Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Protein-Specific Localization of a Rhodamine-Based Calcium-Sensor in Living Cells

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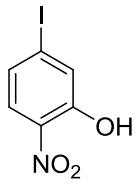
General methods.

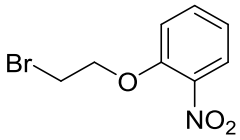
Unless otherwise stated, all the chemicals were purchased from commercial sources and used without any further purification. HO_2CCH_2-[PEG-Halo] was synthesized according to a procedure described in the literature.[1] Reactions were monitored by using thin layer chromatography (0.2 mm silica 60 F_{254} plates, POLIGRAM SIL) and the desired products were purified by either flash chromatography with normal phase silica gel 60 or on a semipreparatory HPLC system (phenomenex Luna C_{18}, 15 x 250 mm, Agilent 1100 series HPLC system, equipped with a diode array detector and eluting with a gradient of 0.1 % TFA in water and 0.1 % TFA in MeCN at a flow rate of 5 mL per minute). NMR spectra were recorded on a Varian Mercury Plus 300 and 500 MHz spectrometer at 25 °C. NMR spectra for RhoCa-Halo and RhoCa-Halo were recorded on a BRUKER AVANCE III 600 MHz spectrometer at 25 °C. Compounds were dissolved in deuterated solvents (Sigma Aldrich and Euriso Top GmbH). The chemical shifts (δ in ppm) were referenced to the solvent signal according to Fulmer *et al*.[2] The assignment of the chemical shifts was performed by 2D NMR measurements (HSQC, COSY and HMBC). For the high resolution mass measurements, a mass spectrometer (Bruker microTOFQII ESI-system) was used and internal calibration (ESI Tunemix) was applied. Measurements were performed within the range of 200-3000 m/z and deconvoluted (maximum entropy deconvolution). Calculated molecular weights refer to the m/z values given by the DataAnalysis software (Bruker Daltonics). Primers were purchased from Integrated DNA Technologies, Inc. Fluorescence scans of the SDS-gels was performed with a Typhoon 9400 imaging system (Amersham Biosciences) and proteins were visualized by Coomassie Stain (Coomassie Brilliant Blue).[3] Calcium titration was performed with Calcium Calibration Buffer Kits (life Technologies) and recorded on a Jasco FP-6500 fluorometer. The quantum yields[4] were obtained by measuring the absorption and emission spectra at defined concentrations of the evaluated fluorophores (at solutions with defined concentrations of free calcium ions ranging from $[Ca2+]_{free}$ = 39 µM and 0,07 ± 0,01 % at $[Ca2+]_{free}$ = 0 µM)) and the reference Rhodamine B (in methanol). The intensity at the emission maxima was plotted against the maximal absorption. The relative quantum yields were obtained by dividing of the slope (reference) through the slope of the rhodamine derivatives and multiplied with the quantum yield of Rhodamine B known from the literature.[5]

Synthetic procedures.



5-iodo-2-nitrophenol. The synthesis was performed according to a method described in the literature with minor changes.[6] 3 g (13.6 mmol) 3-iodophenol was dissolved in 12 mL concentrated acetic acid. A solution of 690 µL (15 mmol) HNO_3 (65 % w/w) in 12 mL concentrated acetic acid was given dropwise to the reaction mixture while stirring at 0 °C. The mixture was then allowed to stir another 30 min at room temperature and was poured into an ice water mixture. Extraction was performed with DCM, the combined organic solvents were dried over $MgSO_4$ and evaporated under reduced pressure. A yellow powder was obtained after purification with silica gel (1:4 ethyl acetate:cyclohexane). Yield: 341 mg (1.29 mmol, 10 %)

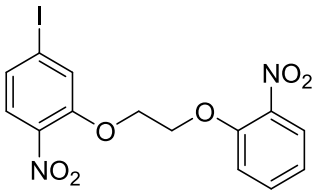
^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C): δ = 10.54 (s, 1H), 7.78 (d, *J* = 8.9 Hz, 1H), 7.61 (d, *J* = 1.8 Hz, 1H), 7.35 (dd, *J* = 8.9, 1.8 Hz, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ = 154.9, 137.7, 129.9, 129.4, 125.8, 105.5. MS (HR-ESI$^-$) m/z 263.9174 (calculated for $[C_6H_3INO_3 - H]^-$ 263.9163).



1

1-(2-bromoethoxy)-2-nitrobenzene.[7] 10 g (71.9 mmol) 2-Nitrophenol, 20 g (143.8 mmol) K_2CO_3 and 135 g (719 mmol, 62.23 mL) 1,2-dibromoethane were dissolved in 215 mL MeCN and refluxed overnight. The mixture was filtered and the precipitate was washed with DCM until it turned colorless. The volatile compounds of the filtrate were removed under reduced pressure. The residue was suspended in hot methanol followed by filtration and removal of the volatile compounds of the filtrate. The residue was then recrystallized in a 1:1 mixture of diethyl ether and cyclohexane to afford the product as slightly yellow crystals. Yield: 14.89 g (60.5 mmol, 85 %).

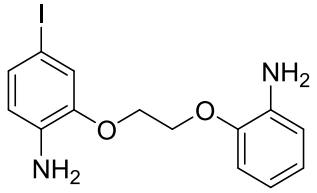
^{1}H-NMR (300 MHz, $CDCl_3$ 25 °C): δ = 7.82 (dd, *J* = 8.4, 1.7 Hz, 1H), 7.53 (ddd, *J* = 8.5, 7.5, 1.7 Hz, 1H), 7.15 – 7.01 (m, 2H), 4.41 (t, *J* = 6.4 Hz, 2H), 3.66 (t, *J* = 6.4 Hz, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ = 151.3, 134.1, 125.7, 121.4, 115.3, 69.6, 28.0. MS (HR-ESI)$^{+}$ 267.9579 (calculated for $[C_8H_8BrNO_3 + Na]^{+}$ 267.9580).



2

4-iodo-1-nitro-2-(2-(2-nitrophenoxy)ethoxy)benzene. 1 g (3.78 mmol) **5-iodo-2-nitrophenol** and 972 mg (7.04 mmol) K_2CO_3 were dissolved in 3 mL DMF and stirred for 30 min at 45 °C. 982 mg (3.78 mmol), **1** was added to reaction mixture and stirred at 130 °C overnight. The mixture was poured into an ice water mixture and the precipitate was filtered and washed with methanol. The product was obtained as a beige powder. Yield: 1.365 g (3.14 mmol, 84 %).

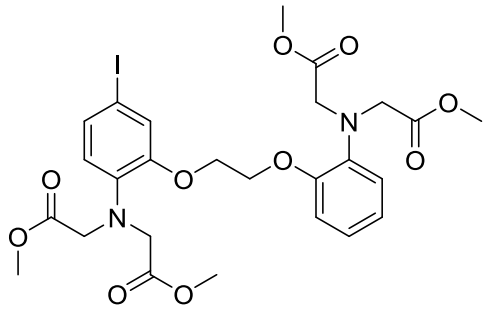
^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.84 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.61 – 7.53 (m, 3H), 7.45 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.22 (dd, *J* = 8.4, 1.2 Hz, 1H), 7.09 (ddd, *J* = 8.4, 7.5, 1.2 Hz, 1H), 4.52 (q, *J* = 1.8 Hz, 4H). ^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ = 152.1, 151.9, 137.7, 134.4, 130.8, 126.8, 125.8, 125.2, 121.7, 116.2, 100.8, 69.1, 68.6. MS (HR-ESI^{+}) m/z 452.9553 (calculated for $[C_{14}H_{11}IN_2O_6 + Na]^{+}$ 452.9554).



3

2-(2-(2-aminophenoxy)ethoxy)-4-iodoaniline. 185 mg (0.43 mmol) **2** were suspended in 2.32 mL ethanol. 1.48 mL concentrated HCl and 227 mg (1.91 mmol) tin powder was added and the suspension refluxed for 1-2 h until the mixture became clear. After cooling to room temperature the solution was poured into ice water and the pH was adjusted 9 with concentrated KOH solution in water. The white precipitate was filtered, dried under vacuum and used without any further purification for the next step. Yield: 159 mg (0.43 mmol, quantitative).

