-SNAP. Imaging was performed using Leica DMI6000B microscope equipped with Hamamatsu-C9100 EM-CCD camera and HCX PL APO 100.0x1.47 OIL objective. Imaging settings were: excitation time step of 4 min, single plane exposure time 150 ms, voxel size 230 x 230 x 692 nm (z-stack of 23 steps). Standard Cy5 filter set was used. Images were processed with Fiji (http://fiji.sc/wiki/index.php/Fiji) to obtain MIP (maximum intensity projections).

Dye bleaching under confocal microscope laser illumination

Bleaching of the fluorophores was measured on methanol fixed U2OS cells expressing SNAP-Cep41. SiR-SNAP staining was combined with primary mouse anti-α-tubulin and secondary anti-mouse-Alexa488 co-staining. SNAP-Surface® 549 staining was combined with primary mouse anti-α-tubulin and secondary anti-mouse-Alexa647, anti-mouse-Atto647N co-staining. Data were acquired on Zeiss LSM 710 (Zeiss, Germany) upverted confocal microscope equipped with Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Images were taken with pixel size 22 x 22 nm, pinhole 30 µm (0.5-0.6 airy unit), zoom 24x, averaging of 8 at maximum framing speed. 75 frames were acquired. 633 nm laser was set at 3% (measured 36 µW arriving to objective) and PMT detection interval was set to 640-758 nm. In addition 488 nm (1%, measured 19 µW coming to objective) and 561nm (1%, measured 51 µW coming to objective) lasers were switched on for reference structure visualization as well as to simulate conditions of three color imaging. All samples

were mounted in 86% glycerol with 4% n-propyl-gallate in PBS. Acquired data analyzed using Fiji (http://fiji.sc/wiki/index.php/Fiji) and Time Series Analyzer plug-in (http://rsbweb.nih.gov/ij/plugins/time-series.html). Florescence decrease on microtubule-like structure was measured. At least three different cells were measured and obtained curves were averaged. Data presented as average value ± standard deviation.

Comparative living cells near-infrared substrate staining

Transient transfection with plasmids (SNAP-Actin, SNAPf-H2B, CLIPf-H2B or Halo-Actin) of U2OS was performed using Lipofectamine™ 2000 (Life Technologies) according to manufacturer recommendations. Afterwards cells were grown for 48 h in a humidified 5% CO_2 incubator at 37 °C. SNAP-tag, CLIP-tag and Halo-tag were labeled using 2.5 μM substrates for 1 h at 37 °C in DMEM supplemented with 10% FBS. Hoechst 33342 counterstaining (0.1 μg/ml) was applied together with substrates. Next, cells were washed 2 times for 5 min with HBSS (Lonza) and once with DMEM supplemented with 10% FBS for 1h at 37 °C. Afterwards, cells were labeled using 1 μM SNAP-Cell® Oregon Green® for 1 h at 37 °C in DMEM supplemented with 10% FBS (Life Technologies). Finally, cells were washed 2 times for 5 min with HBSS (Lonza), once with DMEM supplemented with 10% FBS for 1h at 37 °C and imaged on Leica DMI6000B microscope equipped with Hamamatsu-C9100 EM-CCD camera and HCX PL APO 100.0x1.47 OIL objective. Observations were summarized in **Supplementary Table S1**.

Measurements of UV absorbance spectra

Solution of 2.5 μM SiR-SNAP substrate in TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) or TBS containing 0.1% SDS or ethanol was prepared. Additionally, solution of 2.5 μM BG-TMR substrate in ethanol was prepared. Absorbance spectra were recorded using a SHIMADZU UV spectrophotometer UV-1800 and standard 1cm quartz cuvettes.

Measurements of UV absorbance spectra in water-dioxane mixtures

Solutions of 10 μM SiR-SNAP, BG-6-TMR, SiR-carboxyl, Sir-Methyl and 6-carboxy-TMR in water-dioxane mixtures containing 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90% of dioxane (by volume) were prepared. The absorbance spectra were recorded using a SHIMADZU UV spectrophotometer UV-1800 using 1cm quartz cuvettes. The spectra were integrated from 440 to 650 nm for BG-6-TMR and 6-carboxy-TMR and from 530 to 750 nm for SiR-SNAP, SiR-carboxyl and Sir-Methyl using the software Mathematica. Normalized integrals were plotted against dielectric constant of water-dioxane mixture [8].

In vitro kinetics experiments

Enzymes were diluted in TBS buffer with 0.1 mg/ml BSA to the desired concentration (250 nM for SNAP-tag, 500 nM for CLIP-tag and 100 nM for HaloTag). The reactions were started by the addition of a threefold molar excess of a substrate (as a 50-100 μM solution in DMSO). At each time point, 10 μl of reaction mixture were withdrawn and immediately mixed with 10 μl stop buffer (2x SDS loading buffer supplemented with 150 μM O^6-benzylguanine) pre-heated to 95 °C. The samples were analyzed on a 16% SDS-PAGE gel and in-gel fluorescence was recorded on a Pharos-FX (Biorad) scanner. Fluorescent bands intensity was quantified using Quantity one (Biorad) and plotted against time. Data were fitted to a single exponential equation and labeling rate k was calculated using the formula: $k = \ln 2/(t_{1/2} * c_{tag})$ $M^{-1} \cdot s^{-1}$ where $t_{1/2}$ is the half-life time of labeling and c_{tag} is the concentration of protein tag.

Fluorescence properties of silicon-rhodamine substrates

30 μM stock solution of appropriate substrate in DMSO was directly added into 1500 nM enzyme solution (1:60 dilution) in DMEM or no enzyme containing TBS buffer with 0.1 mg/ml BSA. The samples prepared in 1.5 ml tubes (Eppendorf) were incubated for 2-3 h at 37 °C and fluorescence was measured in a 96-well plate on an Infinite M1000 spectrofluorometer (TECAN). Fluorescence emission detected at 675 nm while exciting at 650 nm. Both the excitation and emission bandwidth for all measurements was set to 10 nm. All samples were

prepared in duplicates and fluorescence was measured over 1-2 h (one measurement each minute) until a stable signal was achieved. Then 20% SDS solution (dilution 1:40) was added to a final concentration of 0.5% and the reading of fluorescence continued for another 1-2 h (one measurement each minute) until signal is stabilized. The last 10 readings of stabilized signals were averaged. Ratios $F_{(+SDS)}/F_{(-SDS)}$ or $F_{(+enzyme)}/F_{(-enzyme)}$ were calculated ($F_{(+SDS)}$ – fluorescence intensity in TBS with 0.5% SDS, $F_{(-SDS)}$ – fluorescence intensity in TBS, $F_{(+enzyme)}$ - fluorescence intensity in DMEM with enzyme and $F_{(+enzyme)}$ - fluorescence intensity in DMEM)

Determination of quantum yield

Fluorescence excitation and emission spectra were recorded on a SREX Fluorolog-3 (Model FL-3–11, Horiba Jobin Yvon, Kyoto, Japan) at 25 ˚C. The instrument was equipped with a R2658P photomultiplier tube (Hamamatsu Photonics, Shizuoka, Japan) as a fluorescence detector. Absolute fluorescence quantum yields were recorded on a Quantaurus QY C11347-12 [9] (Hamamatsu photonics, Shizuoka, Japan) at room temperature.

