

# SUPPORTING INFORMATION

## Fluorescent and bioluminescent calcium indicators with tuneable colors and affinities

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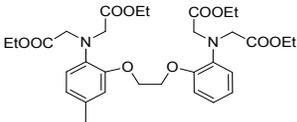
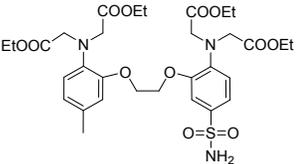
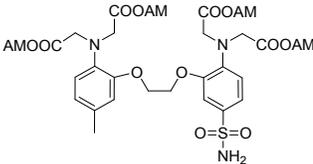
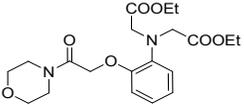
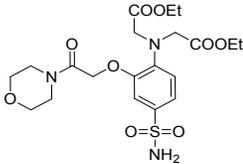
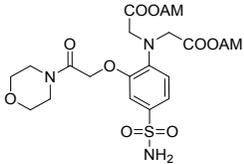
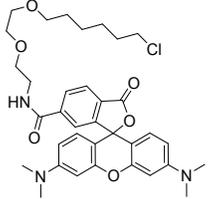
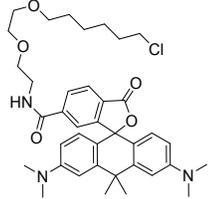
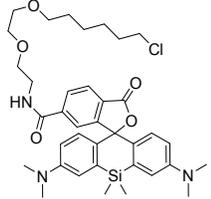
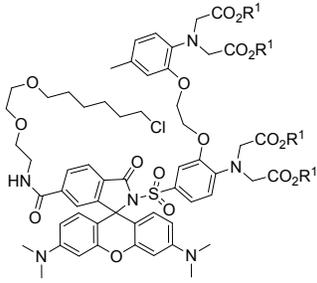
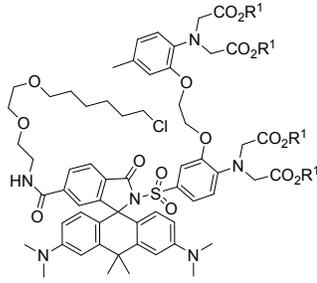
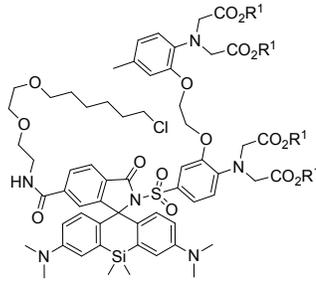
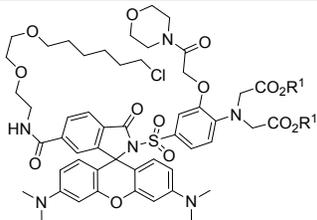
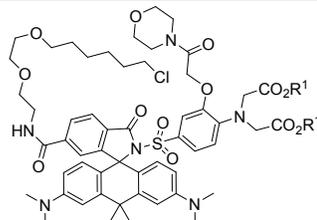
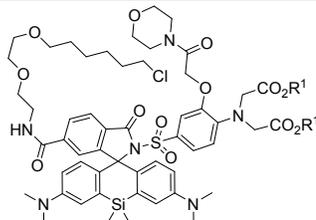
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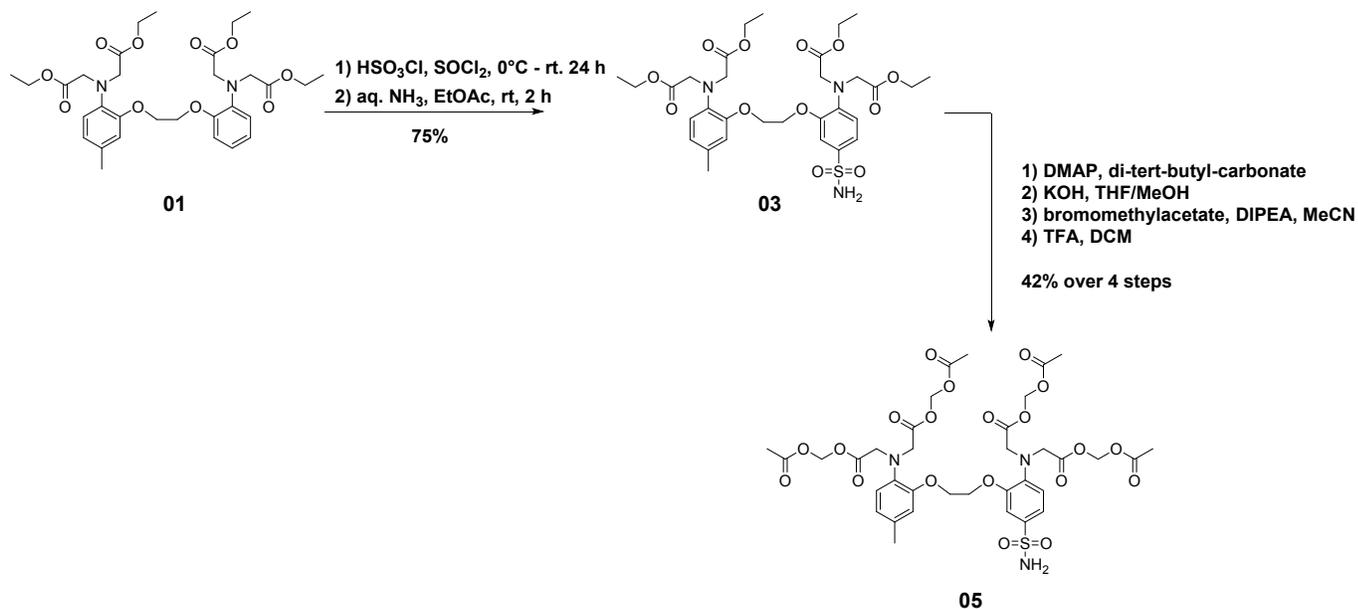
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## Supplementary Figures and Tables

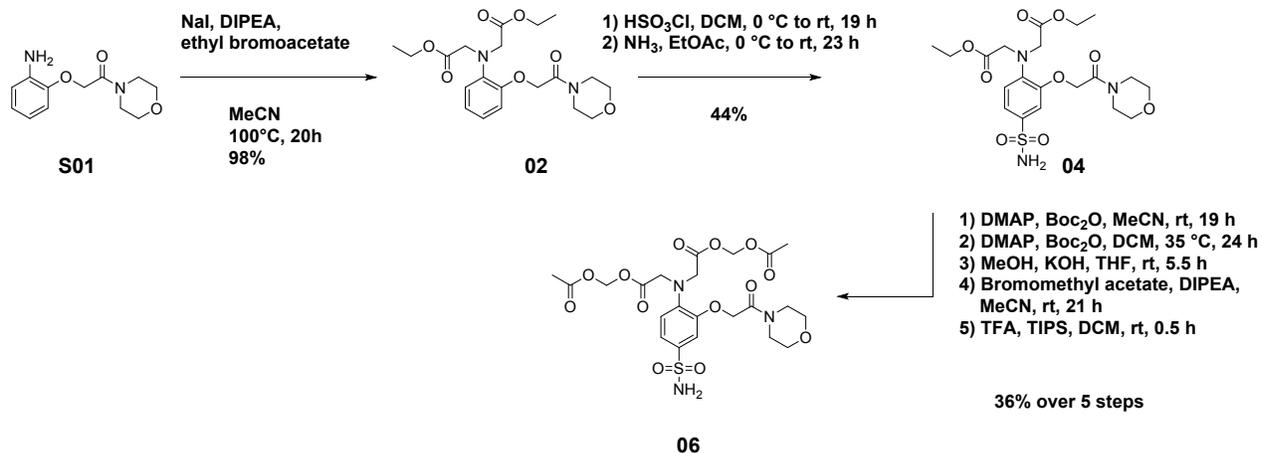
		
<b>01</b>	<b>03</b>	<b>05</b>
		
<b>02</b>	<b>04</b>	<b>06</b>
		
<b>TMR-CA</b>	<b>CPY-CA</b>	<b>SiR-CA</b>
		
<b>R<sup>1</sup>= H   MaPCa-558<sub>high</sub></b> <b>R<sup>1</sup>= AM   MaPCa-558<sub>high</sub> AM</b>	<b>R<sup>1</sup>= H   MaPCa-619<sub>high</sub></b> <b>R<sup>1</sup>= AM   MaPCa-656<sub>high</sub> AM</b>	<b>R<sup>1</sup>= H   MaPCa-656<sub>high</sub></b> <b>R<sup>1</sup>= AM   MaPCa-656<sub>high</sub> AM</b>
		
<b>R<sup>1</sup>= H   MaPCa-558<sub>low</sub></b> <b>R<sup>1</sup>= AM   MaPCa-558<sub>low</sub> AM</b>	<b>R<sup>1</sup>= H   MaPCa-619<sub>low</sub></b> <b>R<sup>1</sup>= AM   MaPCa-619<sub>low</sub> AM</b>	<b>R<sup>1</sup>= H   MaPCa-656<sub>low</sub></b> <b>R<sup>1</sup>= AM   MaPCa-656<sub>low</sub> AM</b>

**Supporting Figure S 1:** Structure of main synthetic molecules in this work.

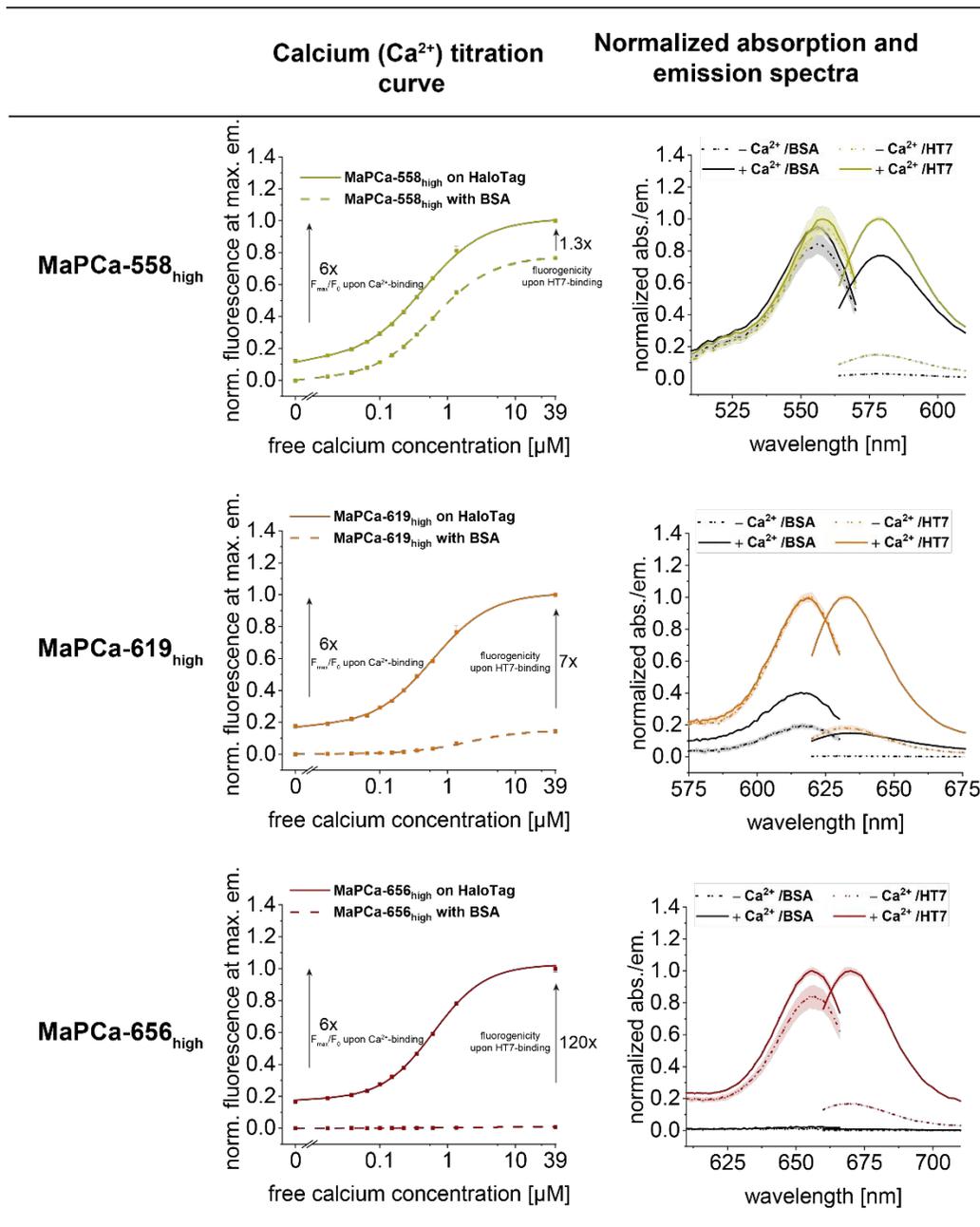
### Synthesis of BAPTA-Sulfonamide:



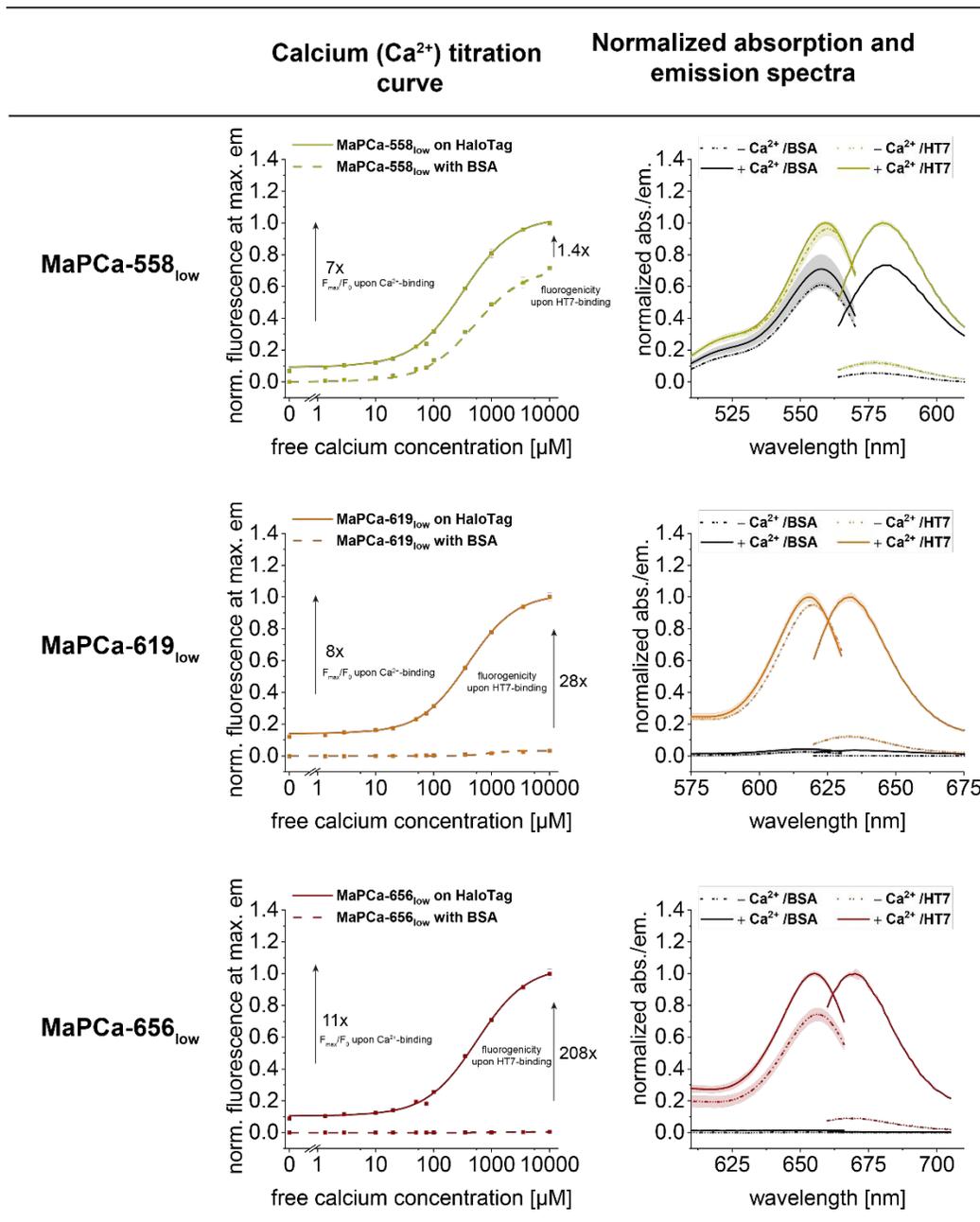
### Synthesis of MOBHA-Sulfonamide:



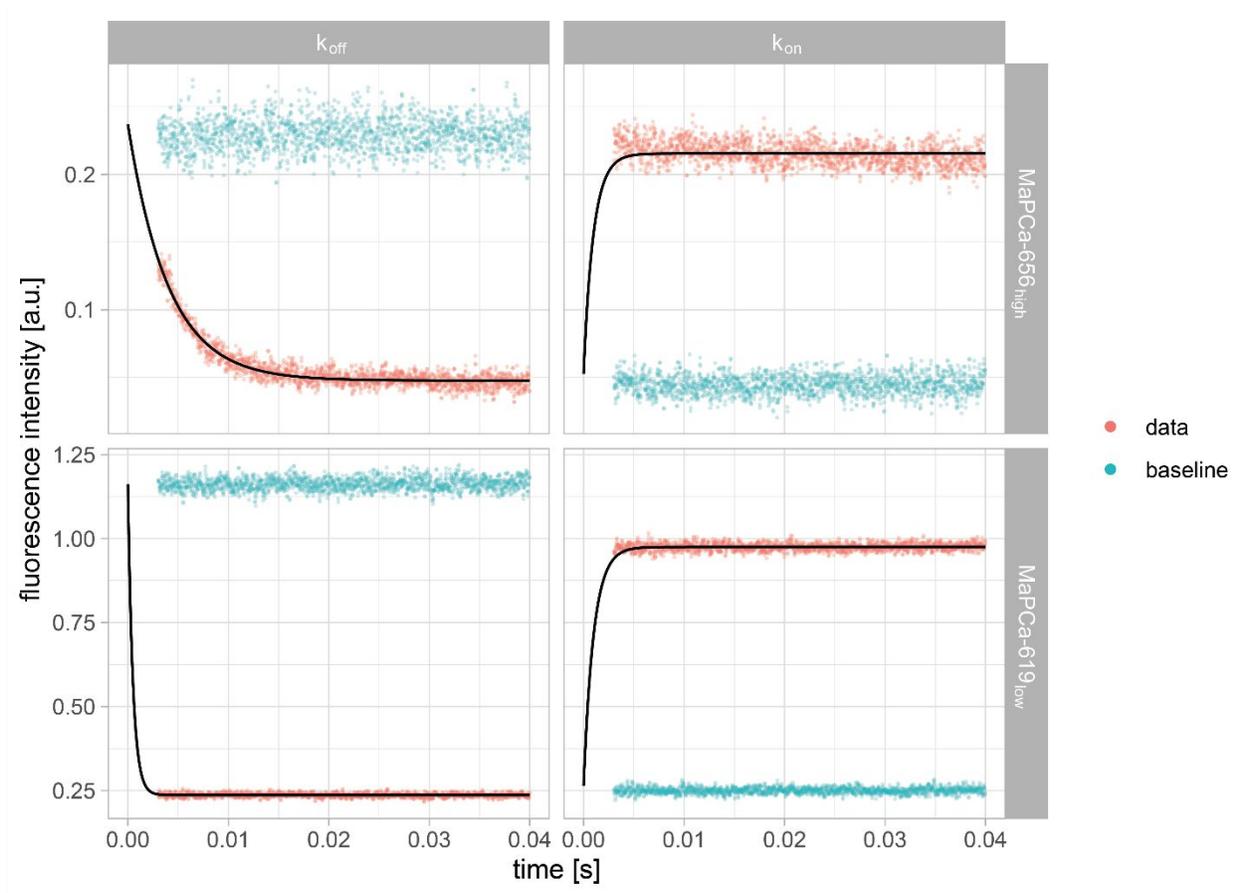
Supporting Figure S 2: Synthetic pathways to BAPTA- and MOBHA-sulfonamide.



**Supporting Figure S 3:** Calcium titration curves and absorption/emission spectra for MaPCa<sub>high</sub> dyes.  $K_D(\text{Ca}^{2+})$ -values are given in Supporting Table 1.

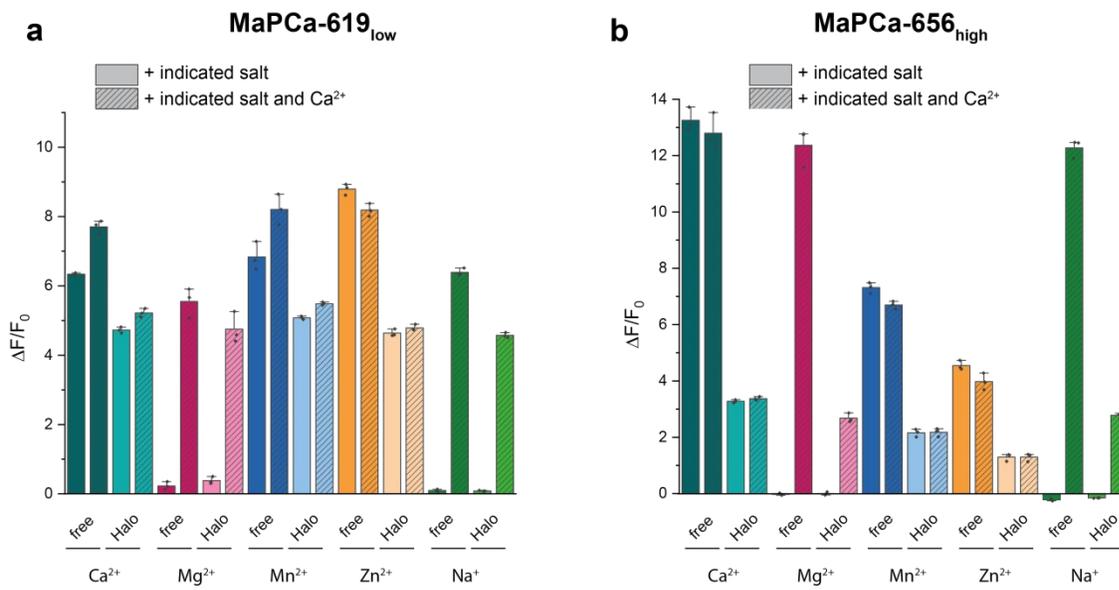


**Supporting Figure S 4:** Calcium titration curves and absorption/emission spectra for MaPCa<sub>low</sub> dyes.  $K_D(\text{Ca}^{2+})$ -values are given in Supporting Table 1.

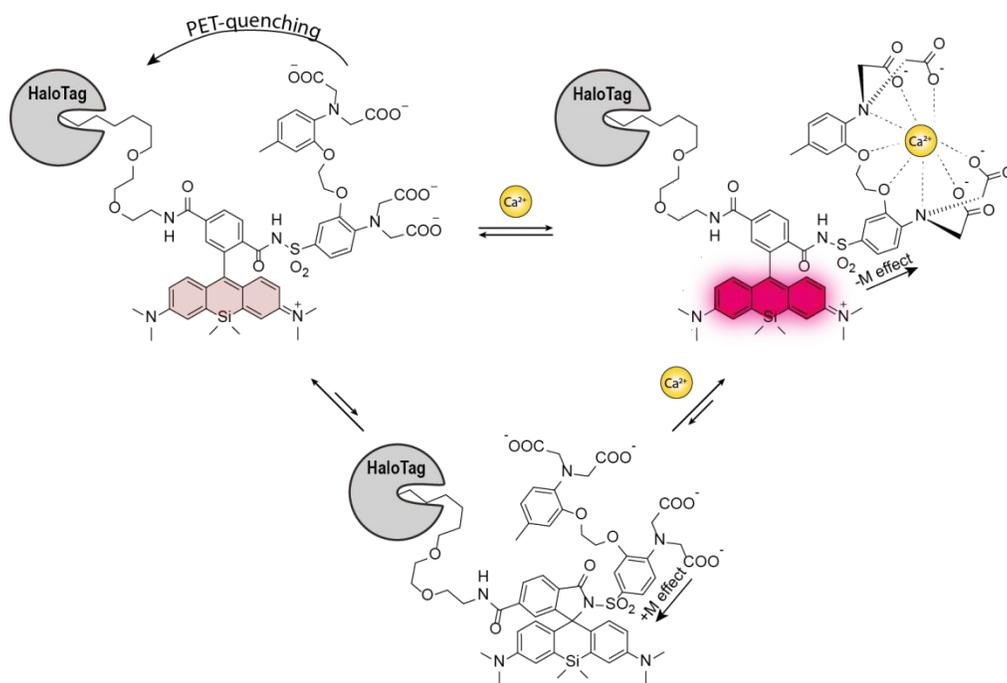


dye	k	measured [s <sup>-1</sup> ]	K <sub>D</sub>	on (calc.) [s <sup>-1</sup> ]
MaPCa-656 <sub>high</sub>	off	248 <i>CI (5%): 242</i> <i>CI (95%): 253</i>	580 nM	4.27 x 10 <sup>8</sup>
	on	> 5 x 10 <sup>7</sup>	580 nM	
MaPCa-619 <sub>low</sub>	off	> 2000	322 μM	
	on	> 2 x 10 <sup>6</sup>	322 μM	

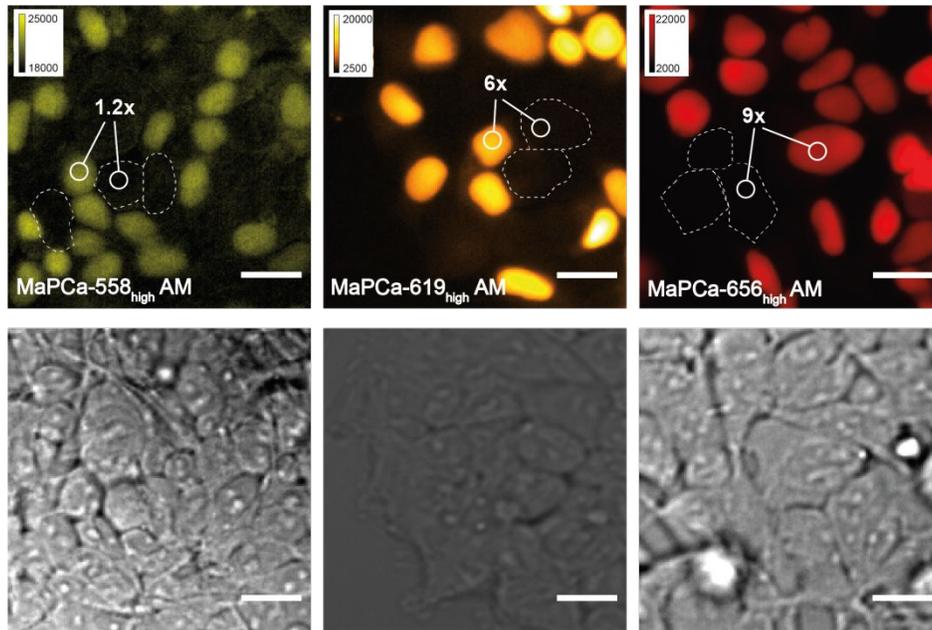
**Supporting Figure S 5:** Calcium binding and unbinding kinetics of MaPCa dyes bound to HaloTag protein demonstrated by stopped-flow measurements for MaPCa-656<sub>high</sub> and MaPCa-619<sub>low</sub>. The  $k_{off}$  of MaPCa-656<sub>high</sub> was measurable, while only minimal values could be estimated for MaPCa-619<sub>low</sub>. A detailed description of parameters is given in the experimental section. CI = confidence interval.



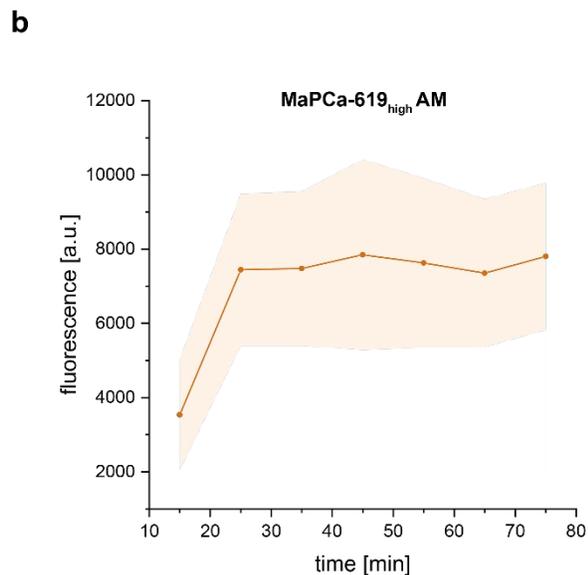
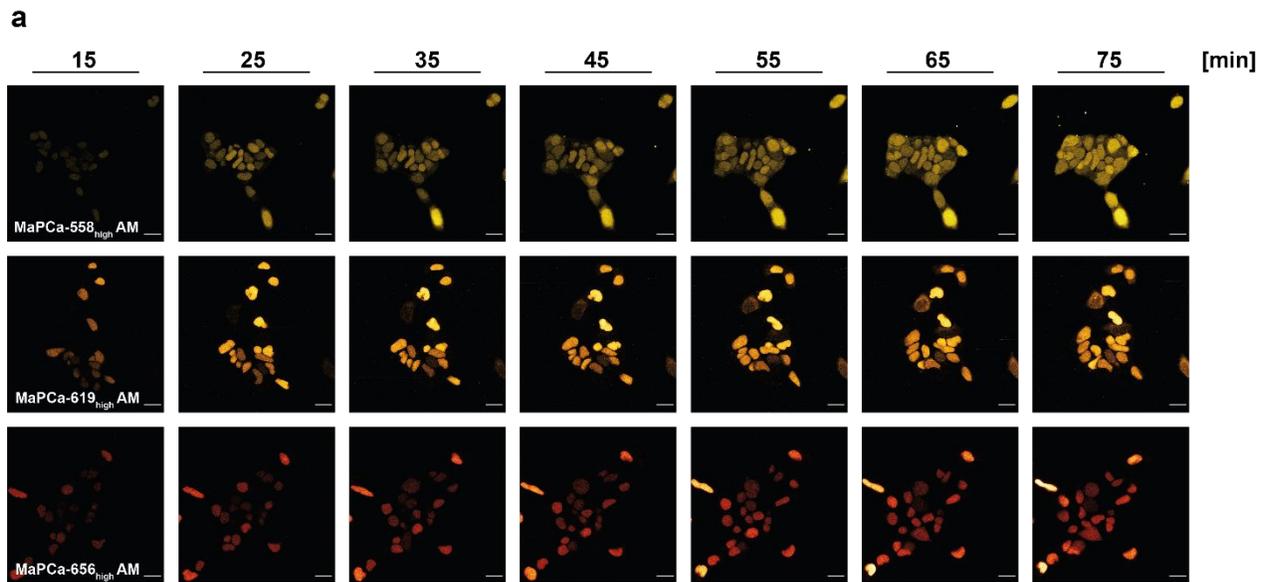
**Supporting Figure S 6:** Selectivity of the MaPCa indicators (exemplary here MaPCa-619<sub>low</sub> (a) and MaPCa-656<sub>high</sub> (b)) against other cations.  $\Delta F/F_0$  of the free indicators (1  $\mu\text{M}$ ) and HaloTag-bound indicators (1  $\mu\text{M}$  dye; 4  $\mu\text{M}$  HaloTag) was measured upon addition of indicated cations (50  $\mu\text{M}$ ) and subsequently upon additional  $\text{Ca}^{2+}$  (50  $\mu\text{M}$ , patterned bars). Buffer: 10  $\mu\text{M}$  EGTA, 30 mM MOPS, 100 mM KCl, pH = 7.2. For the  $\text{Na}^+$  experiment, instead of adding NaCl, the buffer was changed to 100 mM NaCl instead of KCl. Error bars represent standard deviation of  $n = 3$ .