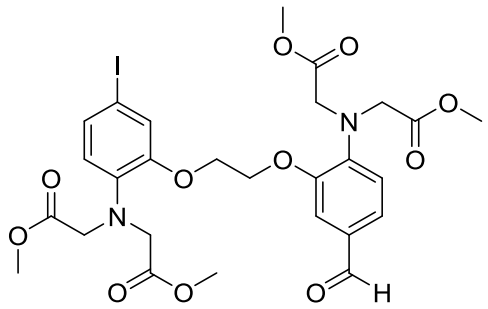
^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.11 (m, 1H), 6.90-6.68 (m, 6H), 4.38-4.27 (m, 4H), 3.87 (s, 4H). ^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ = 147.1, 146.4, 146.2, 136.8, 136.7, 130.7, 122.1, 121.2, 118.6, 116.8, 115.6, 112.6, 78.1. MS (HR-ESI$^+$) m/z 393.0093 (calculated for $[C_{14}H_{15}IN_2O_2 + Na]^+$ 393.0070).



4

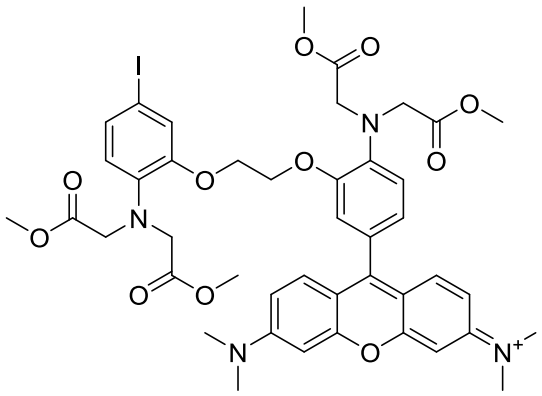
dimethyl 2,2'-((2-(2-(2-(bis(2-methoxy-2-oxoethyl)amino)-5-iodophenoxy)ethoxy)-phenyl)-azanediyl)diacetate. 159 mg (0.43 mmol) **3**, 480 mg *N,N,N′,N′*-Tetramethyl-1,8-naphthalenediamine (2.23 mmol), 50 mg (0.33 mol) NaI and 0.245 mL (2.59 mmol) methyl bromoacetate were dissolved in 1 mL dry MeCN. The mixture was refluxed overnight and filtered. The organic solvents were evaporated under reduced pressure. The product was obtained after purification with silica gel (gradient of 1:2 to 1:1 ethyl acetate:cyclohexane) as a colorless powder. Yield: 79.22 mg (0.12 mmol, 28 %).

^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.17 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.11 (d, *J* = 2.0 Hz, 1H), 6.96-6.79 (m, 4H), 6.55 (d, *J* = 8.4 Hz, 1H), 4.30-4.20 (m, 4H), 4.15 (s, 4H), 4.11 (s, 4H), 3.59 (s, 6H), 3.55 (s, 6H). ^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ = 172.0, 171.7, 151.1, 150.3, 139.4, 139.4, 130.5, 122.4, 121.9, 121.7, 120.6, 119.1, 113.3, 83.8, 67.52, 67.00, 53.4, 53.3, 51.8, 51.8. (HR-ESI$^+$) m/z 681.0920 (calculated for $[C_{26}H_{31}IN_2O_{10} + Na]^+$ 681.0916).



5

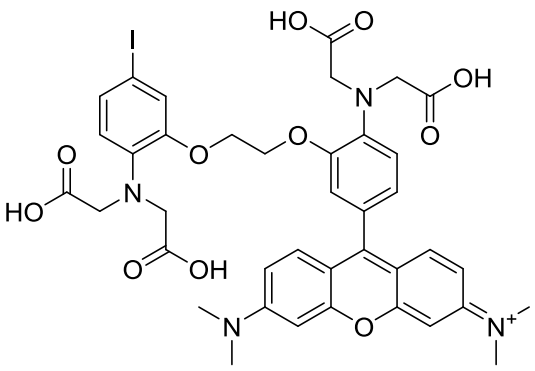
dimethyl 2,2'-((2-(2-(2-(bis(2-methoxy-2-oxoethyl)amino)-5-formylphenoxy)ethoxy)-4-iodo-phenyl)azanediyl)diacetate. 79.22 mg (0.12 mmol) **4** was suspended in a mixture of 12.1 µL (0.15 mmol) pyridine and 116 µL (1.5 mmol) DMF. 87 µL (0.96 mmol) $POCl_3$ was added dropwise at 0 °C and the mixture was stirred for 1 h at 80 °C. The suspension was poured into an ice water mixture and extracted with DCM. The combined organic solvents were dried over $MgSO_4$ and evaporated under reduced pressure. After purification with flash chromatography, (1:1 ethyl acetate: cyclohexane) the product was obtained as a colorless powder. Yield: 22.6 mg (0.033 mmol, 27 %).
^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C): δ = 9.80 (s, 1H), 7.37 (s, 2H), 7.19 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.10 (d, *J* = 1.9 Hz, 1H), 6.76 (d, *J* = 7.9 Hz, 1H), 4.23 (s, 8H), 4.10 (s, 4H), 3.61 (s, 6H), 3.57 (s, 6H). ^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ = 190.6, 171.7, 171.4, 151.1, 149.7, 145.2, 130.8, 130.1, 127.0, 122.1, 120.8, 116.8, 110.9, 83.8, 67.3, 67.3, 53.7, 53.4, 52.2, 51.9. (HR-ESI$^+$) m/z 709.0853 (calculated for $[C_{27}H_{31}IN_2O_{11} + Na]^+$ 709.0865).



6

N-(9-(4-(bis(2-methoxy-2-oxoethyl)amino)-3-(2-(2-(bis(2-methoxy-2-oxoethyl)amino)-5-iodophenoxy)ethoxy)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. The synthesis was performed according to a patent[8] with minor changes. 286 mg **5** and 114 mg (0.83 mmol) 3-(dimethylamino)phenol were dissolved in 4.89 mL propionic acid and stirred at 110 °C for 3 h. The reaction mixture was quenched with 50 mL concentrated aqueous NaOAc solution and the crude product was filtered off and dissolved in 9.5 mL 1:1 v/v chloroform:methanol. 120 mg (0.47 mmol) *p*-chloranil was added and the mixture stirred overnight at room temperature. The crude product was purified by flash chromatography (50:5:1 chloroform:methanol:acetic acid) to obtain a red solid. Yield: 52.4 mg (0.057 mmol, 14 %).

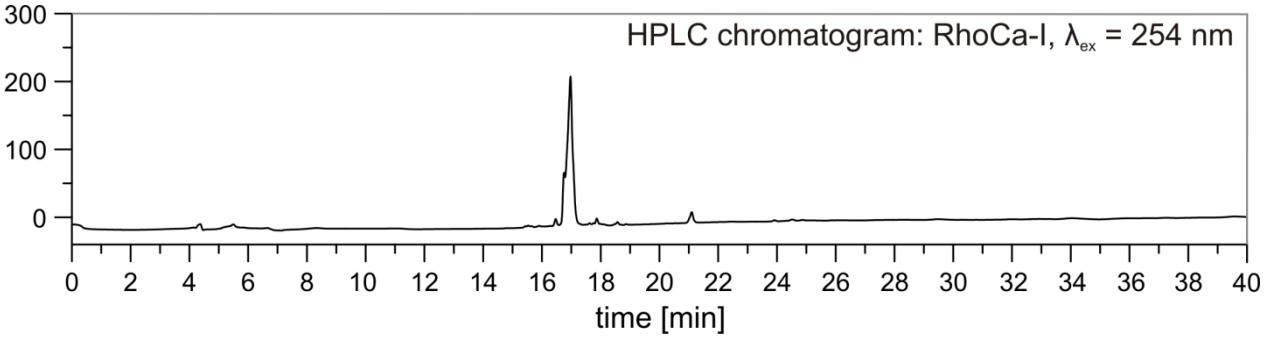
^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.52 (d, *J* = 9.2 Hz, 2H), 7.18 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.11 (d, *J* = 2.0 Hz, 1H), 6.89 (m, 7H), 6.57 (d, *J* = 8.4 Hz, 1H), 4.30 (s, 4H), 4.26 (s, 4H), 4.08 (s, 4H), 3.65 (s, 6H), 3.57 (s, 6H), 3.35 (s, 12H).^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ = 171.6, 171.6, 157.9, 157.2, 151.1, 149.7, 141.6, 139.5, 132.1, 130.9, 124.5, 124.1, 122.5, 121.1, 118.1, 115.2, 114.4, 113.5, 97.2, 84.1, 67.6, 67.4, 53.6, 53.4, 52.2, 51.9, 41.3. MS (HR-ESI$^+$) m/z 923.2336 (calculated for $[C_{43}H_{48}IN_4O_{11}]^+$ 923.2359).