Relative quantum yield of reacted substrates

1000 nM – 2000 nM substrate was incubated with 1.1-2-fold molar excess of SNAP-tag or CLIP-Halo fusion protein in PBS (measured pH 7.35). To minimize aggregation of substrate in water solution, reactions were started by adding 50-100 μM DMSO stock solutions directly into enzyme solution (1:50 - 1:100 dilutions). Reaction was incubated for 3 h at 37 °C and afterwards 4 twofold serial dilutions were prepared. Absorbance spectra were recorded using SHIMADZU UV spectrophotometer UV-1800 in the 200-800 nm interval with 1 nm step and 1 nm fixed bandwidth. Afterwards fluorescence of the same samples was measured in 96 well plate on an Infinite M1000 spectrofluorometer (TECAN). Both the excitation and emission bandwidth for all measurements were set to 10 nm. The spectra were recorded with a step size of 3 nm. The excitation wavelength was set to 600 nm and the emission spectra were recorded in the 620-750 nm interval. Absorbance at 650 nm versus fluorescence intensity at 668 nm for substrates and absorbance 646 nm versus

fluorescence intensity at 665 nm for a free dye were plotted. The ratio of the obtained slope values of reacted substrates to the free dye (SiR-carboxyl) slope value multiplied by measured quantum yield of free dye gave relative quantum yield reported in **Supplementary Table S1**.

STED nanoscope setup description

The STED nanoscope was equipped with a 640 nm laser (≈ 80 ps pulse width, LDH-P-640B, PicoQuant, Berlin, Germany) and a 485 nm laser (≈ 80 ps pulse width, LDH-P-485B, PicoQuant) for excitation of silicon-rhodamine and GFP fluorescence, respectively. The STED beam was provided by a Titanium:Sapphire laser system (MaiTai, Spectra-Physics, Mountain View, CA) operating at 770-780 nm with a repetition rate of 80 MHz. The pulse timing of both lasers was adjusted using a home-built electronic delay unit, where the STED pulses served as the trigger master. The STED laser pulses were stretched to a pulse length of approximately 250-350 ps using a 30 cm optical SF6 glass rod and a 120 m long polarization maintaining single-mode fiber (AMS Technologies, Munich, Germany). Fluorescence excitation and collection was realized using an oil immersion objective (HCXPLAPO *NA* = 1.4, Leica Microsystems). The laser beams were spatially overlaid and the fluorescence light filtered by appropriate (dichroic) filters (AHF Analysentechnik, Tübingen, Germany). The doughnut-shaped focal intensity distribution of the STED beam featuring a central zero was produced by introducing a phase-modifying plate (RPC Photonics, Rochester, NY) into the beam path, imprinting a helical phase ramp ($\exp(i\varphi)$ with $0 \le \varphi \le 2\pi$) onto the wave front. A $\lambda/4$-plate ensured circular polarization of the STED and of the excitation beam. Precise positioning of the laser foci in the sample and sample scanning was realized by a digital beam scanning unit (Yanus, Till-Photonics, Gräfeling, Germany) for lateral directions and a piezo scanning stage (NanoMax-TS Stage, Thorlabs, Newton, USA) for the axial direction. The fluorescence was descanned, split into two channel for silicon-rhodamine and GFP fluorescence detection using a dichroic mirror (AHF Analysentechnik), and coupled into two multi-mode fiber splitters (Fiber Optic Network

Technology, Surrey, Canada) with an aperture size corresponding to 1.4× the magnified excitation spot. The fluorescence signal was then detected by single-photon counting modules (avalanche photo diode SPCM-AQR-13-FC, Perkin Elmer Optoelectronics, Fremont, CA) and the collected fluorescence counts were recorded by a high-speed data acquisition board (NI PCIe-6259 Analog I/O+Counter, National Instruments).

GSDIM/STORM imaging setup description

STORM imaging was performed on a Zeiss Observer D Inverted microscope using a 100x 1.46 objective (Zeiss) and continuous illumination from a laser 640 nm wavelength (Cube 640 nm-100C, Coherent), with a power at the sample of ~1 kW/cm^2. The filters used were a T660lpxr dichroic (Chroma) and a ET700/75 (Chroma) emission filter. To increase stability, the microscope is equipped with a piezo objective scanner (N725, PI) and a linearly encoded sample stage (PRIOR).

Preparation of fixed cells for GSDIM/STORM imaging

Cos7 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS, and were prepared the same way as for live imaging. After staining, the cells were fixed in Methanol containing 5 mM EGTA (Sigma-Aldrich) and imaged in a buffer consisting of PBS containing 50 mM Dithiothreitol (Sigma-Aldrich), 0.5% Mowiol (Sigma-Aldrich) and 2.5 mM 3,4-Dihydroxybenzoic acid (Sigma-Aldrich) (pH 8.0). 10.000 frames were recorded at 30 frames per second.

U2OS cells were prepared the same way as for live imaging, but fixed in 4% paraformaldehyde (Alpha Aesar) after staining, and imaged in the same buffer as tubulin samples. 10.000 frames were recorded at 30 frames per second.

Site-specific labeling of genetically encoded unnatural amino acids with SiR-tetrazine

Recombinant protein expression in E. coli

The previously reported plasmids pEvol tRNA^{Pyl}/PylRS^{AF} and pBAD GFP^{39TAG} were used in this study to express GFP$^{TAG\rightarrow UAA}$ genetically encoding the respective UAAs [5,10]. Briefly,

plasmids were co-transformed into *E. coli* Top10 (Life Technologies, Carlsbad, USA) and grown at 37°C in the presence of ampicillin and chloramphenicol. For small scale expression, typically 0.25 ml of an overnight culture was used to inoculate 25 ml Terrific Broth (TB) medium in a shake flask. Cultures typically grew within 1 – 2 h to an OD of 0.2 – 0.3 at which point the compounds TCO and SCO (stock solution 80 mM in 0.1 M NaOH) were added to a final concentration of 1 mM. pEvol $tRNA^{Pyl}$/$PylRS^{AF}$ containing *E. coli* cultures expressing wild type GFP (termed as GFP^{WT}) in presence of 1 mM TCO served as a negative control in all experiments. The cultures were allowed to grow until OD 0.4 – 0.6, when expression was induced with 0.02% arabinose. Cultures were harvested by centrifugation after 6 – 8 h of shaking at 37˚C. Pellets were resuspended in a 1x phosphate buffered saline (PBS, pH 7.4) solution, incubated at 4°C in the dark in order to wash out excess UAAs for 1 h, and centrifuged. Pellets were frozen at –20°C till next day.