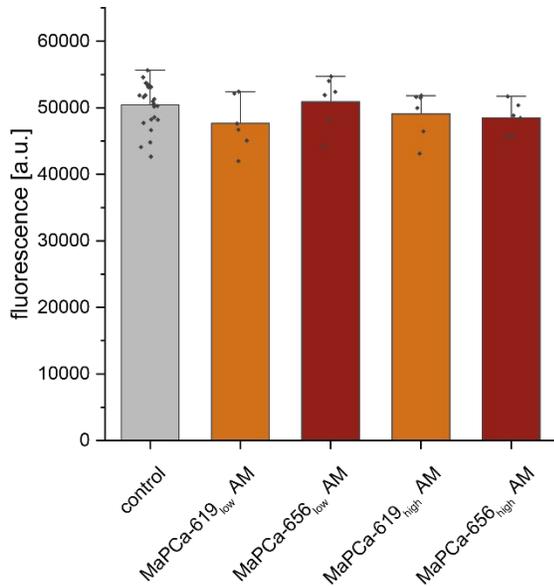
**Supporting Fig. 7:** Schematic representation of the quenching mechanism *via* the influence of calcium on the open/close equilibrium (example of MaPCa-656<sub>high</sub>). The two upper structures do absorb light at 656 nm, while the lower, cyclized structure does not.



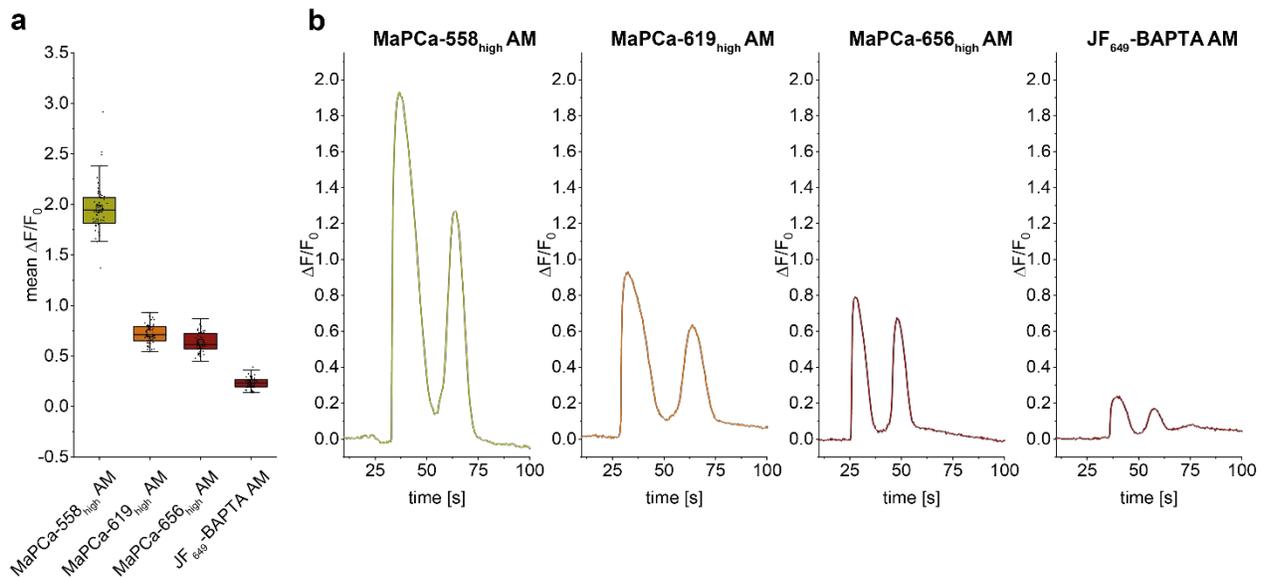
**Supporting Figure S 8:** Fluorescence and transmitted light microscopy images of a co-culture of HaloTag-NLS-expressing and non-expressing 293 cells. Cells were incubated with 1  $\mu$ M MaPCa-558<sub>high</sub> AM (left), MaPCa-619<sub>high</sub> AM (middle) or MaPCa-656<sub>high</sub> AM (right) for 2 h and imaged under no-wash conditions. Turn-on numbers represent average of  $n = 200$  cells. Scale bar, 20  $\mu$ m.



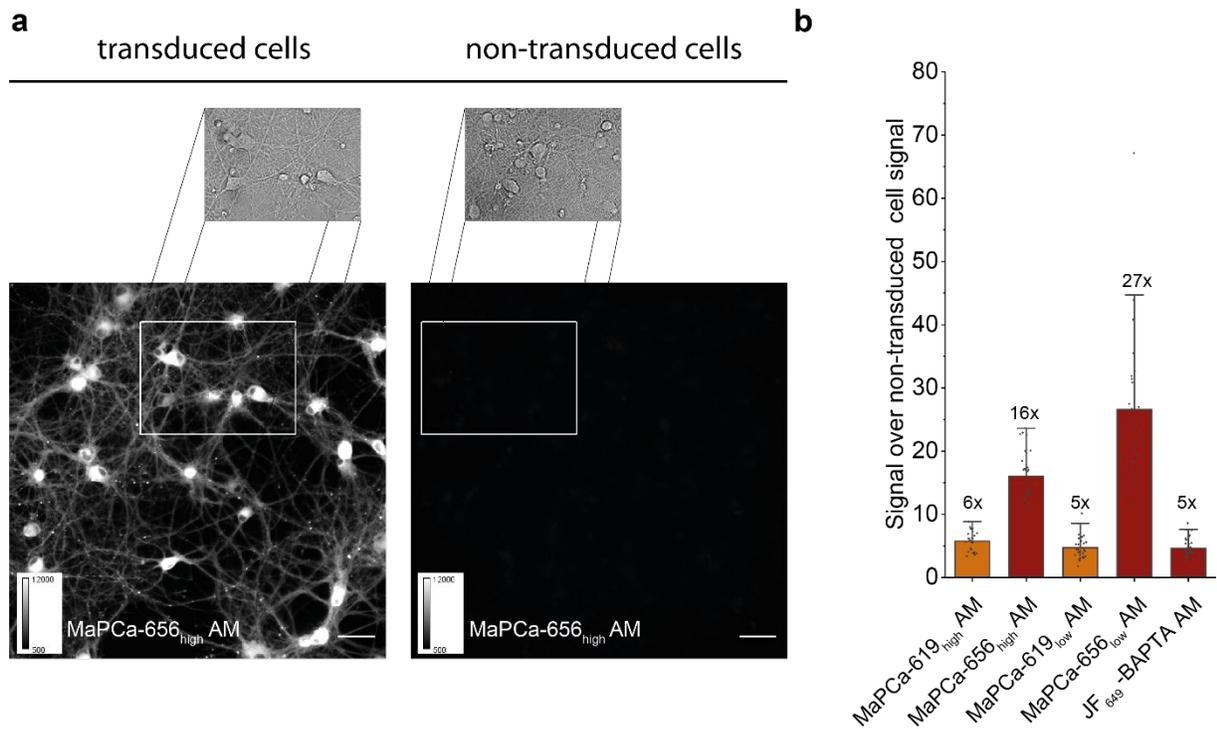
**Supporting Figure S 9:** Labeling kinetics of MaPCa<sub>high</sub> AM. (a) Time-dependent labeling of 293-cells stably expressing a HaloTag-SNAP-tag construct in the nucleus (NLS-tag). The corresponding dye (1  $\mu$ M + 0.04% Pluronic F-127) was added to the cells and imaged on a confocal microscope at the indicated timepoints. Scale bars, 20  $\mu$ m. (b) Analysis of the kinetics of MaPCa-619<sub>high</sub> AM in (a) with  $n = 10$  cells. Depicted is the mean with the corresponding standard deviation.



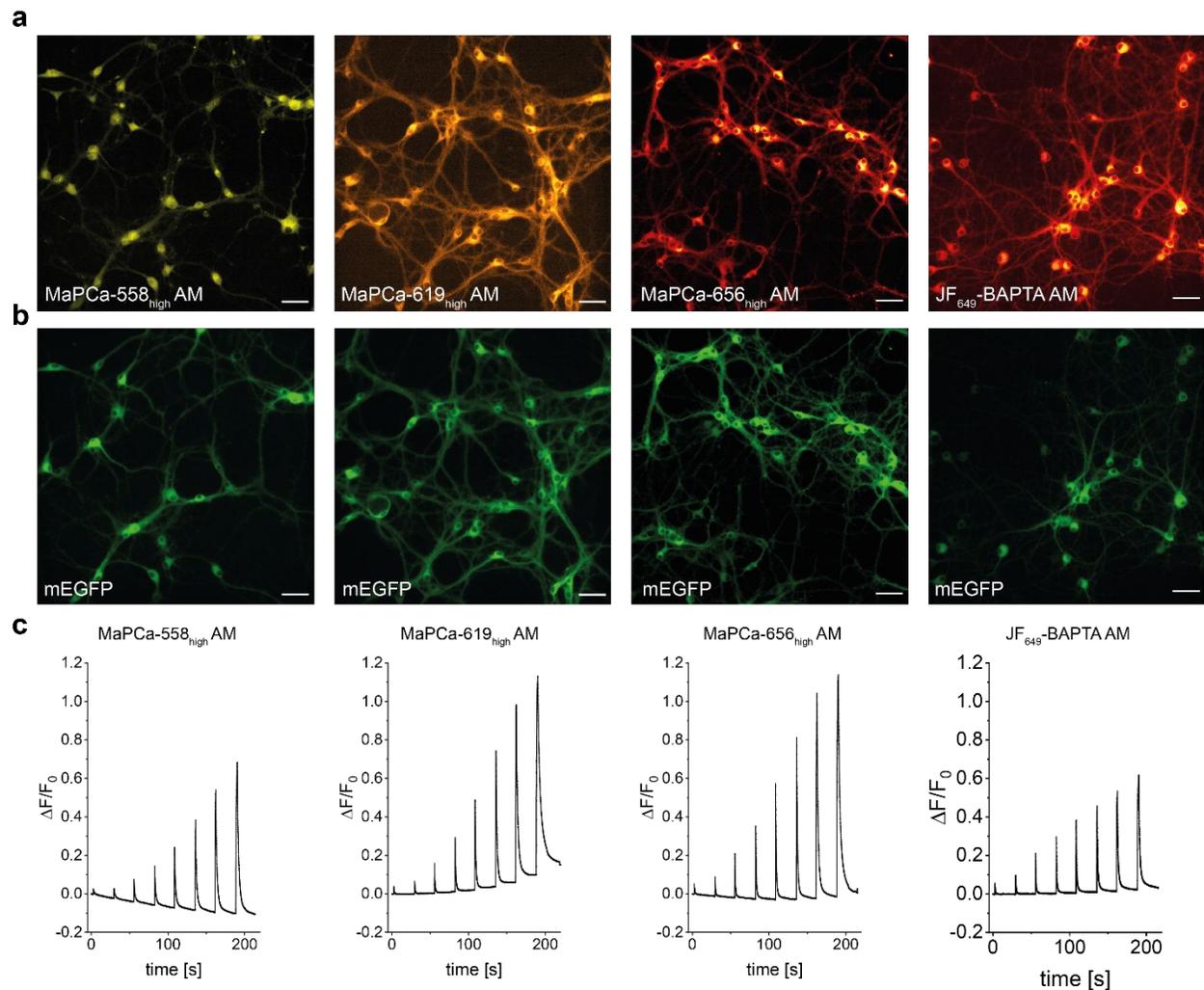
**Supporting Figure S 10:** Cell viability study using alamarBlue HS (Invitrogen). A stable cell line of 293 cells expressing a HaloTag-SNAP-tag in the nucleus (NLS) was incubated with 1  $\mu$ M of the corresponding MaPCa dye or just in medium (control) overnight. The next day, alamarBlue HS was added according to the manufacturers protocol, incubated for 3 h and the fluorescence read out on a plate reader.



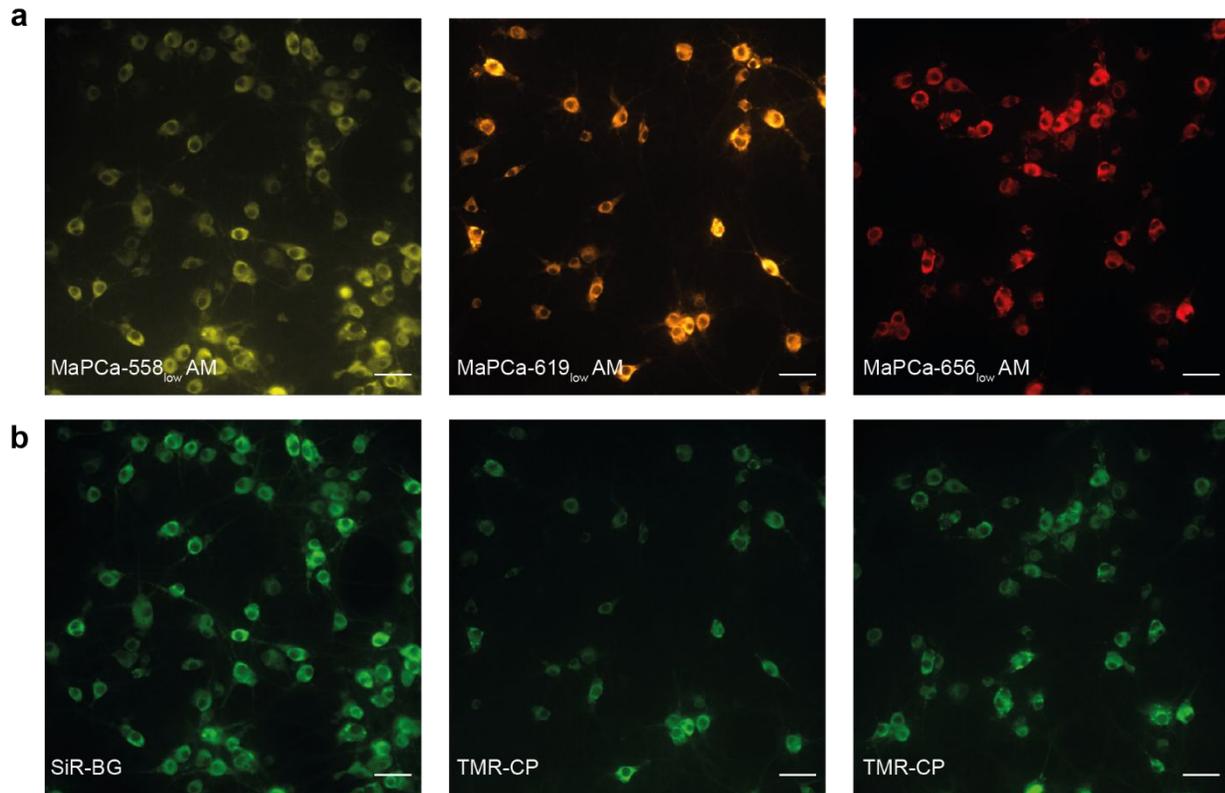
**Supporting Fig. 11:** Analysis of microscopy data of Flp-In 293 cells stably expressing a Halo-SNAP-NLS construct. Live cells were perfused with an HBSS solution and stimulated by fluid exchange with HBSS containing 100  $\mu$ M ATP. (a) mean  $\Delta F/F_0$  of the first peak of the different MaPCa<sub>high</sub> AM-indicators and JF<sub>649</sub>-BAPTA AM upon ATP perfusion ( $n \geq 50$  cells). (b) Exemplary traces of the ATP-perfusion of MaPCa-558<sub>high</sub> AM, MaPCa-619<sub>high</sub> AM, MaPCa-656<sub>high</sub> AM and JF<sub>649</sub>-BAPTA AM. Exposure time: 350 ms.