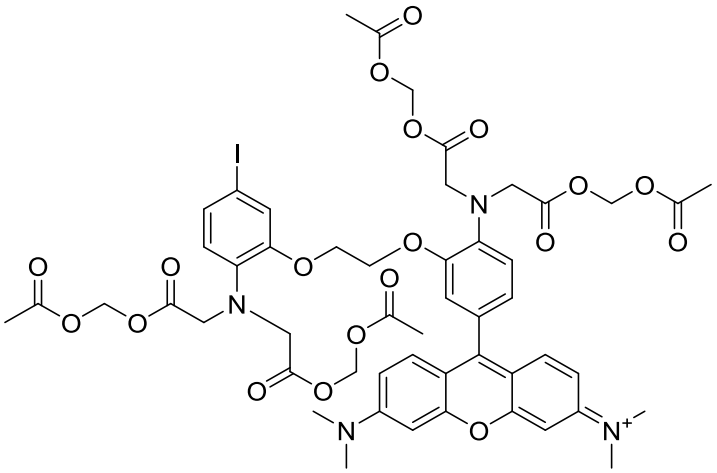
RhoCa-I

N-(9-(4-(bis(carboxymethyl)amino)-3-(2-(2-(bis(carboxymethyl)amino)-5-iodophenoxy)-ethoxy)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. 11 mg (0.012 mmol) **6** were dissolved in a mixture of 1.19 mL DCM and 2.42 mL methanol. 0.71 mL 1 M NaOH solution was added and the mixture was stirred overnight at room temperature. After neutralization with 1 M HCl, extraction was performed with ethyl acetate. The combined organic solvents were dried over $MgSO_4$ and evaporated under reduced pressure. The product was used without any further purification for the next step. For the spectroscopic measurements, a small amount was purified by HPLC (gradient from 90:10 to 40:60 H_2O + 0.1 % v/v TFA:MeCN + 0.1 % v/v TFA over 60 minutes). Yield: 7 mg (8 µmol, 67 %).

^{1}H-NMR (300 MHz, Methanol-d_4, 25 °C): δ = 7.63 (d, *J* = 9.5 Hz, 2H), 7.22 (d, *J* = 1.9 Hz, 1H), 7.18-7.10 (m, 2H), 7.10-6.89 (m, 6H), 6.56 (d, *J* = 8.4 Hz, 1H), 4.37 (s, 4H), 4.28 (s, 4H), 3.99 (s, 4H), 3.30 (s, 12H).



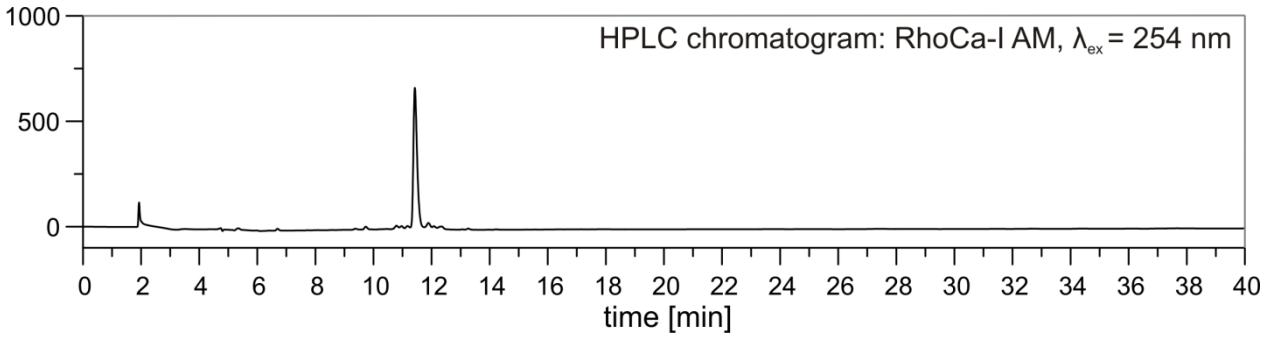
HPLC chromatogram of **RhoCa-I**. Conditions: 90:10 to 20:80 H2O + 0.1 % TFA:MeCN + 0.1 % TFA over 30 min.



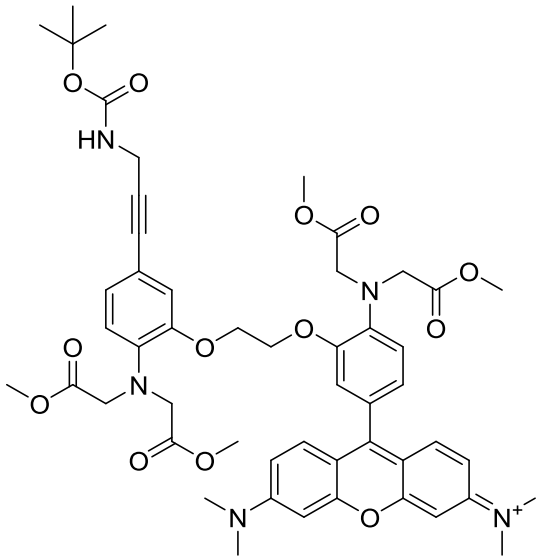
RhoCa-I AM

N-(9-(4-(bis(2-(acetoxymethoxy)-2-oxoethyl)amino)-3-(2-(2-(bis(2-(acetoxymethoxy)-2-oxo-ethyl)amino)-5-iodophenoxy)ethoxy)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. 7 mg (8 µmol) **RhoCa-I** was dissolved in a mixture of 8.31 µL (48 µmol) $(iPr)_2NEt$ and 112 µL DCM and stirred for 30 min at room temperature. 4.70 µL (48 µmol) bromomethyl acetate was added and the mixture stirred overnight at room temperature. The solvents were then evaporated under reduced pressure and the crude product was purified by HPLC (gradient from 70:30 to 20:80 H_2O + 0.1 % v/v TFA:MeCN + 0.1 % v/v TFA over 30 minutes) to obtain the product as a red oil. Yield: 1.2 mg (1 µmol, 13 %).

^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C): δ = 7.71 (dd, *J* = 5.7, 3.3 Hz, 1H), 7.53 (dd, *J* = 5.7, 3.3 Hz, 1H), 7.21 (d, *J* = 8.3 Hz, 1H), 7.16 (s, 1H), 6.97 (d, *J* = 17.4 Hz, 6H), 6.62 (d, *J* = 8.3 Hz, 1H), 5.70 (s, 4H), 5.63 (s, 4H), 4.36 (s, 4H), 4.32 (s, 4H), 4.15 (s, 4H), 3.36 (s, 12H), 2.11 (s, 6H), 2.06 (s, 6H). MS (HR-ESI)$^+$ 1155.2582 (calculated for $[C_{51}H_{56}IN_4O_{19}]^+$ 1155.2578).