Investigation of general suitability of SiR-tetrazine for labeling in E. coli

Cell pellets of cells expressing either GFP^{WT}, $GFP^{TAG→TCO}$, or $GFP^{TAG→SCO}$ were resuspended in 1x PBS and the OD was adjusted to ~4. A total volume of 400 µl of cell suspension (OD ~4) was then incubated with 20 µM SiR-tetrazine (10 mM stock solution in DMSO) at 37°C in the dark shaking for 10 min. Then cells were centrifuged and pellets washed twice with 1x PBS. Subsequently, cells were loaded on a SDS PAGE gel for whole cell analysis. The gel was analysed for fluorescence using a Pharos FX fluorescence scanner (Bio-Rad, München, Germany) by exciting the sample at λ= 635 nm and detecting the emission with an 695 nm band-pass filter. Afterwards, the gel was stained with Coomassie Brilliant Blue and scanned.

Live cell imaging

Cell pellets of cells expressing either GFP^{WT}, $GFP^{TAG→TCO}$, or $GFP^{TAG→SCO}$ were resuspended in 1x PBS and the OD was adjusted to ~4. A total volume of 400 µl of cell suspension (OD ~4) was then incubated with 20 µM SiR-tetrazine (10 mM stock solution in DMSO) at 37°C in the dark shaking for 10 min. Then cells were centrifuged and pellets washed twice with 1x PBS before overnight incubation at 4 °C in 1x PBS containing 5% DMSO to wash out excess

dye. Cells were washed again twice with 1x PBS before before cells were allowed to settle on a coverslip. Cells were then mounted on a Leica SP2 confocal microscope employing a 1.4NA 63x oil objective (Leica, Mannheim, Germany). Images containing 512 x 512 pixels were acquired at a scan speed of 400 Hz and a zoom factor of four yielding a final pixel size of 116.3 nm x 116.3 nm. In addition to a differential interference contrast (DIC) image the sample was sequentially excited using a 488 nm and 633 nm laser, while simultaneously recording the fluorescence signal in the green (500 – 550 nm) or red (655 – 720 nm) channel, respectively.

SUPPLEMENTARY FIGURES AND TABLES

Tables

Supplementary Table S1. Comparison of properties of near infrared fluorophores and their derivatives.

Dye name	Class of dye	$\lambda_{abs}/\lambda_{em, max}$ [nm]	ε_{max} [$M^{-1} cm^{-1}$]	QY	Tested Derivatives*	Background	Intracellular labeling over background	No-wash imaging	References
Dy-615	Cyanine	621/641	200'000	-	BG	Low	Very weak	No	This work
Atto 620	Carbopyronine	619/643	120'000	0.5	BG	Low	Very weak	No	This work and [11]
Atto Rho14	Rhodamine	625/646	140'000	0.8	BG	High	None	No	This work
Atto 633	Carbopyronine	629/657	130'000	0.64	BG	High	Very weak	No	This work and [11]
Atto 647N	Carbopyronine	644/669	150'000	0.65	BG, Halo	High	None	No	This work and [11]
Alexa 647	Cyanine	650/665	239'000	0.33	BG, BC	Low	Very weak	No	This work and [12,13]
Dy-651	Cyanine	656/678	220'000	-	BG	High	Very weak	No	This work
Atto 655	Oxazine	663/684	125'000	0.3	BG, Halo	Low	Very weak	No	This work and [11]
Dy-675	Cyanine	674/699	180'000	-	BG	Low	Very weak	No	This work
Dy-682	Cyanine	690/709	140'000	0.2	BG	High	Very weak	No	This work
SiR-methyl	Si-Rhodamine	648/662	100'000	0.39	BG	High[#]	weak[#]	No	This work
SiR-carboxyl	Si-Rhodamine	645/661	100'000	0.39	BG, BC, Halo	Low[#]	Strong[#]	Yes	This work

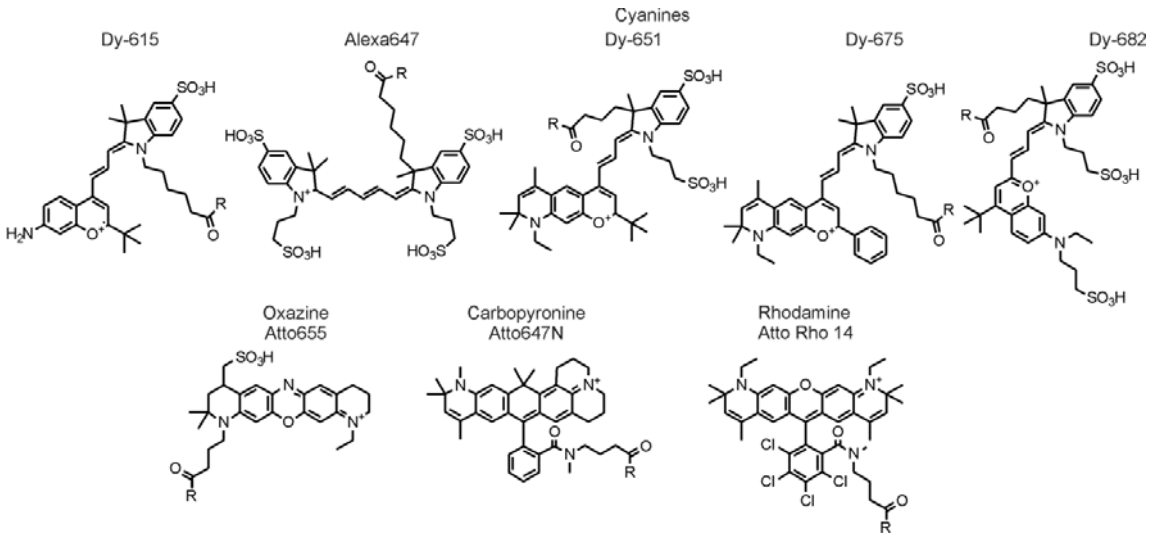
Abbreviations: $\lambda_{abs}/\lambda_{em, max}$– absorption and emission maxima wavelength, QY – quantum yield, BG - benzylguanine, BC – benzylcytosine and Halo – haloalkane. Spectral properties of fluorophores were obtained from the web sites of the following companies: ATTO-TEC (http://www.atto-tec.com), Life Technologies (http://www.lifetechnologies.com) and Dyomics (http://www.dyomics.com). * All BG substrates were tested with nuclear localized SNAP-tag ; Atto647N and Atto 655 were also tested with SNAP-Actin. Halo-tag substrates were tested with Halo-Actin. BC derivatives were tested with nuclear localized CLIP-tag. For details of experiments see page 15 of SI. [#] See Supplementary Figure 2 for representative images.

Supplementary Table S2. Properties of silicon-rhodamine and TMR substrates.