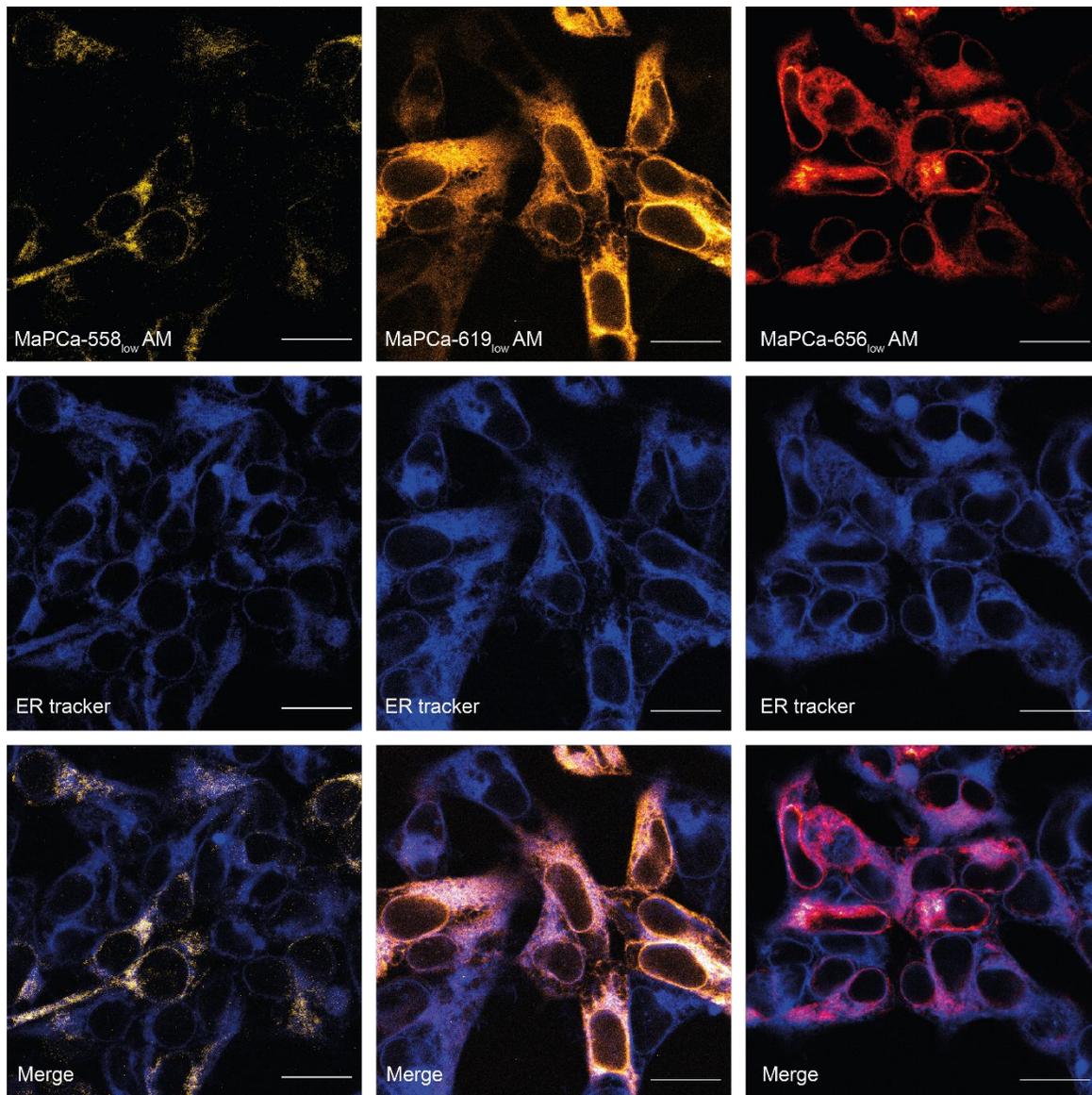
**Supporting Fig. 12:** MaPCa dyes enable no-wash labeling of cytosolic HaloTag expressed in primary rat hippocampal neurons. (a) Widefield fluorescence microscopy data from primary rat hippocampal neurons expressing cytosolically NES-HaloTag-mEGFP and non-transduced cells. Example no-wash fluorescence with brightfield-insert of transduced (left) and non-transduced (right) neurons incubated for 2 h with 1  $\mu$ M MaPCa-656<sub>high</sub> AM. Scale bars, 50  $\mu$ m. (b) Bar plot representing the signal over empty cell signal of the different MaPCa dyes and JF<sub>649</sub>-BAPTA AM. For the MaPCa<sub>low</sub> dyes hippocampal neurons expressing HaloTag in the ER were utilized.  $n \geq 30$  cells.



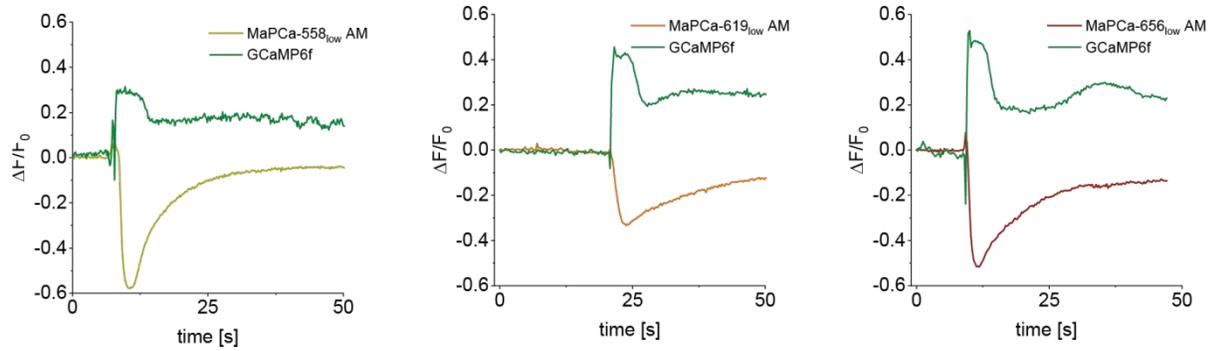
**Supporting Fig. 13:** MaPCa dyes reliably detect induced action potentials in primary rat hippocampal neurons. Widefield microscopy images from primary rat hippocampal neurons (DIV 14-18), expressing NES-HaloTag-mEGFP in the cytosol, incubated with 1  $\mu$ M MaPCa<sub>high</sub> AM for 2 h. (a) Microscopy images of MaPCa-558<sub>high</sub> AM (washed once), MaPCa-619<sub>high</sub> AM (no-wash), MaPCa-656<sub>high</sub> AM (no-wash) and JF<sub>649</sub>-BAPTA AM (no-wash). (b) GFP-channel of (a). (c) Averaged non-corrected traces of electric field stimulation of MaPCa-558<sub>high</sub> AM (washed once), MaPCa-619<sub>high</sub> AM (no-wash), MaPCa-656<sub>high</sub> AM (no-wash) and JF<sub>649</sub>-BAPTA AM (no-wash).  $n \geq 50$  cells. APs: 1,2,5,10,20,40,80,160. Exposure time: 50 ms. Scale bars, 50  $\mu$ m.



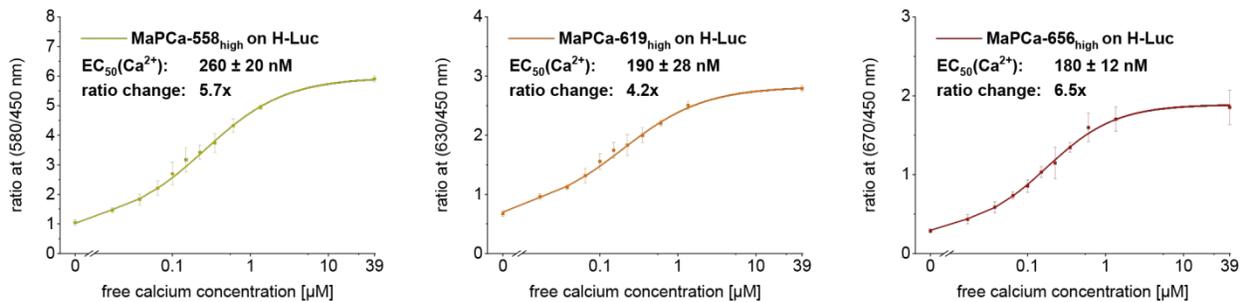
**Supporting Fig. 14:** MaPCa<sub>low</sub> bound to ER-localized HaloTag. Widefield microscopy data from primary rat hippocampal neurons expressing a HaloTag-SNAP construct in the ER, incubated with 1  $\mu$ M of counterstain for 1 h, washed, then incubated with MaPCa<sub>low</sub> AM for 2 h at 1  $\mu$ M. (a) Microscopy images of MaPCa-558<sub>low</sub> AM (left, washed once), MaPCa-619<sub>low</sub> AM (middle, no-wash) and MaPCa-656<sub>low</sub> AM (right, no-wash). (b) counterstain images with SNAP-tag substrates. Left: SiR-BG; middle: TMR-CP; right: TMR-CP. Scale bars 50,  $\mu$ m.



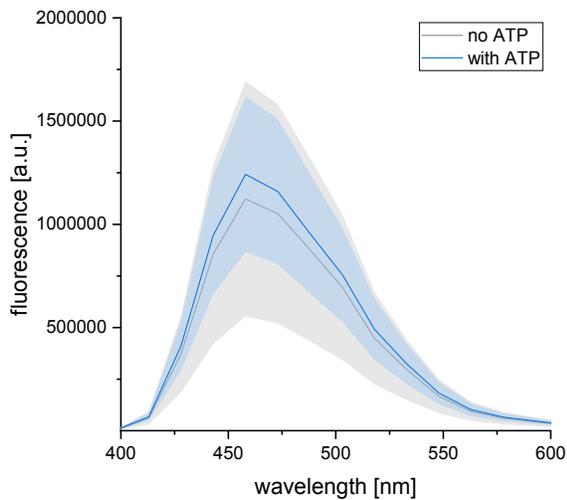
**Supporting Figure S 15:** Colocalization of the MaPCa<sub>low</sub> AM dyes with ER Tracker Blue-White (Invitrogen) in 293 cells stably expressing a HaloTag-SNAP-tag construct in the ER. Cells were incubated with 1  $\mu$ M ER tracker for 30 min and, after washing, with 1  $\mu$ M of the corresponding MaPCa<sub>low</sub> AM dye and imaged after at least 2 h under no-wash conditions. Scala bars, 20  $\mu$ m.



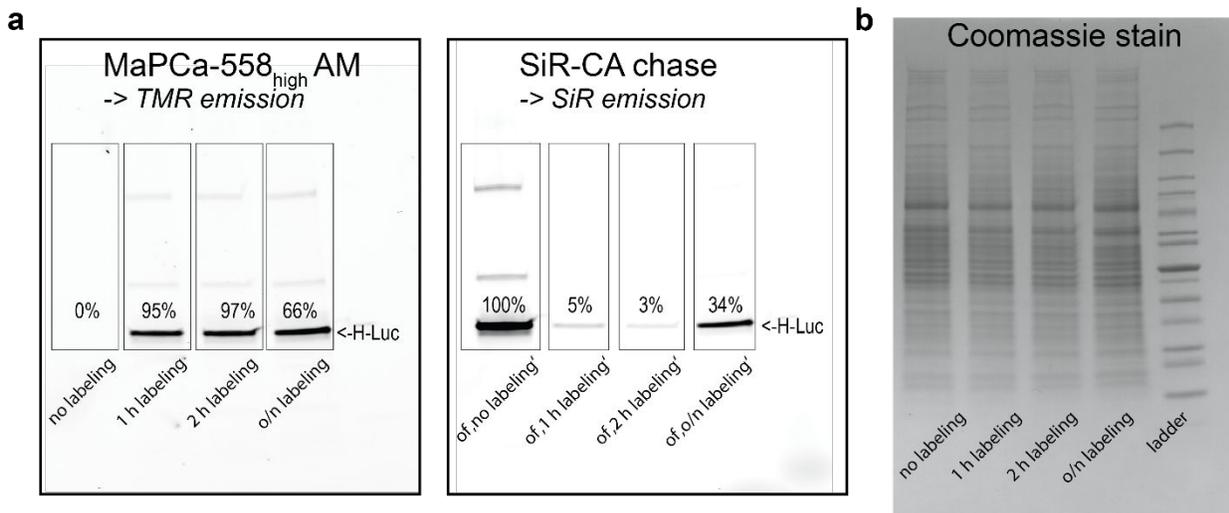
**Supporting Fig. 16:** Representative single-cell fluorescence time trace of rat hippocampal neurons expressing ER-localized HaloTag7 and cytosolic GCaMP6f. Cells were incubated with 1  $\mu\text{M}$  MaPCa<sub>low</sub> AM-dyes for 2 h and imaged under no-wash conditions or washed once (MaPCa-558<sub>low</sub> AM). After several seconds caffeine (final conc.: 20 mM) was added.



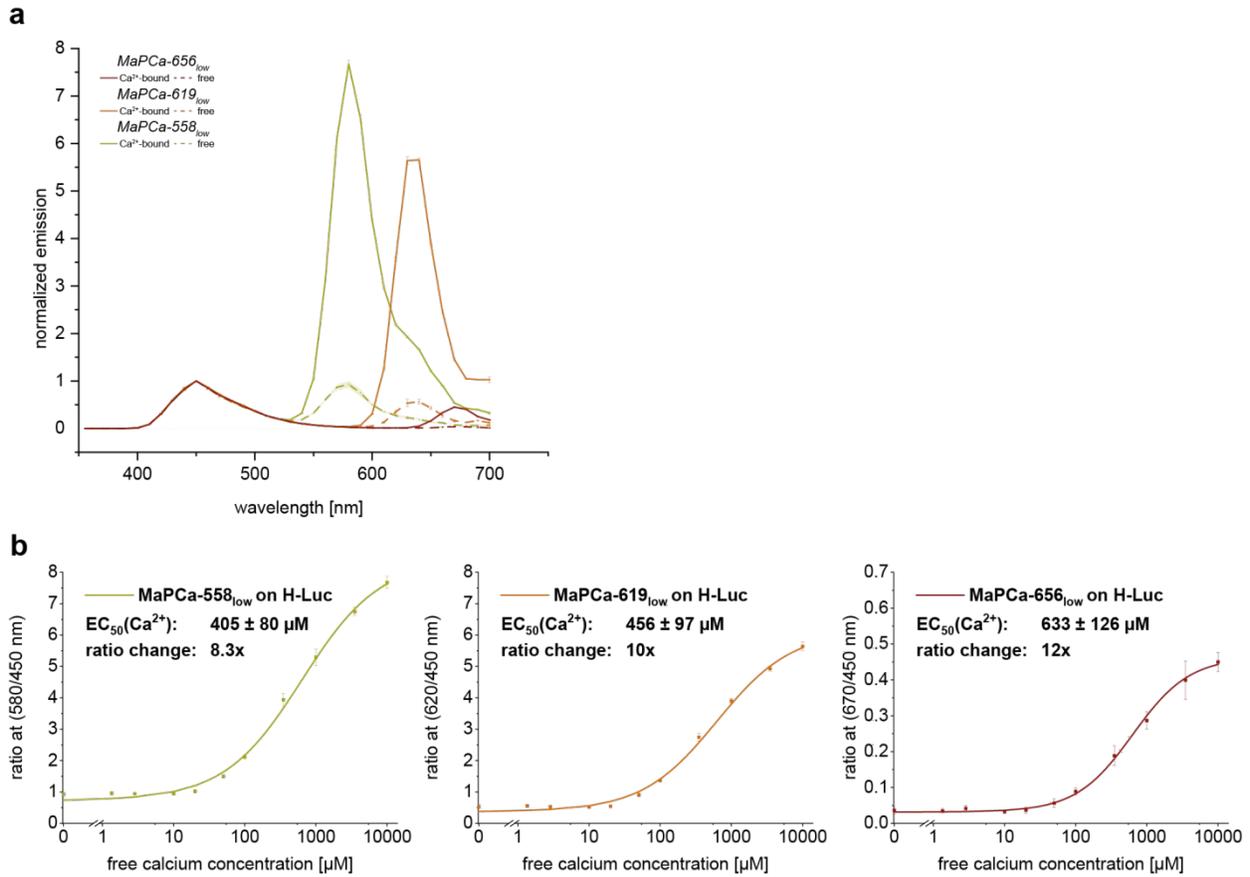
**Supporting Fig. 17:** MaPCa<sub>high</sub> dyes in combination with H-Luc detect calcium changes. *In vitro* calcium titrations of H-Luc labeled with MaPCa<sub>high</sub> indicators. Plotted are the ratios of BRET acceptor/donor.