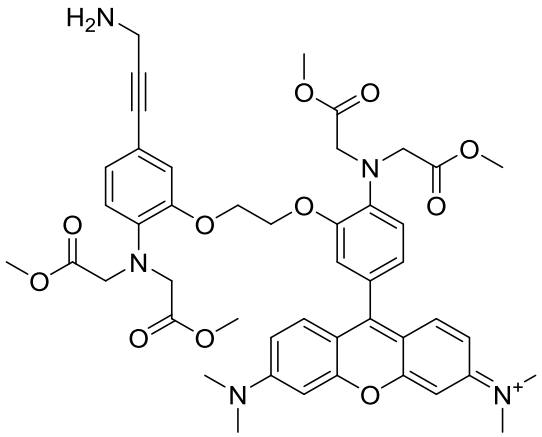
HPLC chromatogram of **RhoCa-I AM**. Conditions: 90:10 to 20:80 H2O + 0.1 % TFA:MeCN + 0.1 % TFA over 30 min.



7a

N-(9-(4-(bis(2-methoxy-2-oxoethyl)amino)-3-(2-(2-(bis(2-methoxy-2-oxoethyl)amino)-5-(3-((tert-butoxycarbonyl)amino)prop-1-yn-1-yl)phenoxy)ethoxy)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. 23 mg **6** (0.025 mmol), 19.3 mg **10** (0.125 mmol), 0.22 mg (1.25 µmol) CuI and 1.4 mg (1.25 µmol) $Pd(PPh_3)_4$ were suspended in 1 mL degassed DMF with 0.17 µL (0.125 mmol) NEt_3 and stirred overnight at room temperature and under argon atmosphere. The crude product was then purified by flash chromatography (50:5:1 chloroform:methanol:acetic acid) and a red oil was obtained. Yield: 16.5 mg (0.017 mmol, 70 %).

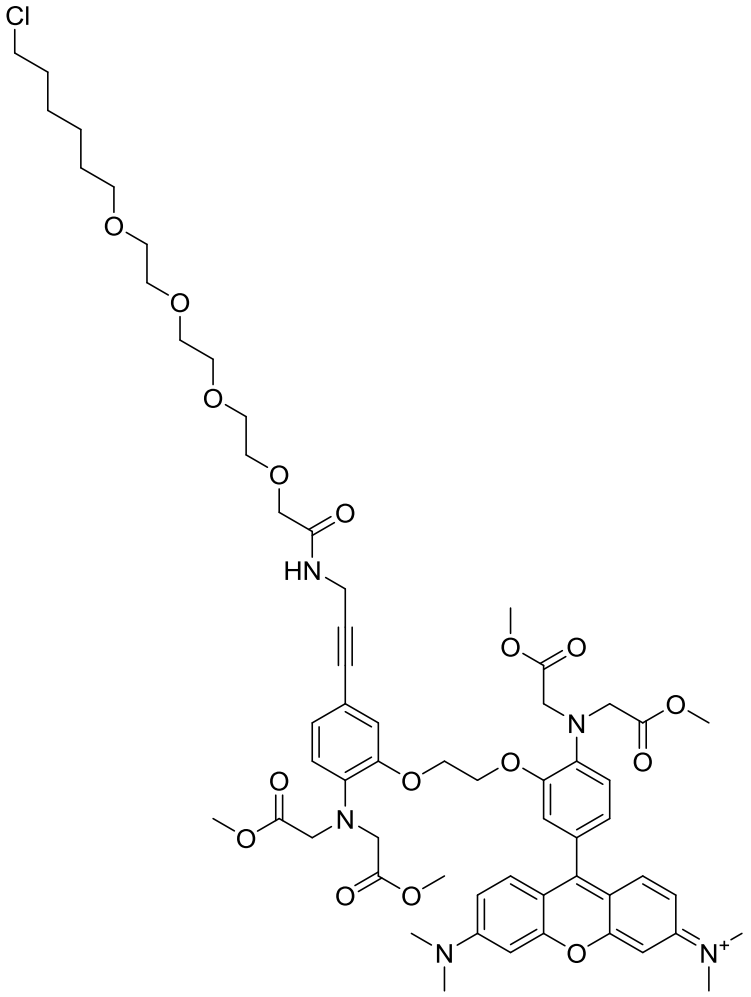
^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C): δ = 7.66 (ddd, *J* = 12.1, 8.3, 1.4 Hz, 1H), 7.59-7.50 (m, 2H), 7.50-7.43 (m, 1H), 7.39 (dd, *J* = 8.9, 1.8 Hz, 1H), 7.02 – 6.81 (m, 6H), 6.69 (d, *J* = 8.2 Hz, 1H), 4.81 (s, 1H), 4.38-4.20 (m, 8H), 4.12 (s, 4H), 3.65 (s, 2H), 3.63 (s, 6H), 3.58 (s, 6H), 3.34 (s, 12H), 1.45 (d, *J* = 5.8 Hz, 12H). ^{13}C-NMR (126 MHz, $CDCl_3$, 25 °C): δ = 171.8, 158.0, 157.2, 149.7, 141.5, 139.9, 132.2, 132.1, 128.7, 128.6, 124.6, 121.7, 118.6, 118.1, 116.1, 114.4, 113.7, 97.4, 84.6, 67.7, 67.2, 53.5, 52.2, 52.0, 41.4, 28.5. MS (HR-ESI$^+$) m/z 950.4154 (calculated for $[C_{51}H_{60}N_5O_{13}]^+$ 950.4182).



7b

N-(9-(3-(2-(5-(3-aminoprop-1-yn-1-yl)-2-(bis(2-methoxy-2-oxoethyl)amino)phenoxy)ethoxy)-4-(bis(2-methoxy-2-oxoethyl)amino)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. 16.5 mg (0.017 mmol) **7a** was dissolved in 4.0 M HCl in dioxane and stirred for 3 h at room temperature. The volatile compounds were removed under reduced pressure and the product was used without any further purification. Yield: 14 mg (0.017 mmol, quantitative).

^{1}H-NMR (300 MHz, Methanol-d_4, 25 °C): δ = 7.54 (dd, J = 10.8, 5.7 Hz, 3H), 7.22 – 6.80 (m, 7H), 6.70 (dd, J = 12.4, 6.2 Hz, 2H), 4.32 – 3.96 (m, 12H), 3.72 – 3.37 (m, 24H), 3.27 (s, 2H). MS (HR-ESI$^+$) m/z 886.3391 (calculated for $[C_{46}H_{52}N_5O_{11}$ + HCl$]^+$ 886.3425).