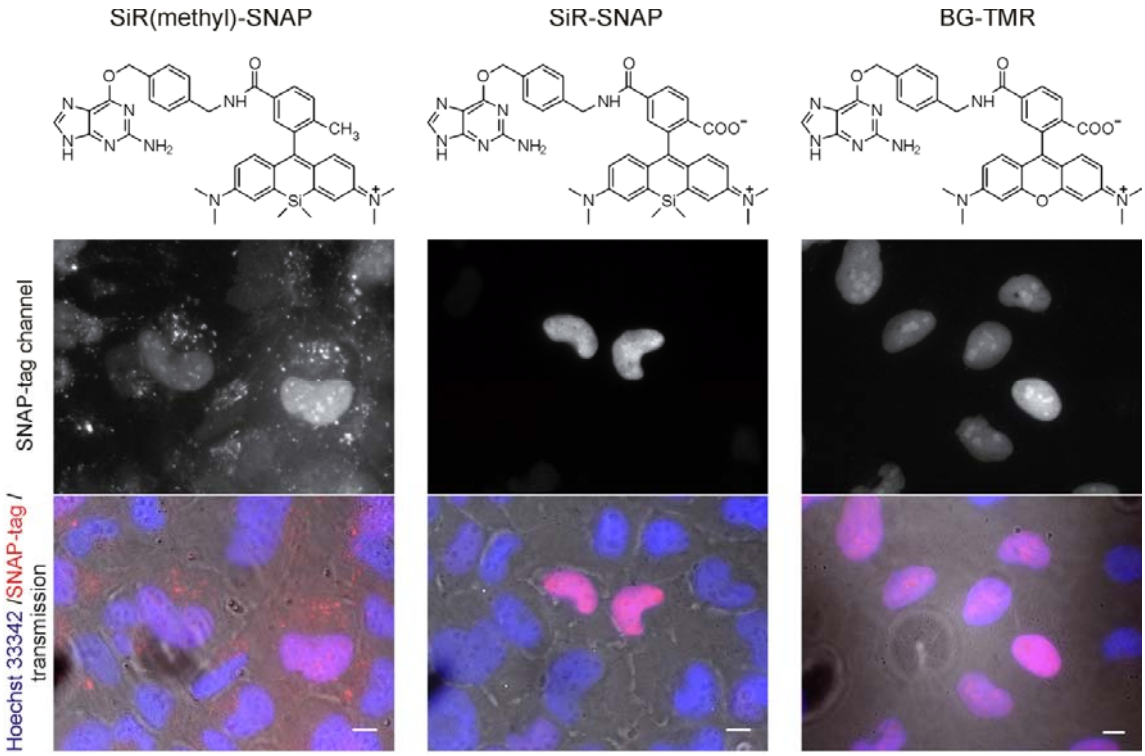
Substrate name	Rate constant for reaction with tag (M^{-1} s^{-1})	*Absorption maximum* (nm)	Emission maximum (nm)	Quantum yield
BG-TMR	42'000	N/d	N/d	0.39* [13]
SiR-SNAP	20'000	650*	668*	0.30*
SiR-CLIP	1000	652*	668*	0.46*
SiR-Halo	>250'000	648*	668*	0.39*
SiR-carboxyl	-	645**	661**	0.39**
SiR-methyl	-	648**	662**	0.39**