**Supporting Figure S 18:** Luminescence of 293 cell stably expressing HLuc in the nucleus (NLS). The luminescence was recorded within one minute after the addition of furimazine (no ATP) or the addition of a solution containing furimazine, ATP (final 100  $\mu$ M) and thapsigargin (final 5  $\mu$ M). n = 18 wells per condition.



**Supporting Figure S 19:** Completeness of labeling of H-Luc in 293 cells using MaPCa-558<sup>high</sup> AM. (a) 293 cells stably expressing H-Luc in the nucleus (NLS) were labeled for the indicated durations with 1  $\mu$ M MaPCa-558<sup>high</sup> AM and subsequently for 30 min with 500 nM SiR-CA. After washing, cells were lysed and the lysate analyzed via PAGE-gel. Fluorescence was read out and intensities quantified based on the SiR-CA labeling signal without MaPCa-558<sup>high</sup> AM labeling (100%). (b) The Coomassie stain depicts equal loading quantities.



**Supporting Fig. 20:** MaPCa<sub>low</sub> dyes in combination with H-Luc *in vitro*. (a) Normalized *in vitro* emission spectra of H-Luc labeled MaPCa<sub>low</sub> dyes, with-and without calcium. (b) *In vitro* calcium titrations of H-Luc labeled with MaPCa<sub>low</sub> indicators. Plotted are the ratios of BRET acceptor/donor.

**Supporting Table 1:** Photophysical properties of MaPCa-dyes.

	$F_{max}/F_0$				$\lambda_{Ex}/\lambda_{Em}$ [nm]	$K_D(Ca^{2+})$ [ $\mu M$ ]		$\epsilon$ (mM <sup>-1</sup> cm <sup>-1</sup> )				$\Phi$			
	Ca <sup>2+</sup> - bound	Ca <sup>2+</sup> - free	HT- bound	HT- free											
	HT- binding	HT- binding	Ca <sup>2+</sup> - binding	Ca <sup>2+</sup> - binding		HT	BSA	HT	BSA	HT	BSA	HT	BSA		
								+Ca <sup>2+</sup>	-Ca <sup>2+</sup>	+Ca <sup>2+</sup>	-Ca <sup>2+</sup>	+Ca <sup>2+</sup>	-Ca <sup>2+</sup>	+Ca <sup>2+</sup>	-Ca <sup>2+</sup>
MaPCa-558 <sub>high</sub>	1.3	4	6	22	558/580	0.41	0.57	81	79	71	68	49%	7%	30%	2%
MaPCa-619 <sub>high</sub>	7	30	6	24	619/632	0.57	2.20	91	91	34	18	60%	11%	59%	4%
MaPCa-656 <sub>high</sub>	120	615	6	31	656/670	0.58	1.80	81	75	<2	<2	41%	7%	NA	NA
MaPCa-558 <sub>low</sub>	1.4	2	7	8	560/580	224	487	68	66	55	45	38%	6%	30%	3%
MaPCa-619 <sub>low</sub>	28	33	8	10	618/633	322	806	93	83	5	3	48%	5%	40%	4%
MaPCa-656 <sub>low</sub>	208	153	11	8	655/670	457	926	34	24	<2	<2	44%	5%	NA	NA

\*  $K_D(Ca^{2+})$ -values were calculated using linear regression of  $\log [(F - F_{min})/(F_{max} - F)]$  vs.  $\log [Ca^{2+}]$ , where x-intercept=  $\log K_D(Ca^{2+})$ .

**Supporting Table 2:** Plasmids and stable cell lines generated for this work.

Addgene#	Plasmid	Gene	Stable cell lines
	pcDNA5	HaloTag7-SNAP-NLS	Flp-In 293 TREx
	pcDNA5	H-Luc-NLS	Flp-In 293 TREx
	pcDNA5	CalR-HT7-SNAP-KDEL	Flp-In 293 TREx
	pAAV2-hSyn	AAV2/1-hSyn-NES-HT7-mEGFP-WPRE-SV40	-
	pAAV2-hSyn	AAV2/1-hSyn-CalR-HT7-Pro30-SNAP-KDEL-WPRE-SV40	-
100837-AAV1		AAV1-Syn-GCaMP6f-WPRE-SV40	-

**Supporting Table 3:** Titers of applied rAAVs.

<b>rAAV(1)-plasmid</b>	<b>titer</b>
pGP-AAV2-hSyn2-GCaMP6f-WPRE	2.8x10 <sup>13</sup> GC/mL
NES-HaloTag-mEGFP	3.95x10 <sup>13</sup> GC/mL
CaIR-HaloTag-SNAP-KDEL	3.79x10 <sup>13</sup> GC/mL

## General Experimental Information

Common reagents were purchased from commercial suppliers (Acros, Bachchem, Fluka, Fluorochem, Merck, Roth, Sigma-Aldrich, TCI and Enamine) and used without further purification. BAPTA-Me (**01**), SiR-CA, CPY-CA and TMR-CA were synthesized according to literature procedures<sup>1-4</sup> by N. Mertes, B. Réssy or D. Schmidt. The composition of mixed solvents are given by the volume ratio (v/v). Reactions in the absence of air were performed in oven-dried glassware under argon atmosphere.

Reaction progress was either monitored by thin layer chromatography (TLC) on TLC-aluminium sheets (Silica gel 60 F<sub>254</sub>, Merck) or liquid chromatography-mass spectrometry (LCMS- Shimadzu MS2020 connected to a Nexera UHPLC system equipped with a C18 80 Å 1.9 µm, 2.1x50 mm column by Supelco). A typical gradient was from 10% to 90% B within 6 min using a 1 mL/min flowrate (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile). Flash column chromatography was performed on an automated system (Biotage Isolera One) using pre-packed columns (12-40g, 130-400 mesh, Silicycle). Preparative RP-HPLC was performed on an UltiMate 3000 system (Thermo Fisher Scientific) or Waters e2695 system using either a C18 5 µm, 10x250 mm column (flow rate 4 mL/min, Supelco) or a C18 5 µm, 21.2 x 250 mm column (flow rate 8 mL/min, Supelco). A typical run was over 60 min, solvent ratios are given in the procedures (solvent A: 0.1% TFA in water, solvent B: MeCN). For large batches, a preparative Shimadzu-system equipped with an SPD-M20A diode array detector for product visualization and an LCMS-2020 for mass detection on either a Shimadzu Shim-pack GIS C18 column (5 µm, 30 x 250 mm) or on a Shimadzu Shim-pack GIS C18 column (5 µm, 50 x 250 mm) was used. Solvent A: 0.1% FA in water, solvent B: 0.1% FA in MeCN. Compounds were dried under high vacuum using a lyophilizer (Christ) equipped with a vacuum pump (Vacuubrand).

<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker Ascend™ 400 at 400 MHz (<sup>1</sup>H) or 101 (<sup>13</sup>C) MHz. All spectra were recorded at 298 K. Chemical shifts δ are reported in ppm downfield from tetramethylsilane using the residual deuterated solvent signals as an internal reference (CDCl<sub>3</sub>: δH = 7.26 ppm, δC = 77.16 ppm; DMSO-d<sub>6</sub>: δH = 2.50 ppm, δC = 39.52 ppm; CD<sub>3</sub>CN: δH = 1.94 ppm, δC = 118.26 ppm). Chemical shifts δ are given in ppm, coupling constants J in Hertz (Hz). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet and bs = broad signal. Data was processed using Mnova from Mestrelab.

High-resolution mass spectrometry was performed by the MS-facility of the Max Planck Institute for Medical Research on a Bruker maXis IITM ETD mass spectrometer coupled to a Shimadzu Nexera system controlled via o-TOF-Control 4.1 and Hystar 4.1 SR2 (4.1.31.1) software (Bruker). The acquisition rate was set to 3 Hz. The following source parameters were used for positive mode electrospray ionization (ESI+): End plate offset = 500 V; capillary voltage = 3800 V; nebulizer gas pressure = 45 psi; dry gas flow = 10 L/min; dry temperature = 250°C. As transfer, quadrupole and collision cell settings are mass range dependent, they were fine-tuned with consideration of the respective analyte's molecular weight. For internal calibration sodium format clusters were used. Samples were desalted via fast liquid chromatography (Titan™ C18 UHPLC Column (Supelco), 1.9 µm, 80 Å pore size, 20 × 2.1 mm, 2 min gradient from 10 to 98% aq. MeCN with 0.1% FA). Sample dilution in 10% aq. MeCN and injection volumes were chosen depending on the analyte's ionization efficiency (on-column loadings: 0.25–5.0 ng). Automated internal re-calibration and data analysis of the recorded spectra were performed with DataAnalysis 4.4 SR1 software (Bruker).

### **Cloning, Protein Expression and Purification**

**General.** Plasmids encoding HaloTag-SNAP for bacterial and mammalian cell protein production at different sub-cellular localizations of mammalian cells (NLS, cyto, ER) were previously described.<sup>5-6</sup> The gene encoding H-Luc was cloned by Gibson assembly<sup>7</sup> in the pCDNA5-FRT vector for mammalian expression (ThermoFisher Scientific) with and without a nuclear localization sequence (NLS, nucleus). For rAVV preparation, mEGFP-HaloTag, SNAP-Halo and H-Luc were analogously cloned in plasmid pAAV2-hSyn paying particular attention to ITR integrity verified by Sanger sequencing (Eurofins) after MaxiPrep DNA extraction (Qiagen). H-Luc was cloned in a pET51b(+) vector (Novagen) featuring an N-terminal His10 for protein purification.

**Protein production and purification.** HaloTag<sup>7</sup> and H-Luc proteins were produced and purified as previously explained.<sup>8</sup> In short, the proteins were expressed in the *Escherichia coli* strain BL21(DE3)-pLysS (Novagen) in lysogeny broth (LB)<sup>9</sup> cultures grown at 37°C to an optical density at 600 nm (OD<sub>600nm</sub>) of 0.8, induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown at 17°C overnight in the presence of 1 mM MgCl<sub>2</sub>. Cells were harvested by centrifugation (4,500 g, 10 min, 4°C), lysed by sonication and the lysate was cleared by centrifugation (75,000g, 10 min, 4°C). The proteins were purified using HisPur Ni-NTA Superflow Agarose (Thermo Fisher Scientific, Waltham, MA, USA) by batch incubation followed by washing and elution steps on a polypropylene column (Qiagen). The proteins were subsequently buffer exchanged using a HiPrep 26/10 Desalting column (Cytiva) on an ÄktaPure FPLC to HEPES 50 mM, NaCl 50 mM pH 7.3 (*i.e.* activity buffer). Proteins were concentrated using Ultra-15 mL

centrifugal filter devices (Amicon, Merck KGaA, Darmstadt, Germany) with a molecular weight cut-off (MWCO) smaller than the protein size to a final concentration of 500  $\mu\text{M}$ . Proteins were aliquoted and stored at  $-80^\circ\text{C}$  after flash freezing in liquid nitrogen or kept in glycerol 45% (w/v) at  $-20^\circ\text{C}$ . Correct size and purity of proteins were assessed by SDS-PAGE and liquid chromatography-mass spectrometry (LC-MS) analysis.

## Optical Spectroscopy

**General** Fluorescent molecules were prepared as stock solutions in anhydrous DMSO and diluted such that the DMSO content did not exceed 1% (v/v) in assays. Fluorescence and absorbance measurements were performed on a plate reader (Spark® 20M, Tecan) equipped with filters and a monochromator, while extinction coefficient determination was carried out on a V-770 Spectrophotometer (Jasco) using quartz cuvettes. Bioluminescence spectra for *in vitro* measurements were obtained using the bioluminescence scan mode of a Cytation 5 plate reader (BioTeck), while cellular data was obtained on a Spark® 20M plate reader (Tecan). Absolute Quantum yields were determined using a Quantaurus-QY spectrometer (model C11374) from Hamamatsu with diluted samples ( $A \approx 0.1$ ). Results were processed and illustrated using Origin software. All reported values are averages of independent measurements or wells ( $n \geq 3$ ).

**Fluorescence/absorbance measurements.** Calcium dyes (100  $\mu\text{M}$ ) were pre-incubated with 400  $\mu\text{M}$  protein (either HaloTag7 or BSA) for 2 h at room temperature (rt) in 1x PBS. Subsequently, the dye-protein mixture was diluted to a final dye concentration of 1  $\mu\text{M}$  in 100  $\mu\text{L}$  of a calcium buffer at varying concentrations (0-39  $\mu\text{M}$  or 0-10'000  $\mu\text{M}$ ) in non-binding black flat-bottom 96-well plates (PerkinElmer). Calcium concentrations of buffers for MaPCa<sub>high</sub> experiments were adjusted by mixing different proportions of buffers from a commercially available kit (Invitrogen) following the vendors protocol [EGTA buffer (30 mM MOPS, 10 mM EGTA, 100 mM KCl, pH 7.2); Ca-EGTA buffer (30 mM MOPS, 10 mM Ca-EGTA, 100 mM KCl, pH 7.2)]. Calcium concentrations of buffers for MaPCa<sub>low</sub> experiments were obtained by self-made buffer solutions (100 mM KCl, 30 mM MOPS, pH 7.2) containing various calcium concentrations (0  $\mu\text{M}$ , 1.35  $\mu\text{M}$ , 2.85  $\mu\text{M}$ , 10  $\mu\text{M}$ , 20  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 350  $\mu\text{M}$ , 1'000  $\mu\text{M}$ , 3'500  $\mu\text{M}$ , 10'000  $\mu\text{M}$ ). Fluorescence intensities were measured using a plate reader (Spark 20M, Tecan) after 30 min of incubation at rt with slow shaking (spectra were recorded with an excitation at 520 nm (TMR-variants), 570 nm (CPY-variants) or 610 nm (SiR-variants); band width (BW): 10 nm, step size: 1 nm). Absorbance measurements were performed analogously but in non-binding flat bottom transparent 96-well plates (ThermoScientific), at 5  $\mu\text{M}$  final dye concentration (BW: 1 nm).