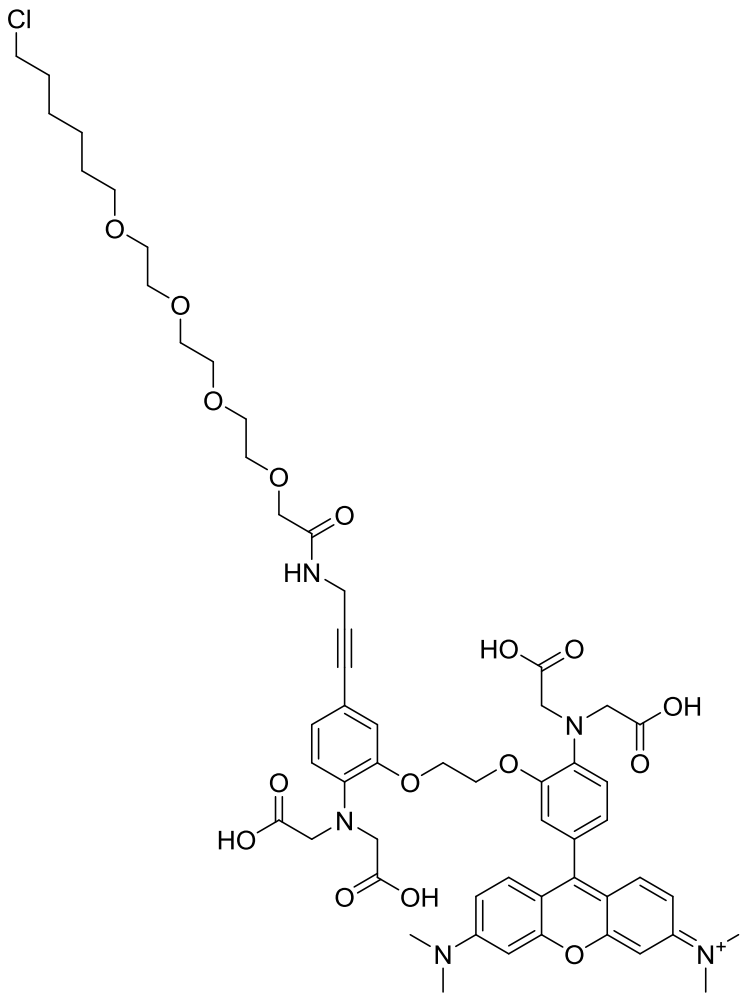


8

N-(9-(4-(bis(2-methoxy-2-oxoethyl)amino)-3-(2-(2-(bis(2-methoxy-2-oxoethyl)amino)-5-(22-chloro-5-oxo-7,10,13,16-tetraoxa-4-azadocos-1-yn-1-yl)phenoxy)ethoxy)phenyl)-6-(dimethyl-amino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. 10 mg (0.012 mmol) **7b**, 2.5 µL (0.0144) $(iPr)_2NEt$, 7.5 mg (0.0144) PyBOP and 4.7 mg (0.0144 mmol) HO_2CCH_2-[PEG-Halo] were dissolved in 400 µL DMF and stirred overnight at room temperature. Purification by flash chromatography (50:5:1 chloroform:methanol:acetic acid) could afford a red oil.

^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C) δ = 8.41 (d, J = 8.4 Hz, 1H), 8.01 (d, J = 8.6 Hz, 1H), 7.80 (t, J = 7.8 Hz, 1H), 7.62 – 7.41 (m, 2H), 7.17 – 6.66 (m, 7H), 4.97 (s, 1H), 4.27 (s, 4H), 4.14 (s, 8H), 3.80 – 3.72 (m, 2H), 3.73 – 3.62 (m, 12H), 3.60 (m, 10H), 3.52 (t, J = 6.7 Hz, 2H), 3.46 (t, J = 6.7 Hz, 2H), 2.08 (s, 12H), 1.81 – 1.72 (m, 4H), 1.59 (m, 4H), 1.48 – 1.31 (m, 4H).

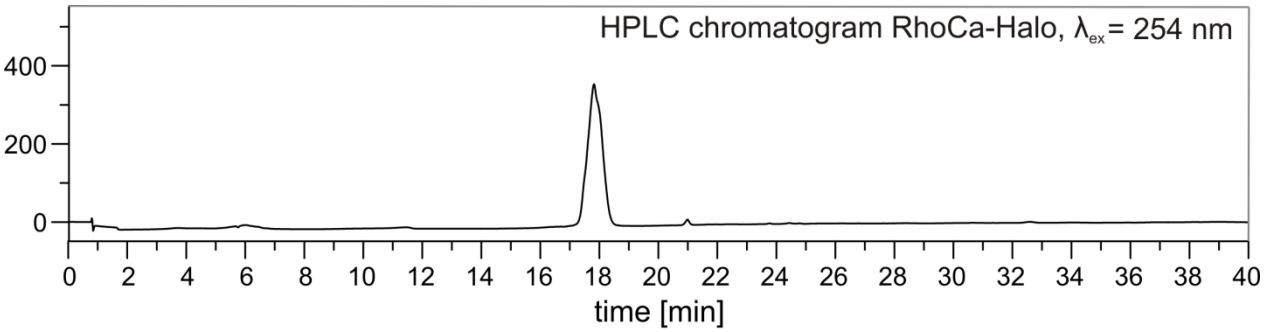
MS (HR-ESI$^+$) m/z 1194.4706 (calculated for $[C_{60}H_{77}ClN_5O_{16} + HCl]^+$ 1194.4815).



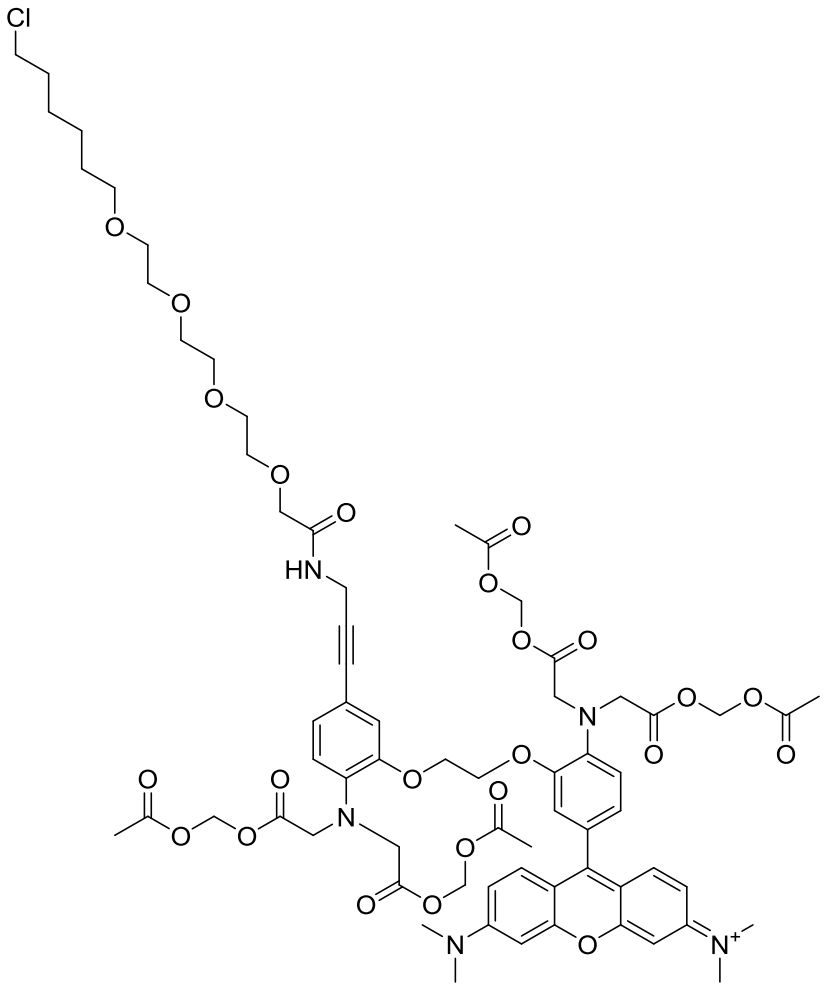
RhoCa-Halo (9a)

N-(9-(4-(bis(carboxymethyl)amino)-3-(2-(2-(bis(carboxymethyl)amino)-5-(22-chloro-5-oxo-7,10,13,16-tetraoxa-4-azadocos-1-yn-1-yl)phenoxy)ethoxy)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. 17.4 mg **8** (0.015 mmol) was dissolved in a mixture of 1.48 mL DCM and 3 mL methanol. 888 µL 1 M NaOH were added and the mixture was stirred for 3 h at room temperature. After neutralization with 1 M HCl, the crude product was extracted with DCM. The combined organic solvents were dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was used for the next step without any further purification. A small amount for the spectroscopic characterization was purified by HPLC (gradient from 70:30 to 20:80 H_2O + 0.1 % v/v TFA:MeCN + 0.1 % v/v TFA over 30 minutes). Yield: 60 %.