*** Values of protein-bound probe;.** - Values of free probe. N/d - not determined.**

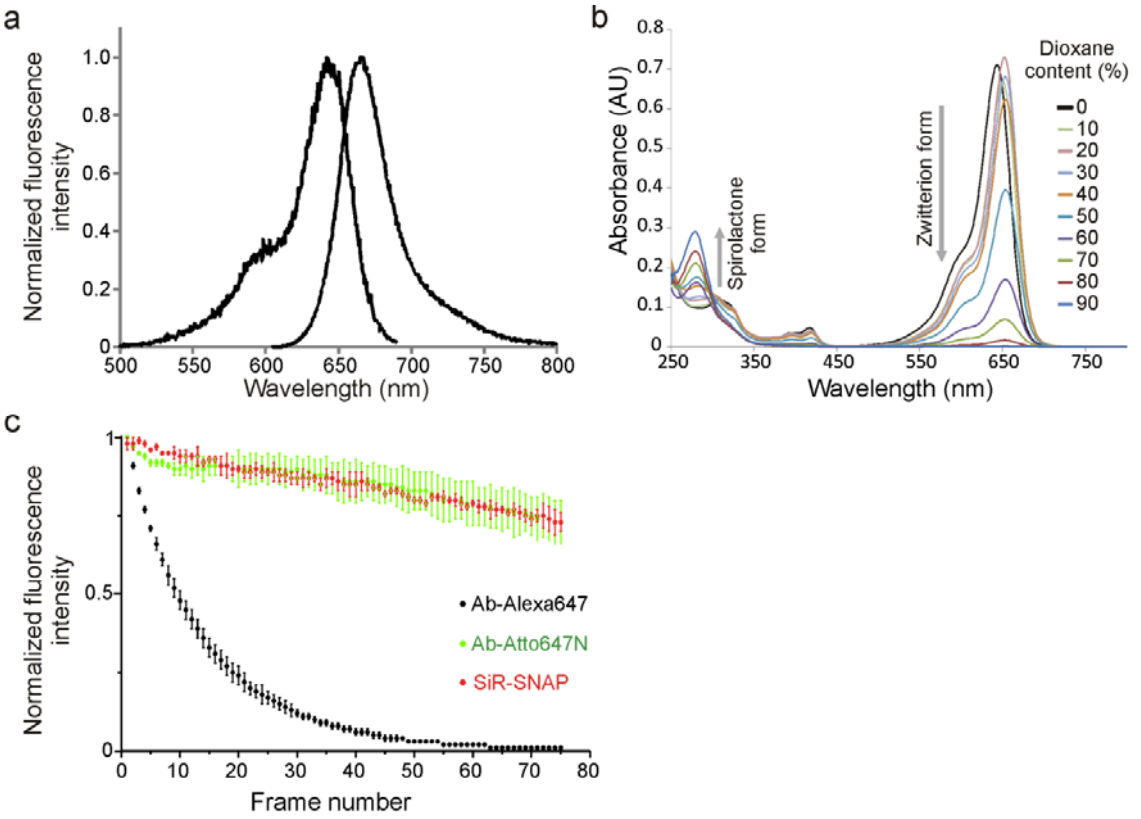
FIGURES



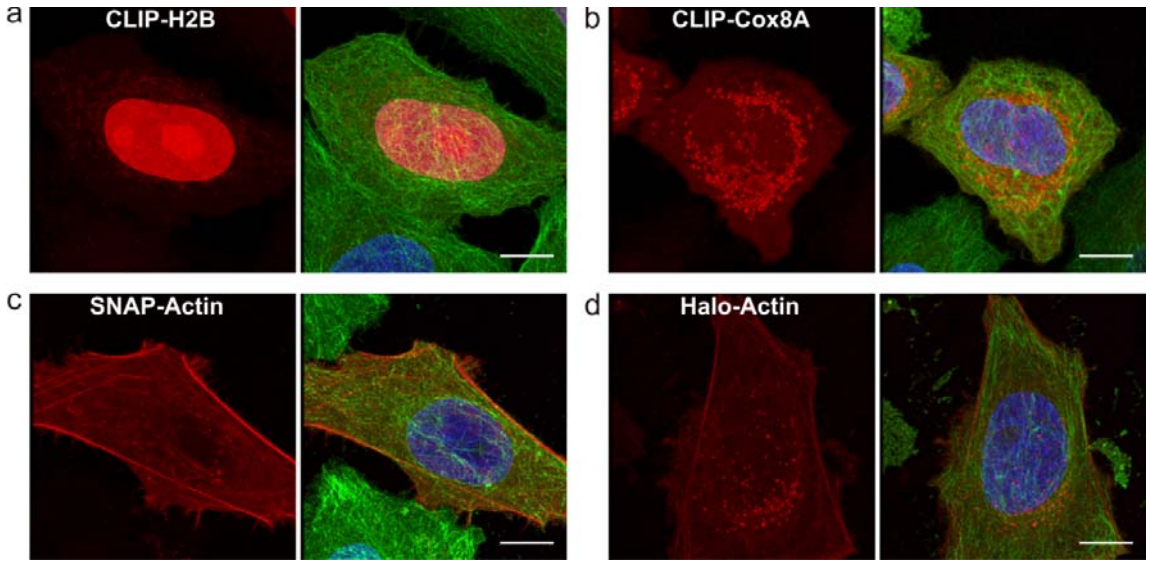
Supplementary Figure S1. Structures of the fluorophores listed in **Supplementary Table S1**. R: site of attachment for substrate for protein labeling. Structures of fluorophores were obtained from the web sites of the following companies: Life Technologies (http://www.lifetechnologies.com) and Dyomics (http://www.dyomics.com). Structure of Atto 655 is described in [14], structure of Atto647N in [15] and structure of Atto Rho14 in [16].



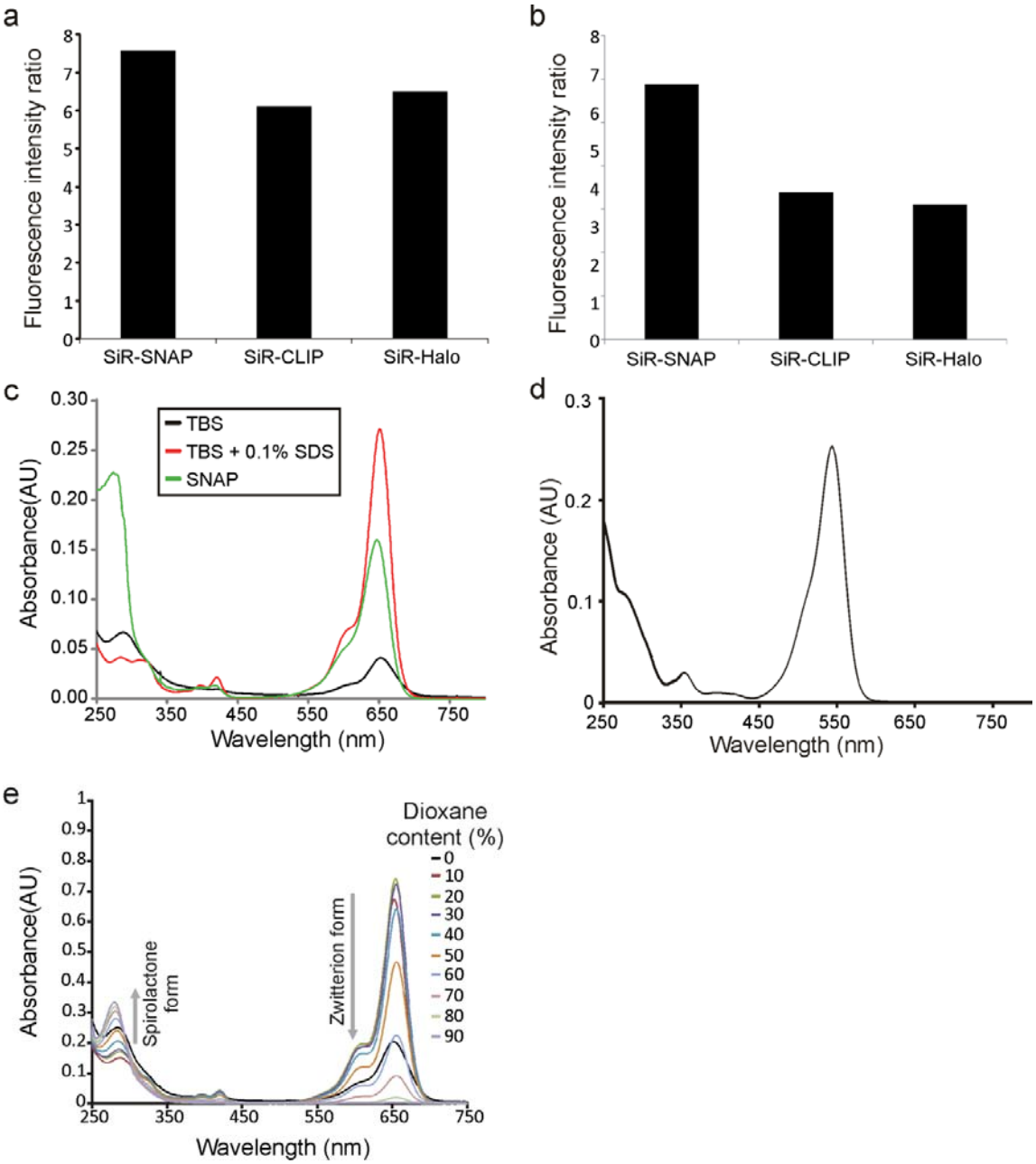
Supplementary Figure S2. Staining performance of SiR(methyl)-SNAP, SiR-SNAP and BG-TMR substrates. Live U2OS cells expressing nuclear localized SNAP-tag construct were stained with the substrates (0.3 - 0.5 µM 60 min in DMEM + 10% FBS) and Hoechst 33342. Imaging was performed after washing two times with HBSS (5 min) and one time DMEM + 10% FBS (60 min) at 37°C. Scale bar 10 µm.