$K_D(\text{Ca}^{2+})$ -values were obtained using linear regression of  $\log [(F - F_{\min})/(F_{\max} - F)]$  vs.  $\log [\text{Ca}^{2+}]$ , where x-intercept =  $\log K_D(\text{Ca}^{2+})$ .

**Selectivity measurements.** Calcium dyes (100  $\mu\text{M}$ ) were pre-incubated with 400  $\mu\text{M}$  HaloTag7 for 2 h at room temperature (rt) in 1x PBS. Subsequently, the dye-protein mixture was diluted to a final dye concentration of 1  $\mu\text{M}$  in 100  $\mu\text{L}$  of a calcium-free buffer (30 mM MOPS, 100 mM KCl, 10  $\mu\text{M}$  EGTA, pH 7.2). After measurement of baseline fluorescence ( $F_0$ ), 50  $\mu\text{M}$  of the divalent cations was added. Lastly, additional 50  $\mu\text{M}$   $\text{CaCl}_2$  was added. For the measurement of the Na-selectivity, a modified base buffer (30 mM MOPS, 100 mM NaCl, 10  $\mu\text{M}$  EGTA, pH 7.2) was used and fluorescence compared to the KCl-containing buffer.

**Bioluminescence measurements.** H-Luc protein (5  $\mu\text{M}$ ) was labeled with excess dye (20  $\mu\text{M}$ ) for at least 30 min at rt. The dye-protein mixture was diluted to 1 nM final protein concentration in 100  $\mu\text{L}$  calcium buffer as previously described (see fluorescence measurements) supplemented with 5 mg/mL BSA. Before measurements, 2  $\mu\text{L}$  of diluted NanoGlow furimazine solution (Promega) was spiked in the mixture (final concentrations: H-Luc: 1 nM, calcium indicator: 4 nM, NanoGlow: 800x, BSA: 5 mg/mL). Luminescence emission profile of each well were recorded on a microplate reader (Spark20M, Tecan) over the range 390-660 nm (18 measurements every 15 nm, BW 25 nm). For the photos of Figure 1, the final H-Luc concentration was at 10 nM with 40 nM dye, 400x NanoGlow and 5 mg/mL BSA.

### **$\text{Ca}^{2+}$ binding and unbinding kinetics of MaPCa-619<sub>low</sub> and MaPCa-656<sub>high</sub> measured via stopped flow**

Stopped Flow measurements were performed on a BioLogic SFM-400 stopped-flow instrument (BioLogic Science Instruments, Claix, France) in a single-mixing configuration at 37 °C. MaPCa-656<sub>high</sub> was excited with 656 nm light and a 695 nm long pass filter was used to detect fluorescence emission. MaPCa-619<sub>low</sub> was excited with 619 nm light and a 645 nm long pass filter was used to detect fluorescence emission.

Calcium dyes (100  $\mu\text{M}$ ) were pre-incubated with 400  $\mu\text{M}$  protein HaloTag7 for 2 h at room temperature (rt) in 1x PBS. Subsequently, the dye-protein mixture was diluted to a final dye concentration of 1  $\mu\text{M}$  in SF-buffer (100 mM KCl, 30 mM MOPS, pH 7.2). For  $k_{\text{off}}$  measurements the buffer was supplemented with 5  $\mu\text{M}$  or 500  $\mu\text{M}$   $\text{CaCl}_2$  for MaPCa-656<sub>high</sub> and MaPCa-619<sub>low</sub>, respectively. The protein-dye conjugates were mixed in 1:1 ratio with 25 mM BAPTA in SF-buffer and fluorescence intensity kinetics were recorded. For  $k_{\text{on}}$  measurements protein-dye conjugates were supplemented with 20  $\mu\text{M}$  EGTA to scavenge calcium ion traces. The protein-dye conjugates were mixed in 1:1 ratio with 40  $\mu\text{M}$   $\text{CaCl}_2$  (MaPCa-656<sub>high</sub>) or 1 mM  $\text{CaCl}_2$  (MaPCa-619<sub>low</sub>) in SF-

buffer and fluorescence intensity kinetics were recorded. Each measurement was repeated at least 50 times.

Baselines for  $k_{\text{off}}$  and  $k_{\text{on}}$  measurements were obtained by mixing protein-conjugates 1:1 with SF-buffer containing the same concentrations of  $\text{CaCl}_2$  or EGTA. Background fluorescence was determined for each dye by injecting SF-buffer without supplements and recording background intensity.

For analysis, data from each experiment were averaged, background fluorescence was subtracted, pre-stop time points ( $t < 10$  ms) were removed and the mixing delay (3.7 ms) was added to the time values. The  $k_{\text{off}}$  trace of MaPCa-656<sub>high</sub> was fitted to a first order kinetic model (equation 1) to obtain the kinetic rate constant  $k_{\text{off}}$ . Confidence intervals of the fitted rate constant were estimated using the Monte Carlo method (1000 MC cycles).<sup>10</sup>

$$\text{equation 1: } F(t) = F_P + (F_0 - F_P) e^{-k_{\text{off}} t}$$

with:

t: time

$k_{\text{off}}$ : first order rate constant

F(t): fluorescence at time t

$F_P$ : fluorescence at the plateau

$F_0$ : fluorescence at  $t = 0$

The  $k_{\text{off}}$  trace of MaPCa-619<sub>low</sub> and the  $k_{\text{on}}$  traces for both dyes did not allow to fit a kinetic model to the data since the reactions were already completed after the mixing delay time of 3.7 ms. Minimal values for these rate constants were estimated by predicting fluorescence intensity traces with  $k_{\text{off}}$  or  $k_{\text{on}}$  values that lead to completion within just 3.7 ms. Predictions were calculated using equation 1 ( $k_{\text{off}}$ ) or equation 2 ( $k_{\text{on}}$ ).

$$\text{equation 2: } F(t) = F_P + \frac{F_0 - F_P}{A_0} * \frac{A_0(A_0 - B_0) e^{(A_0 - B_0) k_{\text{on}} t}}{A_0 * e^{(A_0 - B_0) k_{\text{on}} t} - B_0}$$

with:

t: time

$k_{\text{on}}$ : second order rate constant

F(t): fluorescence at time t

F<sub>p</sub>: fluorescence at the plateau

F<sub>0</sub>: fluorescence at t = 0

A<sub>0</sub>: concentration of protein-dye conjugate at t = 0

B<sub>0</sub>: concentration of Ca<sup>2+</sup> at t = 0

### **Cell culture and stable cell line generation**

**Flp-In 293 cells.** Flp-In 293 cells were maintained in high-glucose DMEM (Life Technologies) medium supplemented with GlutaMAX (Life Technologies), sodium pyruvate (Life Technologies), 10% FBS (Life Technologies) and phenol red in a humidified incubator at 37°C and 5% CO<sub>2</sub> atmosphere. Cells were passaged every 2-3 days or at confluency using 0.05% EGTA-free trypsin solution (Life Technologies). Cell lines were regularly tested for mycoplasma contamination *via* PCR. For live-cell imaging, cells were kept in fully supplemented high-glucose DMEM without additional phenol red and at 37°C and 5% CO<sub>2</sub> atmosphere.

Stable Flp-In T-Rex 293 cell lines were generated from commercially available cell lines (ThermoFisher Scientific, Catalog number: R78007) as previously described.<sup>5</sup> The Flp-In System was used to express a HaloTag-SNAP or H-Luc construct in different compartments (cytosol, nucleus,ER) under control of the Cytomegalovirus (CMV) promoter. In brief, the protein encoding pcDNA5-FRT plasmids (without localization sequence (cyto) or with nuclear localization sequence (NLS, nucleus; CalR and KDEL, ER) and pOG44 that encodes Flippase recombinase were co-transfected into the Flp-In T-REx 293 cell line using Lipofectamine3000 transfection reagent (Life Technologies) following manufacturer's protocol. Homologous recombination of the FRT sites and the host cell chromosome yielded the stable cell lines. Selection was performed using 100 µg/mL Hygromycin B (ThermoFisher Scientific) and 15 µg/mL blasticidine (ThermoFisher Scientific).

### **Rat hippocampal neurons**

**General** 24-well glass-bottom plates were coated with poly-L-ornithine (100 µg/mL) for 20 minutes, washed three times with 1x PBS and coated with laminin dissolved in 1x HBSS (1 µg/mL) for 1 hour. New born pups (0-1 days, WISTAR rats) were sacrificed and the hippocampi were isolated. Tryptic digest was followed by mechanical dissection using a pipette to obtain a homogenous solution. The solution was filtered through a cell strainer (40 µm pore size) and cell numbers were measured considering live and dead cells. Final cell numbers were adjusted to 55'000 live cells per well. 2 h after seeding, medium was removed and fresh phenol-red free

neurobasal medium (NB) supplemented with antibiotics (Penicillin/Streptomycin, Life Technologies), GlutaMAX and B27 was added. Neurons were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub> atmosphere. A third of the medium was exchanged every 5-8 days.

**rAAV production and neuron transduction.** Recombinant AAVs (rAAVs) were generated as described in Zolotukhin *et al.* (2002).<sup>11</sup> In brief, plasmids pRV1 (AAV2 Rep and Cap sequences), pH21 (AAV1 Rep and Cap sequences), pFD6 (Adenovirus helper plasmid) and the AAV plasmid containing the recombinant expression cassette flanked by AAV2 packaging signals (ITRs) were transfected via Polyethylenimine 25000 (PEI25000) into HEK293 cells. 5 days post transfection, the medium and cells were harvested. The cells were lysed using TNT extraction buffer (20 mM Tris pH7.5, 150 mM NaCl, 1% TX-100, 10 mM MgCl<sub>2</sub>). The cell debris was spun down and the cell supernatant treated with Benzonase. The rAAVs were purified from the medium and cell supernatant via FPLC using AVB Sepharose columns, which were subsequently concentrated using centrifugal filter devices (Amicon, Merck KGaA, Darmstadt, Germany) with a MWCO of 100 kDa. rAAV1 GCaMP6f are commercially available (Addgene 100837- AAV1).

The rAAV titers were evaluated by qPCR and are summarized in Supporting Table 3.

Hippocampal neurons were transduced with rAAVs after 7-8 days in culture. 0.5 µL of the respective rAAV were diluted into 10 µL of phenol-red free NB medium and subsequently added to corresponding samples. After the 13-18 days *in vitro* (DIV), the neurons were labelled with dyes as described for Flp-In 293 cells.

## Microscopy

**General.** For most microscopy images, a DMI8 widefield microscope (Leica) equipped with a HC PL APO 20x/0.8 (dry) was used. Excitation/Emission settings: TMR-based dyes:  $\lambda_{ex}$  = 515/30 nm, detection  $\lambda_{det}$  = 609/54 nm; CPY/SiR-based dyes:  $\lambda_{ex}$  = 635/18 nm, detection  $\lambda_{det}$  = 700/75 nm. For images of the Supporting Figures 10 and 15 a confocal Leica SP8 microscope equipped with a Leica TCS SP8 X scanhead; a SuperK white light laser, a 355 nm CW laser (Coherent) and a HC PL APO 63x oil objective was used. Excitation/Emission settings: TMR-based dyes:  $\lambda_{ex}$  = 555 nm, detection  $\lambda_{det}$  = 570-620 nm; CPY-based dyes:  $\lambda_{ex}$  = 610 nm, detection  $\lambda_{det}$  = 625-660 nm; SiR-based dyes:  $\lambda_{ex}$  = 645 nm, detection  $\lambda_{det}$  = 660-740 nm. Imaging data was processed using Fiji.<sup>12</sup> ROIs were drawn manually and average fluorescence intensities extracted and plotted using Origin software. HaloTag in this work corresponds to the variant HaloTag7.

**Signal/background measurements Flp-In 293 cells.** Flp-In 293 cells stably expressing HaloTag-SNAP-NLS were co-cultured with non-expressing cells on a 10-well-plate (Greiner bio-

one; cellview cell culture slide; glass bottom). After 2 h incubation with 1  $\mu\text{M}$  MaPCa<sub>high</sub> AM (or JF-indicator) and 0.04% Pluronic-F127, cells were imaged without any washing steps. For analysis,  $n \geq 200$  cells from at least 4 different wells were selected. Raw intensities over ROI of identical size were averaged (AI) for expressing (AI<sub>E</sub>) and non-expressing (AI<sub>NE</sub>) cells and signal/background were calculated by measuring the ratio AI<sub>E</sub>/AI<sub>NE</sub>.

**Labeling Kinetics.** Flp-In 293 cells stably expressing HaloTag-SNAP-NLS were seeded in Ibidi  $\mu$ -Slides (VI 0.4, Poly-L-Lysine coated, part No: 80604) 24 h before start of the experiment. Then, the medium was exchanged with imaging medium containing 1  $\mu\text{M}$  of the corresponding dyes and 0.04% Pluronic-F127. The dish was imaged every 10 min on the confocal microscope (63x oil objective).

**ATP perfusion experiments.** Flp-In 293 stably expressing HaloTag-SNAP-NLS were seeded in Ibidi  $\mu$ -Slides (VI 0.4, Poly-L-Lysine coated, part No: 80604) 24 h before start of the experiment. SNAP-tag was co-stained by incubating cells with TMR-CP or SiR-BG at 1  $\mu\text{M}$  for 30 min to 1h at 37°C. After washing twice with medium, cells were stained with calcium dyes (1  $\mu\text{M}$  + 0.04% Pluronic-F127, 2 h) through HaloTag labeling. The chamber was placed on the microscope and attached to a gravity-flow perfusion system. Cells were perfused with HBSS and, upon trigger, with HBSS containing 100  $\mu\text{M}$  ATP. Image acquisition was performed every 350 ms.

**ER-staining in Flp-In 293 cells.** Flp-In 293 cells stably expressing HaloTag-SNAP-tag in the ER (CalR and KDEL) were cultured on a 10-well-plate (Greiner bio-one; cellview cell culture slide; glass bottom). Cells were incubated with 1  $\mu\text{M}$  ER tracker (ER-Tracker™ Blue-White DPX; Invitrogen™) for 30 min and, after washing, with 1  $\mu\text{M}$  of the corresponding MaPCa<sub>low</sub> AM dye and imaged after at least 2 h under no-wash conditions on the confocal microscope (63x oil objective).

**Live-cell labeling and imaging in primary hippocampal neurons.** Primary hippocampal neurons were prepared from postnatal P0-P2 Wistar rats as previously described<sup>13</sup> and cultured in 24-well glass-bottom plates for 14-18 days. The procedure was conducted in accordance with the Animal Welfare Law of the Federal Republic of Germany (Tierschutzgesetz der Bundesrepublik Deutschland, TierSchG) and the regulation for animals used in experiments (1 August 2013, Tierschutzversuchsverordnung). To euthanize rodents for the subsequent preparation of any tissue, all the regulations given in Section 4 of the TierSchG were followed. As the euthanization of animals is not an experiment on animals according to Section 7 paragraph 2 sentence 3 of the TierSchG, no specific authorization or notification is required. On day 7-8, neurons were transduced with 0.5  $\mu\text{L}$  of the corresponding rAAVs for hSyn1 driven expression of NES-HaloTag-mEGFP; CalR-HaloTag-Snap-tag-KDEL (ER-localizing) or GCaMP6f (Addgene

100837- AAV1). On day 14-18 the neurons were incubated for at least 2 h with 1  $\mu$ M of the corresponding MaPCa dyes containing 0.04% Pluronic-F127. While the medium of MaPCa-558 was subsequently exchanged once, the other dyes were imaged under no-wash conditions. In case a co-stain was utilized, this was added before the MaPCa-staining (or JF-indicator) at 1  $\mu$ M for 1 h followed by a single medium exchange afterwards. For stimulation of the ER-localized MaPCa dyes, a caffeine-solution (final concentration: 20 mM) was administered manually during recording (Exposure time: 250 ms). For electric field stimulation, synaptic blockers NBQX (10  $\mu$ M, Santa Cruz) and APV (25  $\mu$ M, Sigma Aldrich) were added in order to suppress natural spiking activity. Then, a custom-built<sup>14</sup> electrode was inserted into the wells and APs (1,2,5,10,20,40,80,160: with 25 s pauses) were evoked with following settings: Pulse width: 1 ms; Amperage 100 mA; Frequency 80 Hz. Data represents the average of  $N \geq 50$  manually selected ROI of different neurons from at least three different wells. Exposure time: 50 ms.