^{1}H NMR (600 MHz, Methanol-d_4, 25 °C) δ = 7.70 – 7.45 (m, 2H), 7.43 – 6.90 (m, 9H), 6.82 (dd, J = 46.5, 6.1 Hz, 1H), 5.34 (t, J = 4.9 Hz, 1H), 4.39 (d, J = 20.0 Hz, 1H), 4.27 (s, 1H), 4.19 (d, J = 24.6 Hz, 1H), 4.03 (s, 2H), 3.93 (s, 1H), 3.76 – 3.54 (m, 14H), 3.55 – 3.46 (m, 4H), 3.42 (dd, J = 3.6, 1.9 Hz, 2H), 2.19 (t, J = 7.6 Hz, 1H), 2.10 – 1.97 (m, 2H), 1.94 (s, 6H), 1.81 – 1.64 (m, 2H), 1.57 (d, J = 43.4 Hz, 4H), 1.45 (d, J = 9.0 Hz, 2H), 1.39 (d, J = 18.4 Hz, 4H), 1.23 (d, J = 8.1 Hz, 2H), 0.90 (t, J = 6.9 Hz, 2H).
MS (HR-ESI^{+}) m/z 1138.4154 (calculated for $[C_{56}H_{69}ClN_5O_{16} + HCl]^{+}$ 1138.4189).



HPLC chromatogram of **RhoCa-Halo**. Conditions: 70:30 to 20:80 H2O + 0.1 % TFA:MeCN + 0.1 % TFA over 30 min.



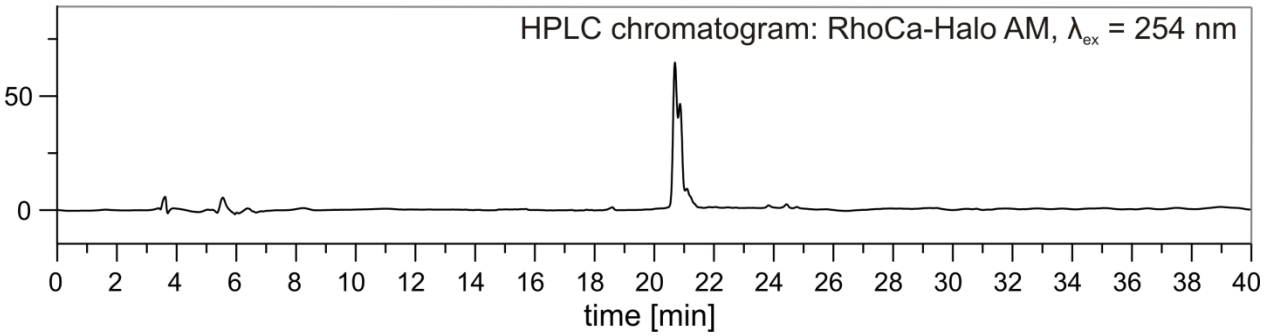
RhoCa-Halo AM (9b)

N-(9-(4-(bis(2-(acetoxymethoxy)-2-oxoethyl)amino)-3-(2-(2-(bis(2-(acetoxymethoxy)-2-oxoethyl)amino)-5-(22-chloro-5-oxo-7,10,13,16-tetraoxa-4-azadocos-1-yn-1-yl)phenoxy)-ethoxy)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium. 7 mg of the crude **RhoCa-Halo** and 4.96 mg (0.038 mmol) $(iPr)_2NEt$ were dissolved in DCM und stirred for 30 min at room temperature. After the addition of 3.72 µL (0.038 mmol) bromomethyl acetate the mixture was stirred overnight at room temperature. Die volatile compounds were evaporated under reduced pressure and the dark red oil was purified by HPLC (gradient from 70:30 to 20:80 H_2O + 0.1 % v/v TFA:MeCN + 0.1 % v/v TFA over 30 minutes) yielding a red powder as product. Yield: 35 %.

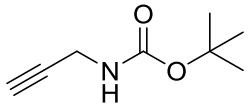
^1H NMR (600 MHz, DMSO-d_6) δ = 7.49 (d, J = 9.5 Hz, 2H), 7.17 – 7.11 (m, 2H), 7.08 (dd, J = 9.4, 2.7 Hz, 2H), 7.06 – 7.01 (m, 1H), 6.95 (d, J = 2.4 Hz, 2H), 6.92 (d, J = 8.2

Hz, 1H), 6.68 (d, J = 8.5 Hz, 2H), 5.62 (s, 4H), 5.55 (s, 4H), 5.32 (t, J = 5.0 Hz, 1H), 4.31 (s, 4H), 4.25 (s, 4H), 4.14 (s, 4H), 4.03 (t, J = 5.9 Hz, 2H), 3.90 (d, J = 3.9 Hz, 2H), 2.03 (s, 6H), 2.05 – 1.95 (m, 6H), 1.99 (s, 6H), 1.75 (s, 12H), 1.66 (dd, J = 14.2, 6.8 Hz, 2H), 1.44 (q, J = 7.2 Hz, 8H), 1.39 – 1.31 (m, 4H), 1.27 (d, J = 7.2 Hz, 2H).

MS (HR-ESI$^+$) m/z 1426.5001 (calculated for $[C_{68}H_{85}ClN_5O_{24} + HCl]^+$ 1426.5034).



HPLC chromatogram of **RhoCa-Halo**. Conditions: 70:30 to 20:80 H2O + 0.1 % TFA:MeCN + 0.1 % TFA over 30 min.



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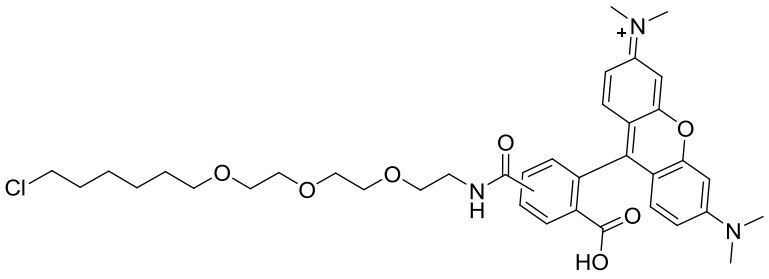
***tert*-butyl prop-2-yn-1-ylcarbamate.** 1 g (0.018 mmol) propargylamine and 4.71 g (0.0216 mmol) di-*tert*-butyl dicarbonate were dissolved in a mixture of 45 mL methanol and 5 mL NEt_3 and stirred overnight at room temperature. The volatile compounds were evaporated under reduced pressure and the product could be obtained as reddish crystals. Yield: 2.58 g (0.0166 mmol, 93 %).

^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C): δ = 4.74 (s, 1H), 3.91 (s, 2H), 2.21 (t, *J* = 2.5 Hz, 1H), 1.44 (s, 9H). ^{13}C-NMR (126 MHz, $CDCl_3$, 25 °C): δ = 155.36, 80.22, 71.36, 30.53, 28.47. MS (HR-ESI)$^+$ m/z 178.0834 (calculated for $[C_8H_{13}NO_2 + Na]^+$ 178.0838).



Halo rhodamine. The compound was synthesized according to a procedure described in the literature.[9]

MS (HR-ESI)$^+$ m/z 680.3088 (calculated for $[C_{37}H_{47}ClN_3O_7]^+$ 680.3097).

Design and synthesis of a Halo-fusion proteins.

H2B-GFP was a gift from Geoff Wahl (Addgene plasmid # 11680)[10]

pEGFP-Halo.

The target vector containing the HaloTag-sequence for C-terminal fusion constructs (pFC14K, Barnase-Halo; Promega GmbH) was purchased from Promega. Molecular cloning was then performed by the FlexiVector system according to the manufacturer's protocol. The target vector was linearized by mixing 200 ng plasmid DNA with 20 % v/v 5 x Flexi Digest Buffer and 10 % v/v Flexi Enzyme Blend (SgfI/EcoICRI) in a final volume of 200 µL and incubated for 30 min at 37 °C followed by heat deactivation at 65 °C.