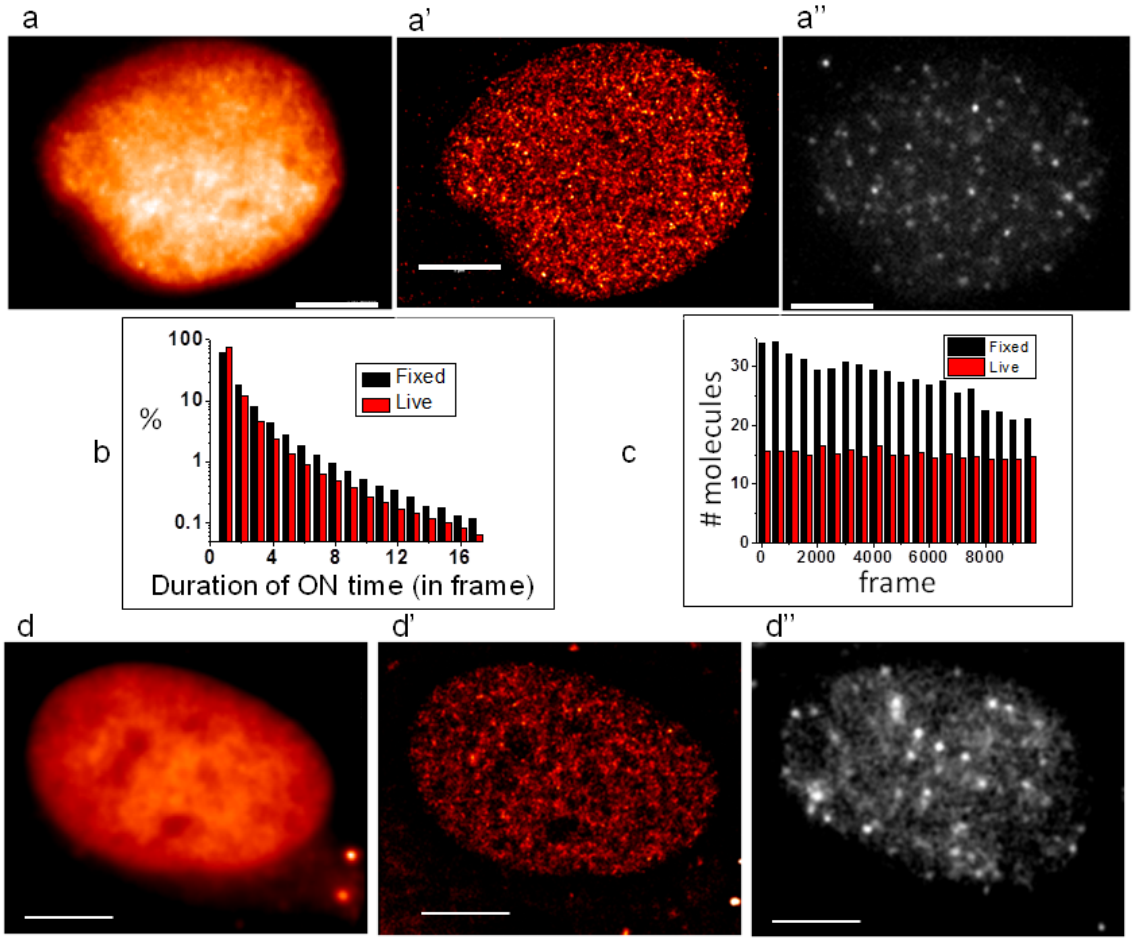
Supplementary Figure S3. Properties of silicon-rhodamine. (**a**) Excitation and emission spectra of SiR-carboxyl. (**b**) Changes of absorbance spectrum of SiR-carboxyl (7 μM) in mixtures of dioxane and water. Note, absorbance peak is shifted in dioxane mixtures compared to water as a result of fluorophore interaction with dioxane. (**c**) Relative photostability of silicon-rhodamine labeled SNAP and antibodies labeled with Alexa647 or Atto647N.



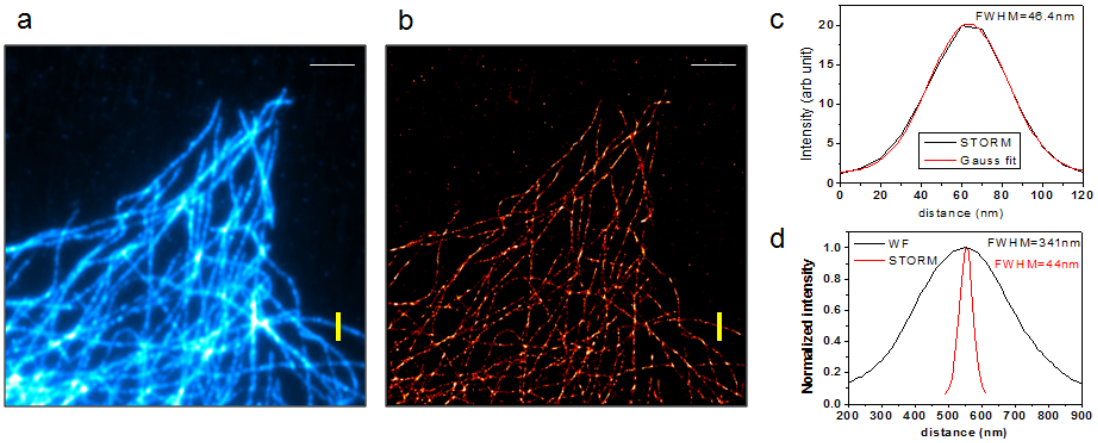
Supplementary Figure S4. Three color confocal fluorescence microscopy of SNAP, CLIP and Halo-tagged proteins (red) in living HeLa cells expressing EGFP-α-tubulin (green) and H2B-mCherry (blue) [7]. Note that substrate was not washed before imaging (2.5 μM SiR-SNAP, 6.8 μM SiR-CLIP and 1.5 μM SiR-Halo). Z-stacks were deconvolved using Huygens Essentials package and presented as MIP. Scale bar 10 μm.