**Signal/background measurements hippocampal neurons.** Primary rat hippocampal neurons were transduced with a rAAV encoding a cytosolic (NES) HaloTag-eGFP while others were left non-transduced. All wells were labeled on DIV 14-18 with 1  $\mu$ M MaPCa<sub>high</sub> AM-indicator (or JF-indicator) and in presence of 0.04% Pluronic-F127 for at least 2 h. Transduced and untransduced neurons were subsequently imaged as previously explained for Flp-In 293 cells and data treatment was performed analogously.

### **Other live-cell experiments**

**Live-cell measurements with bioluminescent readout.** The Flp-In System (ThermoFisher Scientific) was used to generate stable 293 cells expressing H-Luc in the nucleus. The cells stably expressing H-Luc-NLS were plated on a black flat glass bottom 96-well plates (Eppendorf; tissue culture treated). They were incubated with 1  $\mu$ M MaPCa<sub>high</sub> AM dyes and 0.04% Pluronic-F127 (or without anything for control experiment) for at least 2 h in 100  $\mu$ L at 37°C. 50  $\mu$ L of a mix substrate/ extracellular inhibitor were added in each well to measure only the luminescence coming from intact cells [NanoBRET™ Nano-Glo® Substrate/ Extracellular NanoLuc® Inhibitor solution (final dilution substrate: 1'000x; final dilution inhibitor: 3'000x)]. Half the wells were treated with an additional 10  $\mu$ L of mixture ATP/thapsigargin (final concentrations ATP: 100  $\mu$ M; thapsigargin: 5  $\mu$ M). Measurements were performed stepwise (max three wells at a time, max two minutes delay between pipetting and imaging) in order to ensure identical conditions. Spectra from whole sample wells were measured on a plate reader (Spark20M, Tecan). Data represents averaged results from  $N=4$  experimental replicates with standard deviations. The average ratios between BRET

acceptor and BRET donor of ATP/thapsigargin-treated wells were then compared to the non-treated wells.

The z-factor was calculated with the formula

$$z - factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p + \mu_n|}$$

With  $\sigma$  = standard deviation;  $\mu$  = mean; p = positive control and n = negative control.

**Labeling completeness H-Luc.** Flp-In 293 cells stably expressing H-Luc-NLS were plated on 6-well plate and treated with 1  $\mu$ M MaPCa-556<sub>high</sub> AM (+ 0.04% Pluronic) for the indicated times. Then, medium was exchanged with medium containing 500 nM SiR-CA for 30 min. After 1x washing with PBS, cells were lysed using Cell Lytic M (Merck KGaA) with complete protease inhibitor (Roche). The centrifuged cell-lysate was run on SDS-PAGE-gel and analyzed using Typhoon laser-scanner platform Amersham Inc (wavelengths pre-set for Cy3 or Cy5, automatic PMT adjustment). Lastly, the gel was stained with coomassie blue overnight and imaged on a ChemiDoc imaging system.

**Cell viability.** The cell viability study was executed according to the manufacturers protocol using alamarBlue™ HS Cell Viability Reagent (Invitrogen™). In brief, 293 cell stably expressing a HaloTag-SNAP-tag construct in the nucleus (NLS) were treated with 1  $\mu$ M of the corresponding MaPCa dye (+0.04% Pluronic F-127) overnight or left untreated (ctrl). Then, to the 90  $\mu$ L medium 10  $\mu$ L of alamarBlue™ HS was added and fluorescence read out after 3 h on a plate reader. The fluorescence of the control wells was compared to the treated cells. The TMR-based constructs were not measured due to an overlap of wavelength with the alamarBlue™ HS reagent.

**General statistics.** All measurements were measured at least in triplicates and presented as mean with s.d. if not stated otherwise. Cellular imaging experiments were performed at least twice on different days.

## Protein Sequences

Protein Amino Acid sequence of NES-HaloTag7-mEGFP expressed in cultured neurons

MLQNELALKLAGLDINKTGGSGSEIGTGFPDPHYVEVLGERMHYVDVGPRDGTPLVFLHGNP  
TSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLYFFDDHVRFMDFIEALGLEEVVLIHDW  
GSALGFHWAKRNPERVKGIAFMFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGT  
LPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVP  
KLLFWGTPGVLIPPAEAARLAKSLPNCKAVDIGPGLNLLQEDNPDIGSEIARWLSTLEISGMVSK  
GEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGV  
QCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEKDTLVNRIELKGIDFKE  
DGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPD  
NHYLSTQSKLSKDPNEKRDHMLLEFVTAAGITLGMDELYK

Protein Amino Acid sequence of CalR-HaloTag7-P30-SNAP-KDEL expressed in cultured neurons and mammalian cells

MLLSVPLLLGLLGLAVAGGSGGSEFGSEIGTGFPDPHYVEVLGERMHYVDVGPRDGTPLVFL  
HGNPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLYFFDDHVRFMDFIEALGLEEVVLI  
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Protein Amino Acid sequence of HaloTag7-P30-SNAP-3xNLS expressed in mammalian cells

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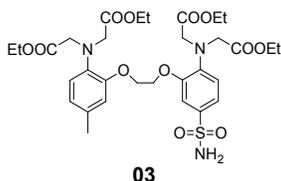
Protein Amino Acid sequence of HLuc-NLS expressed in mammalian cells

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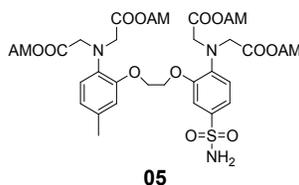
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## Chemical synthesis and characterization



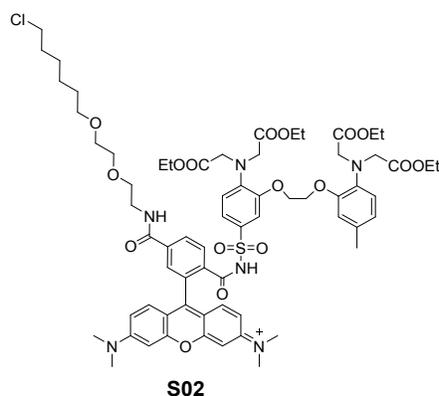
A mixture of thionyl chloride (169  $\mu$ L, 2.32 mmol, 7.0 eq.) and chlorosulfonic acid (122  $\mu$ L, 1.83 mmol, 5.5 eq.) was cooled on an ice bath for 15 minutes. Then, a solution of N-[2-[2-[2-[Bis(2-ethoxy-2-oxoethyl)amino]-5-methylphenoxy]ethoxy]phenyl]-N-(2-ethoxy-2-oxoethyl)glycine ethyl ester\* **01** (200 mg, 332  $\mu$ mol, 1.0 eq.) in DCM (1.6 mL) was added slowly. The solution was allowed to warm to room temperature and stirred for 24 h. The solution was slowly added to a cooled mixture of EtOAc and aqueous ammonia ( $\approx$  10 mL). The resulting mixture was further stirred for 2 h. The product was extracted with EtOAc and the combined organic layers dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography (25 g SiO<sub>2</sub> column, 20-60% EtOAc in hexanes) to give **03** as an off-white powder (141 mg, 62%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.46 (s, 1H), 6.93 – 6.79 (m, 4H), 6.74 (s, 1H), 4.92 (d, J = 4.5 Hz, 2H), 4.30 (hept, J = 2.7 Hz, 4H), 4.11 (d, J = 6.4 Hz, 8H), 4.03 (p, J = 7.0 Hz, 8H), 2.57 (s, 3H), 1.15 (td, J = 7.1, 4.2 Hz, 12H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 171.5, 171.1, 153.1, 150.2, 139.5, 137.0, 132.1, 131.09, 122.3, 121.8, 119.3, 119.3, 116.2, 113.75, 7.46, 67.0, 61.0, 60.8, 53.4, 19.9, 14.1, 14.0. HRMS (ESI<sup>+</sup>) m/z calcd. for C<sub>31</sub>H<sub>43</sub>N<sub>3</sub>O<sub>12</sub>S [M]<sup>+</sup>, 682.2640; found 682.2635.

\* synthesized according to published procedures<sup>15</sup>



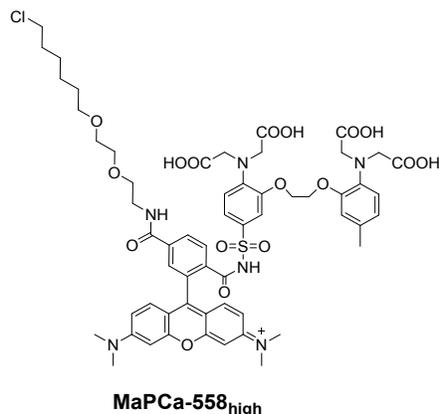
Sulfonamide **03** (110 mg, 161  $\mu$ mol, 1 eq.) was dissolved in anhydrous DCM (1.1 mL). Then 4-dimethylaminopyridine (29.6 mg, 242  $\mu$ mol, 1.5 eq.) and di-tert-butyl dicarbonate (276  $\mu$ L, 1.29 mmol, 8.0 eq.) were added and the solution was stirred for 24 h at 35°C. The product was extracted with DCM and the combined organic layers dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography (25 g SiO<sub>2</sub> column, 20-70% EtOAc in hexanes) to give Boc-protected **03** as an off-white powder (80 mg, 59%). It was directly re-dissolved in methanol (500  $\mu$ L) and tetrahydrofuran (4 mL).

Subsequently, potassium hydroxide (1 M, 382  $\mu\text{L}$ , 382  $\mu\text{mol}$ , 4 eq.) was added followed by potassium hydroxide (0.1M, 2.86 mL, 286  $\mu\text{mol}$ , 3 eq.). The solution was stirred at room temperature for 2 h. In the following, the solution was neutralized using 0.1 M HCl and acetic acid. The mixture was dried by lyophilization and the crude re-suspended in MeCN (2 mL). Then add DIPEA (316  $\mu\text{L}$ , 1.91 mmol, 20 eq.) and bromomethyl acetate (65.5  $\mu\text{L}$ , 668  $\mu\text{mol}$ , 7 eq.) was added. After 48 h, the solvents were evaporated and the crude residue was suspended in DCM (2 mL) and TFA (200  $\mu\text{L}$ ). The resulting solution was stirred for 2 h before being neutralized using  $\text{Na}_2\text{CO}_3$ . The product was extracted with EtOAc and the combined organic layers were dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude mixture was purified by HPLC (50 mL/min; A/B 30-80%; 60 min) to obtain **05** as white powder (48 mg, 61%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  = 7.43 (d,  $J$  = 2.4 Hz, 1H), 7.01 – 6.84 (m, 4H), 6.77 (s, 1H), 5.58 (d,  $J$  = 2.8 Hz, 8H), 5.19 (s, 2H), 4.33 (dq,  $J$  = 7.6, 4.3, 3.5 Hz, 4H), 4.16 (d,  $J$  = 4.4 Hz, 8H), 2.60 (d,  $J$  = 2.5 Hz, 3H), 2.16 – 1.99 (m, 12H);  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  = 170.3, 170.3, 169.9, 169.8, 153.2, 150.5, 138.8, 136.0, 132.7, 132.1, 123.3, 122.0, 120.1, 120.1, 116.2, 113.6, 79.5, 79.3, 77.5, 77.4, 77.2, 76.8, 67.4, 67.0, 53.6, 53.4, 20.9, 20.8, 20.2. HRMS (ESI $^+$ )  $m/z$  calcd. for  $\text{C}_{35}\text{H}_{43}\text{N}_3\text{O}_{20}\text{S}$  [M+Na] $^+$ , 880.2053; found 880.2052.

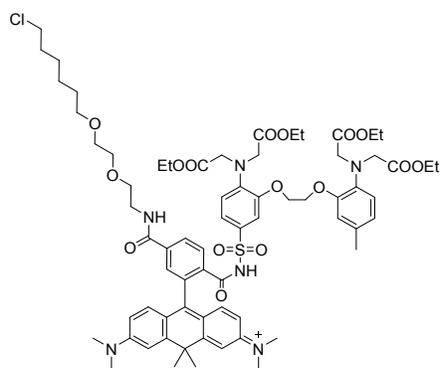


TMR-CA (1.0 mg, 1.57  $\mu\text{mol}$ , 1.0 eq.) and sulfonamide **03** (1.3 mg, 1.89  $\mu\text{mol}$ , 1.2 eq.) were solubilized in DCM (1 mL). Then, DMAP (1.5 mg, 12.6  $\mu\text{mol}$ , 8.0 eq.) and EDC-HCl (2.4 mg, 12.6  $\mu\text{mol}$ , 8.0 eq.) were added and the tube was sealed. The solution was stirred at 60°C overnight. The crude mixture was purified by HPLC (4 mL/min; A/B 30-90%; 60 min) to obtain rhodamine conjugate **S02** as red powder (1.2 mg, 56%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 7.96 – 7.91 (m, 2H), 7.55 – 7.50 (m, 1H), 7.39 (s, 1H), 7.03 – 6.74 (m, 5H), 6.67 – 6.57 (m, 5H), 4.30 (s, 4H), 4.11 – 3.93 (m, 14H), 3.70 – 3.56 (m, 6H), 3.55 – 3.45 (m, 4H), 3.40 (t,  $J$  = 6.7 Hz, 2H), 3.11 (s, 12H), 2.31 (s, 3H), 1.81 – 1.66 (m, 2H), 1.54 (p,  $J$  = 6.8 Hz, 2H), 1.47 – 1.37 (m, 2H), 1.36 –

1.27 (m, 2H), 1.13 (td,  $J = 7.1, 3.8$  Hz, 12H).  $^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ )  $\delta = 170.8, 170.4, 170.1, 164.6, 158.3, 158.0, 153.5, 152.4, 149.5, 138.9, 136.1, 128.6, 128.4, 121.3, 121.3, 120.3, 118.1, 115.5, 113.7, 108.5, 98.3, 70.1, 69.5, 69.4, 68.6, 67.4, 66.9, 60.2, 60.1, 60.1, 53.1, 52.9, 45.4, 32.0, 29.0, 26.1, 24.9, 19.5, 13.8$ . HRMS (ESI $^+$ )  $m/z$  calcd. for  $\text{C}_{66}\text{H}_{83}\text{ClN}_6\text{O}_{17}\text{S}$   $[\text{M}]^{2+}$ , 650.2685; found 650.2680.