The DNA fragment containing the EGFP gene flanked by SgfI and PmeI restriction sites was amplified from a EGFP-eDHFR plasmid (kindly provided by Virginia Cornish) by PCR (10 % v/v 10 % Hybrid buffer including 15 mM $MgCl_2$, 4 % DMSO, 0.2 mM dNTPs, 0.5 µM fw primer, 0.5 µM rv primer, 1 U Hybrid DNA-polymerase, and 10 pg template plasmid DNA in a total volume of 50 µL) at the annealing temperature of 67 °C. PCR products were then purified by using SV Minicolumns (Promega GmbH) according to the manufacturer's protocol. For the digestion, 500 ng PCR fragment was incubated with 20 % v/v 5 x Flexi Digest Buffer and 20 % Flexi Enzyme Blend (SgfI/PmeI) in a final volume of 20 µL at 37 °C for 30 min followed by heat deactivation at 65 °C for 20 min. The PCR product was again purified with SV Minicolumns.

The PCR-product and the linearized vector were ligated by incubating 40-80 ng each with 50 % v/v Flexi Ligase Buffer and 20 U T4 DNA ligase (HC; Promega) in a total volume of 20 µL according to the manufacturer's protocol.

Halo-H2B.

Cloning of the **Halo-H2B** construct was performed by the FlexiVector system according to the manufacturer's protocol. H2B with flanked SgfI and PmeI cleavage site was amplified from the H2B-eDHFR template[11] and ligated with the linearized pFN21A vector (Promega GmbH; digested with PmeI and SgfI) as described for **pEGFP-Halo**.

GFP-Halo.

pET His6 GFP TEV LIC cloning vector (1GFP) was a gift from Scott Gradia (Addgene plasmid # 29663) and was linearized with SspI. The reaction was analyzed by agarose-gel analysis (1 %), the according band was excised and purified by gel-extraction. The insert-DNA for the HaloTag was amplified from **pEGFP-Halo** by using the High-Fidelity DNA Polymerase kit (Thermo Fisher Scientific Inc.). The according primers were purchased from Integrated DNA Technologies and used for the PCR procedure. The product was excised from agarose-gel (1 %) and purified. Seamless cloning was then performed by Gibson assembly:[12] 20 ng linearized plasmid-DNA and 20 ng insert-DNA were incubated in 15 µL 1.33x Gibson assembly master mix for 1 h at 50 °C. *Escherichia Coli* (*E. coli*) DH5α-cells were transformed with the plasmid and incubated overnight at 37 °C (30 µg/mL kanamycin). After harvesting the cells and purification of the plasmids, the correct sequence was verified by sequencing.

For protein expression, *E. coli* BL21(DE3)+pLys (Promega GmbH) were transformed with the plasmid and incubated overnight at 37 °C (30 µg/mL kanamycin) and transferred into 1 L TB-medium (30 µg/mL kanamycin). Cells were grown at 37 °C and protein expression was induced by 0.5 mM IPTG at OD_{600} = 0.6. At OD_{600} = 6, the suspension was centrifuged at 1792 x g at 4 °C. The residue was suspended in PBS and centrifuged again at 1792 x g and 4 °C. Cells were then suspended in 25 mL lysis buffer (500 mM NaCl, 30 mM imidazole, 20 mM, 20 mM sodium phosphate (pH = 7.4), 0.2 % Triton X-100, 50 µg/mL lysozyme (chicken egg), 1 cOmplete protease inhibitor per 50 mL buffer (Hoffmann-La Roche AG)) with subsequent sonification. The suspension was centrifuged at 35000 x g at 4 °C. The supernatant was filtered through a 0.22 µm filter unit.

The residue of the filtration was loaded onto a His-Trap column, equilibrated with binding buffer (50 mM Tris (pH = 7), 300 mM NaCl, 30 mM imidazole). The protein was eluted

with elution buffer (50 mM Tris (pH = 7), 300 mM NaCl, 100 mM imidazole) by a gradient of 95:5 binding buffer:elution buffer to 100 % elution buffer over 120 min. Fractions were then analyzed by SDS-gel analysis. Dialysis was then performed against PBS (10000 MWCO, Carl Roth GmbH). The protein was concentrated by centrifugal filter units (10000 MWCO, VIVASPIN, Sartorius AG) according to the manufacturer's instructions and sterile filtered. The final concentration was determined by spectroscopic measurements (NanoDrop) and stored at -80 °C.

In vitro experiments with GFP-Halo

10 µM protein in PBS (pH = 7.4) was incubated with **RhoCa-Halo** at a concentration between 10-50 µM for 2 h at room temperature. 10 µM BSA and 50 µM **RhoCa-Halo** was used as a negative control and 10 µM GFP-Halo with 10 µM Halo rhodamine as a positive control, respectively. A defined volume of the reaction mixture was mixed with the same volume of Laemmli buffer[13] and heated to 95 °C for 5 min. The in-gel fluorescence scan was performed with a Typhoon 9400 reader (λ_{ex} = 532 nm and λ_{em} = 670 nm) and the SDS gel was stained with Coomassie Brilliant-Blue[3].

For the spectroscopic evaluation of the **RhoCa-Halo** labeled GFP-Halo, 10 µM protein was incubated with 50 µM fluorophore for 2 h at room temperature. Excess **RhoCa-Halo** was removed by spin filtration (Amicon centrifugal filters, 10 K) and the spectroscopic measurements were performed as described before.

Live cell fluorescent imaging

Live cell experiments with RhoCa-I AM and NP-EGTA

HeLa cells were incubated with 10 µM NP-EGTA AM for 1 h at 37 °C and 5 % CO_2. Calcium release was then stimulated with 100 µM ATP over 1 h at 37 °C and 5 % CO_2. Incubation with 10 µM fluorophore was performed by using the RHOD-3 Calcium imaging kit (Life Technologies) in Ringers buffer according the manufacturer's protocol. Cells were washed three times with DPBS. Live cell imaging was performed with a Olympus IX83 laser scanning microscope at 37 °C and 5 % CO_2. A Hamatsu C9100-50 EM CCD-camera and an Olympus Plan-APON microscope objective 60 × (NA 1.4, oil immersion) were used. A 559 nm laser (120 mW/cm^2, 2.0 %) served as light source and pictures were recorded with FluoView (version 4.2). A pulsed 405 nm laser line (10 MHz)

was applied for uncaging experiments. For uncaging experiments, circular regions of interest (ROIs) of 4-10 µm diameter were pre-defined. Pre-activation images were captured for 5 frames (5s/frame), followed by 30 s of activation within the ROI. Recovery images were captured for 45 min at a frame rate of 5 s/frame.

Live cell experiments with RhoCa-Halo AM

NIH/3T3 and HeLa cells were transiently transfected with pHalo-H2B and pH2B-GFP. 24 h after transfection cells were incubated with 1 µM fluorophore by using the RHOD-3 Calcium imaging kit (Life Technologies) in Ringers buffer according the manufacturer's protocol over 1 h. Cells were washed three times with Ringers buffer. Live cell imaging was performed with a Nikon Ti-E epifluorescence microscope, a S Plan Fluor ELWD 40x NA 1.3, oil immersion microscope objective, a Hamatsu Orca AG C4742-80-12AG camera and GFP HQ, Bs 495, em 500-545 filter (green channel) and a Texas Red, Bs 595, em 630/60 filter (red channel). Pictures were recorded with NIC-Elements (Version 4.13.04, 64 bit).

Calcium influx was stimulated with 100 µM ATP in Ringers buffer and imaging was performed with a Olympus IX83 laser scanning microscope as described for the NP-EGTA experiment.