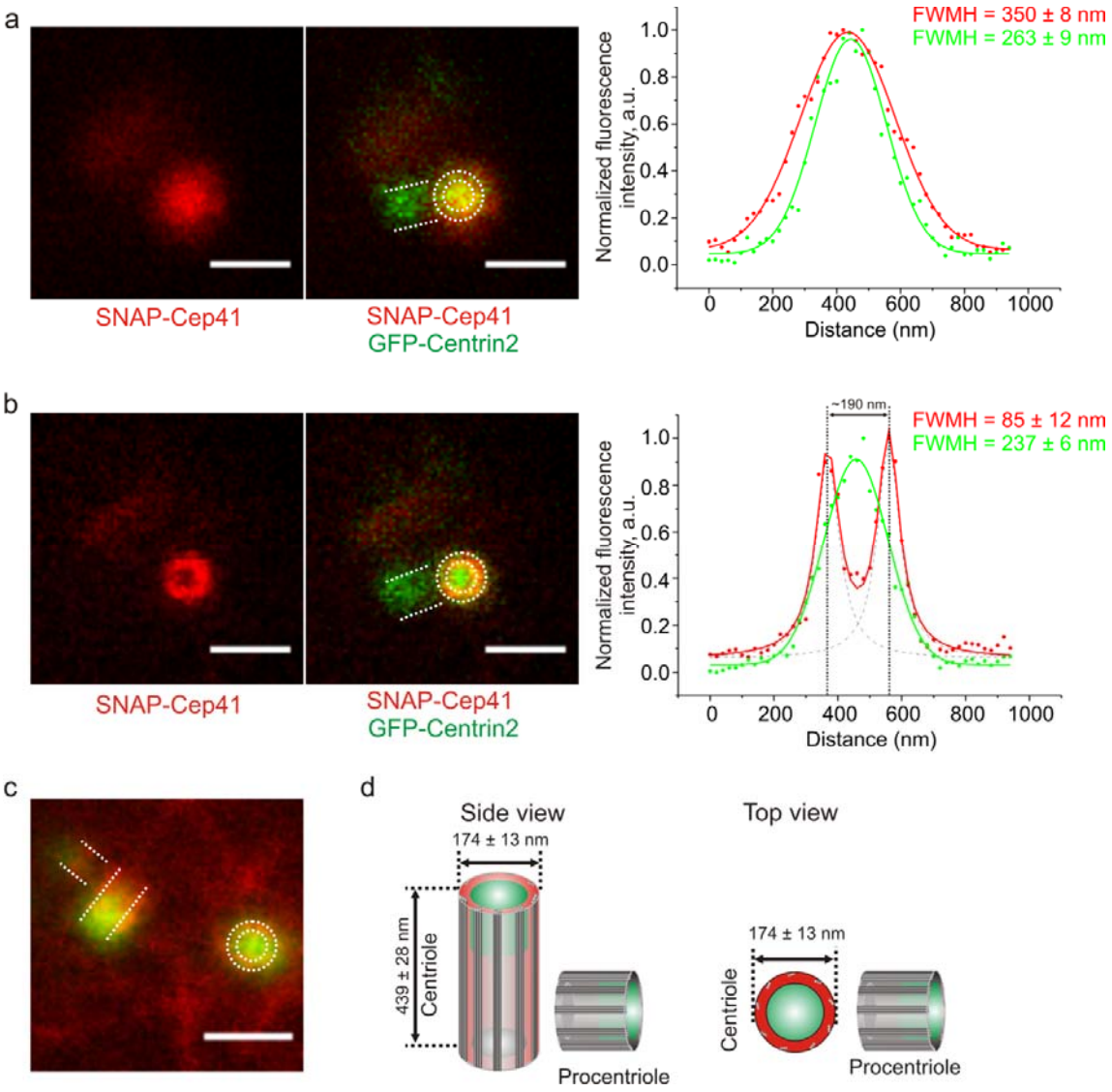
Supplementary Figure S5. Comparison of silicon-rhodamine (SiR) and tetramethylrhodamine (TMR) properties. (**a**) Fluorescence intensity increase upon conjugation with corresponding tag in DMEM with 10% FBS. (**b**) Fluorescence intensity increase upon addition of 0.5% SDS to TBS with 0.1 mg/ml BSA. (**c**) SiR-SNAP (2.5 μM) absorbance spectrum change upon addition of 0.1% SDS to TBS or reaction with SNAP-tag (threefold excess). (**d**) Absorbance spectrum of 2.5 μM BG-TMR in ethanol. (**e**) Changes of absorbance spectrum of SiR-SNAP (7 μM) in mixtures of dioxane and water. Note absorbance at 0% of dioxane is significantly different due to aggregation of SiR-SNAP.



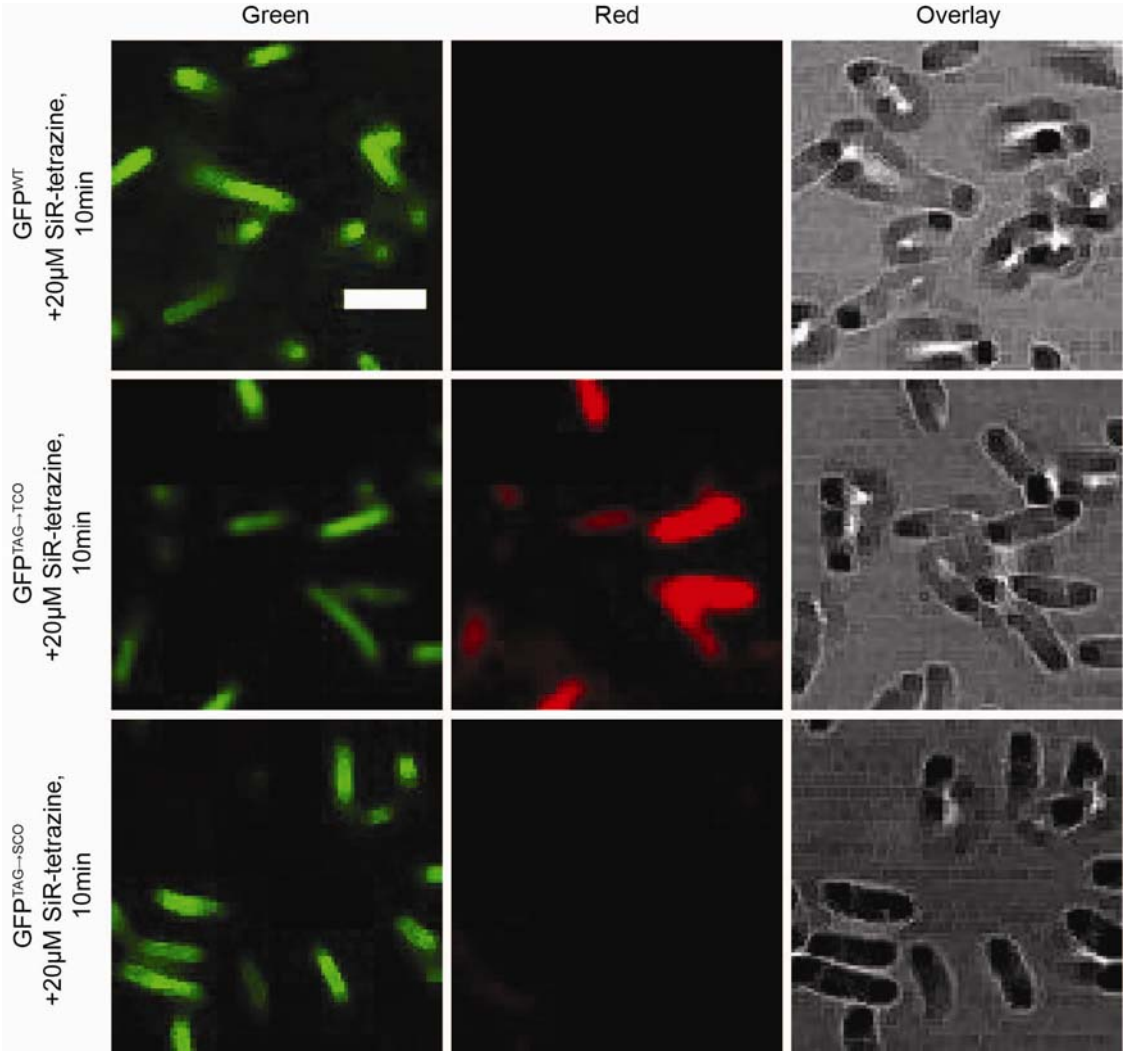
Supplementary Figure S6. Comparison between live cell and fixed cell imaging of SNAP-H2B stained with SiR-SNAP. (Top) - fixed cell imaging with (**a**) wide-field image, (**a'**) GSDIM/STORM image and (**a''**) single frame from the raw GSDIM/STORM data. (**b**) distribution of ON times of individual SiR-SNAP molecules for live imaging and on fixed cells. (**c**) Number of individual molecules localized per frame, showing sustainable blinking in both cases. (bottom) – live cell imaging: (**d**) widefield image, (**d'**) GSDIM/STORM image, and (**d''**) single frame. Scale bar 5 μm. Imaging buffer for fixed cells is PBS containing 50 mM DTT, 0.5% PVA and 2.5 mM PCA (pH 8.0).