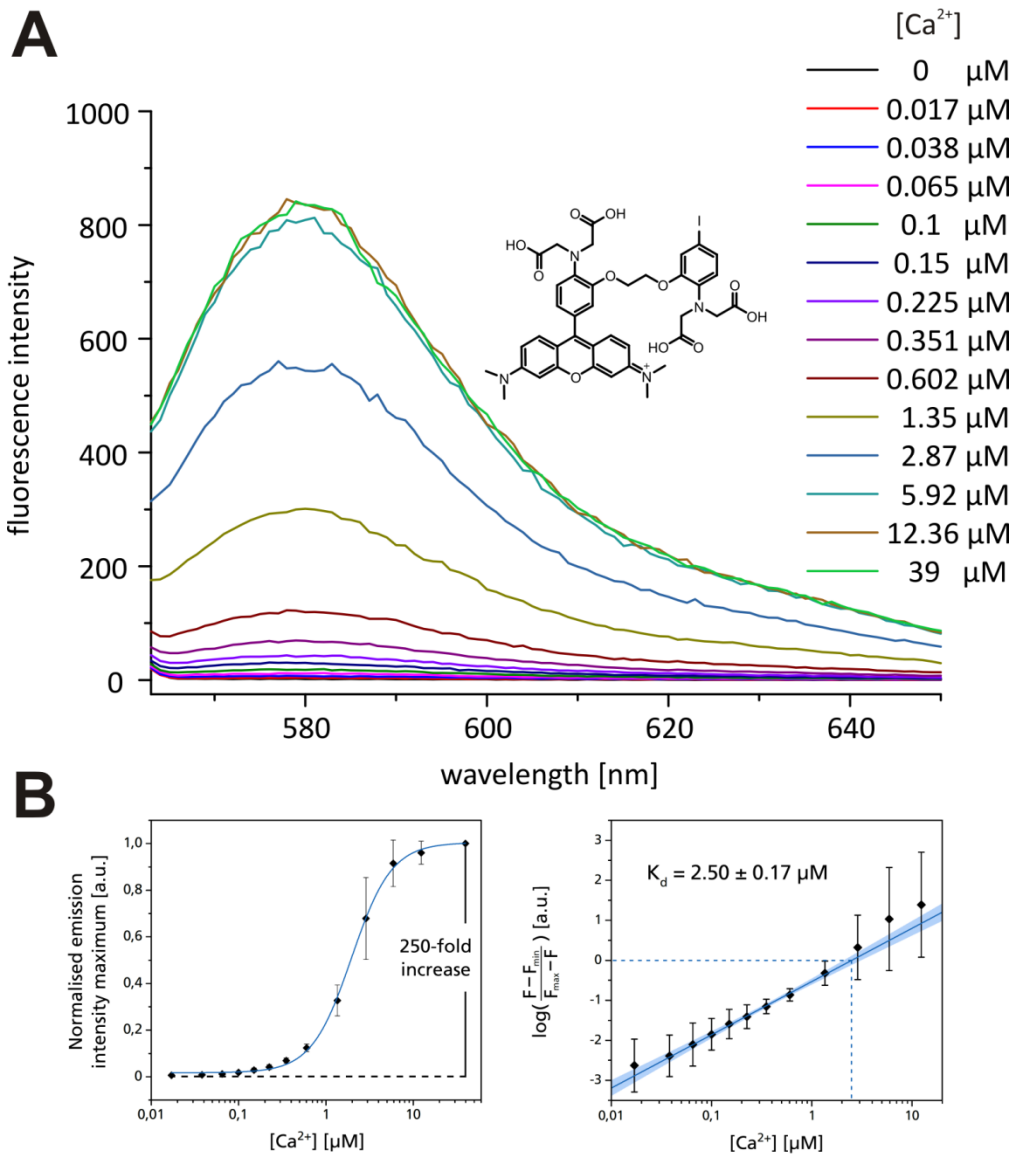


Figure S1. (A) Calcium titration of **RhoCa-I** with concentrations of free calcium ions ranging from 0 µM to 39 µM. The fluorophore was excited at 552 nm. (B) A 215-fold enhancement upon calcium binding was observed (left). The K_d-value was determined to 2.50 ± 0.17 µM (right).

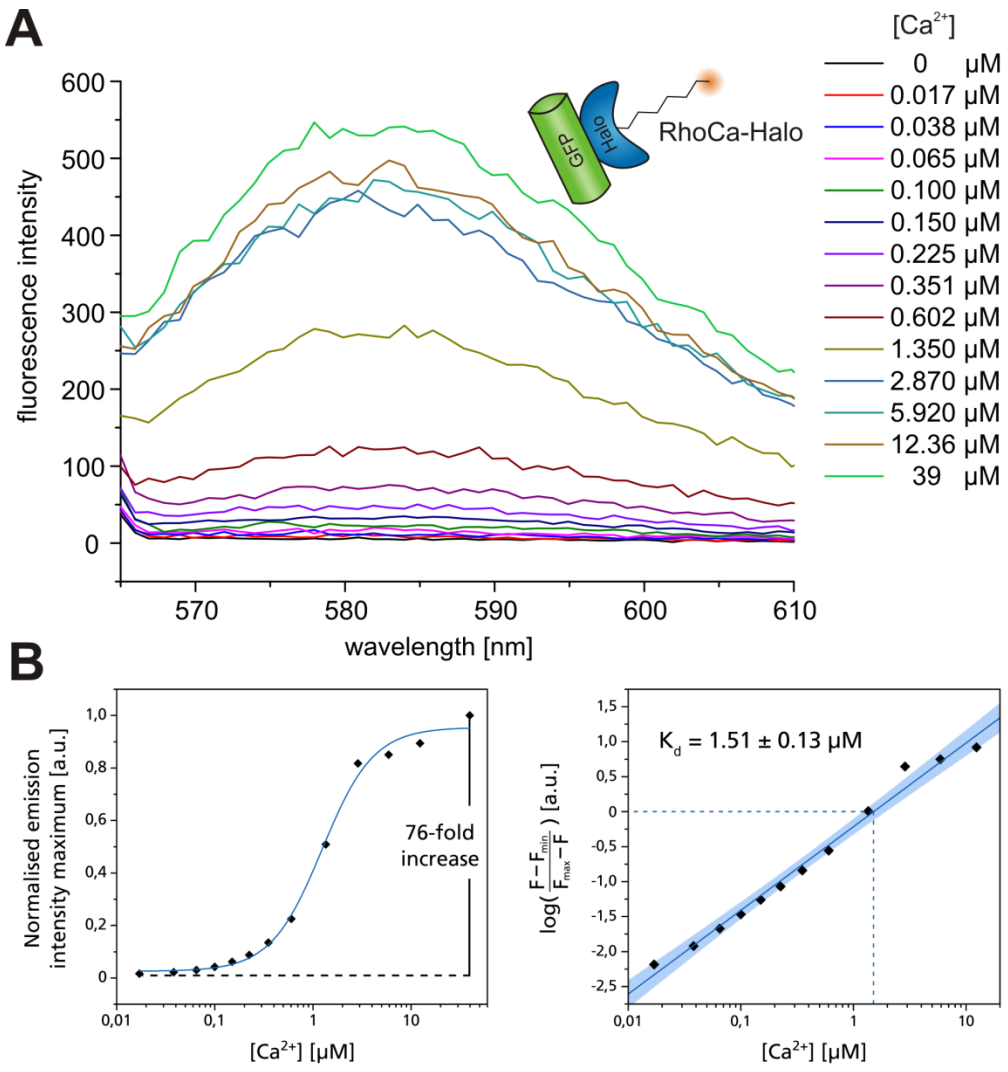


Figure S2. (A) Calcium titration of the purified **RhoCa-Halo**-labeled GFP-Halo protein with concentrations of free calcium ions ranging from 0 µM to 39 µM. The fluorophore was excited at 552 nm. (B) A 76-fold enhancement upon calcium binding was observed (left). The K_d-value was determined to 1.51 ± 0.13 µM (right).

Primer sequences

pEGFP-Halo

SgfI-EGFP_fw

ATTGCGATCGCGTGACCGTCAGATCCGCTAG

EGFP-PmeI_rv

TTAGTTTAAACCTTGTACAGCTCGTCCATGCCGAGAGTGATCC

Halo-H2B

SgfI-Halo-H2B_fw

ATTGCGATCGCCATGCCTGAACCCTCTAAGTCTGCTC

PmeI-Halo-H2B_rv

TTATGTTTAAACCTTAGAGCTAGTGTACTTGGTAACTGCCTTAGTG

GFP-Halo

GFP-Halo_fw

TACTTCCAATCCAATGCATCCGAAATCGGTACTGGCTTTC

GFP-Halo_rv

TTATCCACTTCCAATGTTATTAACCGGAAATCTCCAGAGTAGAC

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