Supplementary Figure S7. Fixed cell GSDIM/STORM imaging of SNAP-tubulin. (**a**) Widefield image and (**b**) corresponding GSDIM/STORM image. (**c**) Intensity profile on the single microtubule shown in yellow, and Gaussian fit with a FWHM of 46.4 nm. (**d**) Comparison between the resolution of the widefield image and GSDIM/STORM image. Scale bar 5 μm. Imaging buffer is PBS containing 50 mM DTT, 0.5% PVA and 2.5 mM PCA (pH 8.0)



Supplementary Figure S8. Combination of single color confocal and single color STED imaging of Cep41 protein localization in fixed U2OS cells. (**a**) Two-color confocal image. (**b**) Combination of single color confocal (GFP) and single color STED (SNAP) imaging. SNAP-Cep41 (red) expressing cells stained with SiR-SNAP substrate and centrosomal marker GFP-Centrin2 (green) localizing to distal end of centriole. Centriole walls are marked by white dashed lines in the images. Intensity profiles are plotted in the right panels. The full width at half maximum diameter (FWHM) of the imaged structures was obtained by fitting fluorescence intensity profiles to Gauss or Lorenz distributions (OriginPro 8.1, http://www.originlab.com/). Two separated Lorenz distributions are indicated by grey dashed lines in case of STED profile fitting. Distance between peaks of double Lorenz fitting was taken as diameter of the structure. (**c**) Single color confocal and single color STED image of differently oriented centrosome enables to estimate its length. (**d**) Schematic presentation of the centrosome structure: Cep41 localization indicated in red, Centrin2 localization indicated in green. Scale bar 500 nm.



Supplementary Figure S9. Site-specific labeling of genetically encoded unnatural amino acids (UAAs) with SiR-tetrazine. Live *E. coli* cells expressing GFP^{WT} (first row), $GFP^{TAG→TCO}$ (second row), and $GFP^{TAG→SCO}$ (third row; as additional control compared to $GFP^{TAG→TCO}$ of **Figure 6** in the main text) were incubated for 10 min with 20 μM SiR-tetrazine at 37°C. After washing, cells were imaged for green (first column) or red (second column) fluorescence (third column: overlay of both channels with DIC image). While GFP fluorescence was observed for all samples, red fluorescence originating from covalently reacted SiR-tetrazine was detected exclusively for $GFP^{TAG→TCO}$ (second row) even though $tRNA^{Pyl}/Pyl^{AF}$ and TCO were present in GFP^{WT} (first row) expressing cells. For $GFP^{TAG→SCO}$ (last row) no substantial red fluorescence above background was observed and thus, no labeling occurred. Scale bar 5 μm.

Movies

Supplementary Movie S1. Long-term imaging of SNAP-Cep41 expressing U2OS cell in the presence of 2.5 µM SiR-SNAP. Scale bar 5 µm. Time lapse clearly shows SNAP-Cep41 localization to the microtubules and midbody during cell division.

Supplementary Movie S2. Dynamic live-cell SiR-SNAP STED imaging: 5x5 μm^2 time lapse STED images of Cep41-SNAP expressing live U2OS cells. SNAP-tag was stained with 2.5 µM SiR-SNAP substrate in H-DMEM (Life Technologies) + 10% FBS for 30 min at room temperature. 5-10 min before imaging medium was replaced to H-DMEM + 10% FBS without substrate. Scale bar 500 nm. Time as indicated in seconds.

Supplementary references

1 Keppler, A. *et al.* A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat Biotechnol* **21**, 86-89, (2003).

2 Gautier, A. *et al.* An engineered protein tag for multiprotein labeling in living cells. *Chem Biol* **15**, 128-136, (2008).

3 Los, G. V. *et al.* HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol* **3**, 373-382, (2008).

4 Yang, J., Karver, M. R., Li, W., Sahu, S. & Devaraj, N. K. Metal-catalyzed one-pot synthesis of tetrazines directly from aliphatic nitriles and hydrazine. *Angew Chem Int Ed Engl* **51**, 5222-5225, (2012).

5 Plass, T. *et al.* Amino acids for Diels-Alder reactions in living cells. *Angew Chem Int Ed Engl* **51**, 4166-4170, (2012).

6 Bach, M. *et al.* Fast set-up of doxycycline-inducible protein expression in human cell lines with a single plasmid based on Epstein-Barr virus replication and the simple tetracycline repressor. *FEBS J* **274**, 783-790, (2007).

7 Held, M. *et al.* CellCognition: time-resolved phenotype annotation in high-throughput live cell imaging. *Nat Methods* **7**, 747-754, (2010).

8 Åkerlöf, G. & Short, A. O. The Dielectric Constant of Dioxane—Water Mixtures between 0 and 80°. *J Am Chem Soc* **58**, 1241–1243, (1936).

9 Suzuki, K. Quantaurus-QY: Absolute photoluminescence quantum yield spectrometer. *Nature Photonics* **5**, (2011).

10 Plass, T., Milles, S., Koehler, C., Schultz, C. & Lemke, E. A. Genetically encoded copper-free click chemistry. *Angew Chem Int Ed Engl* **50**, 3878-3881, (2011).

11 Stohr, K. *et al.* Quenched substrates for live-cell labeling of SNAP-tagged fusion proteins with improved fluorescent background. *Anal Chem* **82**, 8186-8193, (2010).

12 Campos, C., Kamiya, M., Banala, S., Johnsson, K. & Gonzalez-Gaitan, M. Labelling cell structures and tracking cell lineage in zebrafish using SNAP-tag. *Dev Dyn* **240**, 820-827, (2011).

13 Keppler, A., Arrivoli, C., Sironi, L. & Ellenberg, J. Fluorophores for live cell imaging of AGT fusion proteins across the visible spectrum. *Biotechniques* **41**, 167-170, 172, 174-165, (2006).

14 Zilles, A., Arden-jacob, J., Drexhage, K.-H., Kemnitzer, N. & Hamers-schneider, M. SULFONIC ACID DERIVATIVES OF POLYCYCLIC DYES USED FOR ANALYTICAL APPLICATIONS. DE20031029860 (2005).

15 Kolmakov, K. *et al.* A Versatile Route to Red-Emitting Carbopyronine Dyes for Optical Microscopy and Nanoscopy. *European Journal of Organic Chemistry*, 3593-3610, (2010).

16 Arden-jacob, J., Drexhage, K.-H., Hamers-schneider, M., Kemnitzer, N. & Zilles, A. CARBOXAMIDE-SUBSTITUTED DYES FOR ANALYTICAL APPLICATIONS. DE20021059374 (2004).