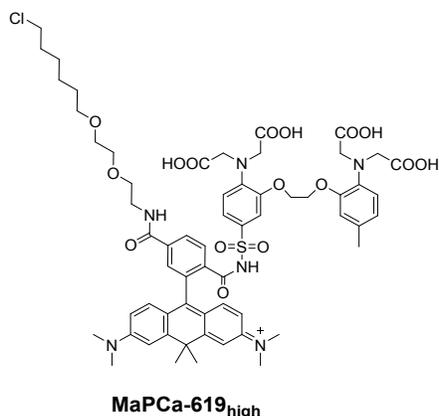


To a solution of **S02** (500  $\mu\text{g}$ , 0.348  $\mu\text{mol}$ , 1.0 eq.) in MeOH (100  $\mu\text{L}$ ) and THF (100  $\mu\text{L}$ ) was added an aqueous solution of potassium hydroxide (0.1M, 192  $\mu\text{L}$ , 19.2  $\mu\text{mol}$ , 50 eq.) and the resulting solution stirred for 6 h at rt. In the following, the solution was neutralized by the addition of 0.1 M HCl and acetic acid. The solvents were evaporated and the crude mixture purified by HPLC (4 mL/min; A/B 10-90%; 60 min) to obtain **MaPCa-558<sub>high</sub>** as red powder (0.3 mg, 66%).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta = 12.39$  (s, 4H), 8.69 (t,  $J = 5.7$  Hz, 1H), 8.02 (d,  $J = 8.1$  Hz, 1H), 7.91 (d,  $J = 7.9$  Hz, 1H), 7.42 (s, 1H), 7.01 – 6.92 (m, 2H), 6.90 – 6.70 (m, 4H), 6.50 – 6.36 (m, 6H), 4.27 (dq,  $J = 9.9, 5.6$  Hz, 4H), 3.97 (d,  $J = 32.2$  Hz, 8H), 3.58 (t,  $J = 6.6$  Hz, 2H), 3.44 (s, 6H), 3.28 (d,  $J = 6.5$  Hz, 4H), 2.94 (s, 12H), 2.21 (s, 3H), 1.66 (p,  $J = 6.7$  Hz, 2H), 1.40 (p,  $J = 7.1$  Hz, 2H), 1.37 – 1.24 (m, 2H), 1.24 (dq,  $J = 8.9, 4.5, 3.9$  Hz, 2H).  $^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ )  $\delta = 172.5, 171.9, 165.4, 164.7, 153.4, 152.3, 151.3, 149.4, 140.3, 139.5, 136.6, 133.3, 128.8, 128.6, 121.7, 121.1, 118.2, 115.2, 108.5, 106.3, 98.2, 70.1, 69.5, 69.3, 69.1, 68.6, 67.2, 53.5, 52.9, 45.4, 40.1, 39.9, 39.7, 39.5, 39.3, 39.1, 38.9, 32.0, 29.00, 26.1, 24.9, 19.6$ . HRMS (ESI $^+$ )  $m/z$  calcd. for  $\text{C}_{58}\text{H}_{68}\text{N}_6\text{O}_{17}\text{ClS}$   $[\text{M}]^{2+}$ , 594.2059; found 594.2057. HRMS (ESI $^+$ )  $m/z$  calcd. for molecule attached to the HaloTag-protein, 36616.6677; found 36616.3602.

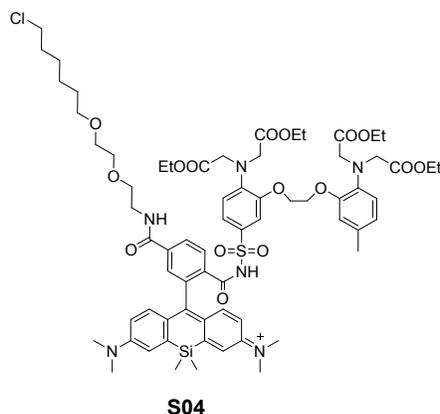


**S03**

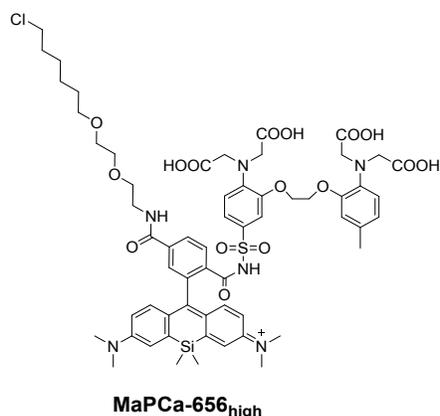
To a solution of CPY-CA (5.0 mg, 7.54  $\mu\text{mol}$ , 1.0 eq.) in anhydrous DCM (1.5 mL) was added thionyl chloride (8.2  $\mu\text{L}$ , 113  $\mu\text{mol}$ , 15 eq.) followed by pyridine (4.9  $\mu\text{L}$ , 60.3  $\mu\text{mol}$ , 8.0 eq.). The solution was stirred for 30 minutes at room temperature. In the following, the mixture was heated to 60°C until most of the solvent had evaporated. Then, the vial was closed and the crude dried under high vacuum. After 1 h, a solution of BAPTA-Et-sulfonamide **03** (6.2 mg, 9.05  $\mu\text{mol}$ , 1.2 eq.), DIPEA (37  $\mu\text{L}$ , 226  $\mu\text{mol}$ , 30 eq.) and 4-dimethylaminopyridine (1.8 mg, 15.1  $\mu\text{mol}$ , 2.0 eq.) in anhydrous MeCN (2.4 mL) was added under argon atmosphere. The solution was heated to 60°C and stirred for 1 h. The solvents were evaporated and the crude mixture purified by HPLC (8 mL/min; A/B 50-90%; 60 min) to obtain **S03** as blue powder (4.2 mg, 42%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 7.92 (d,  $J$  = 8.0 Hz, 1H), 7.75 (dd,  $J$  = 8.0, 1.4 Hz, 1H), 7.48 (d,  $J$  = 2.5 Hz, 2H), 7.39 (s, 1H), 7.08 (d,  $J$  = 1.4 Hz, 1H), 6.96 – 6.83 (m, 6H), 6.74 (d,  $J$  = 8.8 Hz, 2H), 6.58 (s, 1H), 4.31 (s, 4H), 4.09 – 3.95 (m, 16H), 3.61 – 3.47 (m, 10H), 3.39 (t,  $J$  = 6.7 Hz, 2H), 3.12 (s, 12H), 2.11 (s, 3H), 1.91 (d,  $J$  = 3.9 Hz, 6H), 1.73 (dt,  $J$  = 14.7, 6.8 Hz, 2H), 1.53 (p,  $J$  = 6.9 Hz, 2H), 1.47 – 1.36 (m, 2H), 1.36 – 1.27 (m, 2H), 1.13 (q,  $J$  = 7.0 Hz, 12H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$ = 171.8, 171.2, 166.6, 166.2, 161.9, 161.5, 154.8, 154.3, 150.3, 146.5, 145.9, 141.1, 129.5, 129.1, 127.4, 124.8, 123.3, 122.0, 121.8, 119.3, 116.0, 115.4, 71.3, 70.3, 70.0, 69.6, 67.3, 61.3, 61.1, 53.9, 45.2, 43.8, 40.0, 38.4, 36.1, 33.3, 32.6, 29.9, 29.4, 26.7, 25.5, 20.4, 14.2, 14.1, 1.2. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for  $\text{C}_{69}\text{H}_{89}\text{ClN}_6\text{O}_{16}\text{S}$   $[\text{M}]^{2+}$ , 663.2945; found 663.2939.



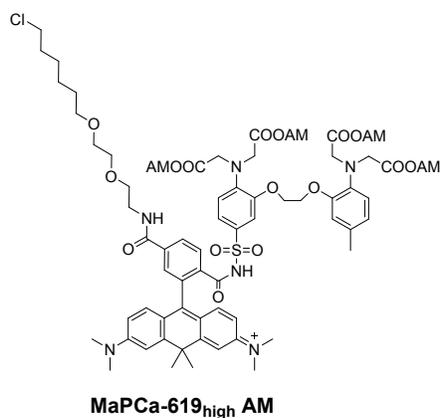
To a solution of **S03** (5.2 mg, 3.92  $\mu\text{mol}$ , 1.0 eq.) in methanol (1.0 mL) and tetrahydrofuran (1.0 mL) was added potassium hydroxide (0.1M, 1.2 mL). The mixture was stirred at room temperature for 8 h. The solution was neutralized by the addition of 0.1 M HCl and acetic acid. The solvents were evaporated and the crude mixture purified by HPLC (4 mL/min; A/B 40-80%; 60 min) to obtain **MaPCa-619<sub>high</sub>** as blue powder (2.0 mg, 42%).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-d}_6$ ):  $\delta$  = 8.67 (t,  $J$  = 5.8 Hz, 1H), 7.90 (d,  $J$  = 2.1 Hz, 2H), 7.14 (s, 1H), 7.04 – 6.79 (m, 5H), 6.79 – 6.71 (m, 2H), 6.53 (dd,  $J$  = 9.0, 2.5 Hz, 2H), 6.45 (dd,  $J$  = 8.9, 5.4 Hz, 2H), 4.33 – 4.24 (m, 4H), 3.99 (d,  $J$  = 27.8 Hz, 8H), 3.58 (t,  $J$  = 6.6 Hz, 2H), 3.40 (ddd,  $J$  = 17.1, 6.1, 3.5 Hz, 6H), 3.28 (t,  $J$  = 6.6 Hz, 2H), 2.94 (s, 12H), 2.11 (s, 3H), 1.85 (s, 6H), 1.65 (p,  $J$  = 6.7 Hz, 2H), 1.46 – 1.17 (m, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-d}_6$ )  $\delta$  = 172.5, 171.9, 166.4, 164.9, 158.2, 157.9, 153.2, 149.4, 144.9, 140.4, 139.5, 136.5, 133.1, 129.1, 128.3, 128.0, 123.9, 122.4, 121.6, 121.1, 118.2, 115.8, 112.3, 71.9, 70.1, 69.5, 69.4, 68.6, 67.1, 53.5, 52.9, 45.3, 37.6, 35.7, 32.8, 32.0, 29.0, 26.1, 24.9, 19.7. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for  $\text{C}_{61}\text{H}_{74}\text{ClN}_6\text{O}_{16}\text{S}$   $[\text{M}]^{2+}$ , 607.2319; found 607.2311. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for molecule attached to the HaloTag-protein, 36904.4347; found 36904.4396.



To a solution of SiR-CA (5.0 mg, 7.36  $\mu\text{mol}$ , 1.0 eq.) in anhydrous DCM (1.4 mL) was added thionyl chloride (8.1  $\mu\text{L}$ , 110  $\mu\text{mol}$ , 15 eq.) followed by pyridine (4.8  $\mu\text{L}$ , 58.9  $\mu\text{mol}$ , 8.0 eq.). The solution was stirred for 30 minutes at room temperature. In the following, the mixture was heated to 60°C until almost no solvent was left. Then, the vial was closed and the crude dried under high vacuum. After 1 h, a solution of BAPTA-Et-sulfonamide **03** (6.2 mg, 9.05  $\mu\text{mol}$ , 1.2 eq.), DIPEA (37  $\mu\text{L}$ , 226  $\mu\text{mol}$ , 30 eq.) and 4-dimethylaminopyridine (1.84 mg, 15.1  $\mu\text{mol}$ , 2.0 eq.) in anhydrous MeCN (2.4 mL) was added under argon atmosphere. The solution was heated to 60°C and stirred for 1 h. The solvents were evaporated and the crude mixture purified by HPLC (8 mL/min; A/B 50-90%; 60 min) to obtain **S04** as pale green-blue powder (4.2 mg, 41%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 7.92 (d,  $J$  = 8.1 Hz, 1H), 7.72 (d,  $J$  = 9.4 Hz, 1H), 7.58 – 7.43 (m, 2H), 7.12 – 7.02 (m, 3H), 6.95 – 6.77 (m, 6H), 6.73 – 6.63 (m, 1H), 6.53 (s, 1H), 4.30 (s, 4H), 4.16 – 3.96 (m, 16H), 3.62 – 3.47 (m, 10H), 3.40 (t,  $J$  = 6.7 Hz, 2H), 3.10 (s, 12H), 1.97 (s, 3H), 1.80 – 1.68 (m, 2H), 1.54 (p,  $J$  = 6.8 Hz, 2H), 1.47 – 1.37 (m, 2H), 1.38 – 1.27 (m, 2H), 1.17 – 1.12 (m, 12H), 0.65 (d,  $J$  = 18.6 Hz, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$ = 171.9, 167.0, 166.2, 156.1, 154.1, 150.4, 147.2, 141.0, 135.9, 134.1, 130.4, 129.7, 129.5, 126.9, 124.7, 122.9, 122.1, 122.0, 119.4, 117.9, 116.6, 115.3, 75.6, 71.3, 70.4, 70.1, 69.7, 67.3, 66.7, 61.5, 61.3, 54.3, 45.2, 41.8, 40.0, 32.6, 29.8, 29.5, 26.8, 25.5, 22.8, 20.2, 14.2, 1.2, 0.1, -0.6. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for  $\text{C}_{68}\text{H}_{89}\text{ClN}_6\text{O}_{16}\text{SSi}$   $[\text{M}]^{2+}$ , 671.2830; found 671.2821.

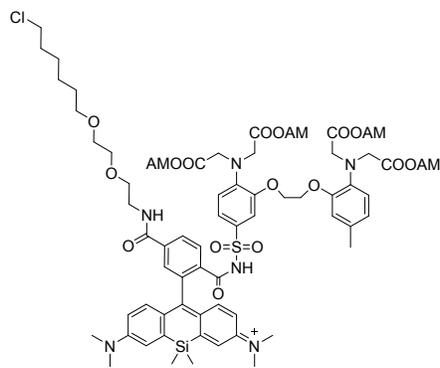


To a solution of **S04** (6.0 mg, 4.47  $\mu\text{mol}$ , 1.0 eq.) in methanol (1.0 mL) and tetrahydrofuran (1.0 mL), was added potassium hydroxide (0.1 M, 1.3 mL). The mixture was stirred at rt for 8 h. The solution was neutralized by the addition of 0.1 M HCl and acetic acid. The solvents were evaporated and the crude mixture purified by HPLC (4 mL/min; A/B 30-70%; 60 min) to obtain **MaPCa-656<sub>high</sub>** as pale green-blue powder (3.1 mg, 56%).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  = 8.65 (t,  $J$  = 5.7 Hz, 1H), 7.95 – 7.84 (m, 2H), 7.22 (s, 1H), 7.04 – 6.92 (m, 4H), 6.85 (q,  $J$  = 6.6, 6.0 Hz, 2H), 6.79 – 6.69 (m, 2H), 6.59 (d,  $J$  = 9.1 Hz, 2H), 6.47 (d,  $J$  = 8.9 Hz, 2H), 4.26 (h,  $J$  = 5.9 Hz, 4H), 3.98 (d,  $J$  = 28.3 Hz, 8H), 3.58 (t,  $J$  = 6.6 Hz, 2H), 3.47 – 3.36 (m, 6H), 3.32 – 3.24 (m, 4H), 2.92 (s, 12H), 1.96 (s, 3H), 1.65 (p,  $J$  = 6.9 Hz, 2H), 1.46 – 1.37 (m, 2H), 1.37 – 1.24 (m, 2H), 1.25 – 1.15 (m, 2H), 0.56 (d,  $J$  = 21.8 Hz, 6H).  $^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ )  $\delta$  = 172.5, 171.9, 166.5, 164.9, 158.3, 158.0, 156.0, 153.1, 149.4, 148.1, 140.3, 139.4, 136.5, 134.5, 133.0, 129.3, 128.9, 128.3, 121.6, 121.1, 118.2, 115.1, 70.1, 69.5, 69.4, 68.6, 67.1, 53.5, 52.9, 45.3, 32.0, 30.7, 29.0, 26.1, 24.9, 19.3, -0.1, -0.7. HRMS (ESI $^+$ )  $m/z$  calcd. for  $\text{C}_{60}\text{H}_{73}\text{ClN}_6\text{O}_{16}\text{SSi}$  [ $\text{M}$ ] $^{2+}$ , 615.2204; found 615.2196.



To a solution of CPY-CA (5.0 mg, 7.54  $\mu\text{mol}$ , 1.0 eq.) in anhydrous DCM (1.4 mL) was added thionyl chloride (8.2  $\mu\text{L}$ , 113  $\mu\text{mol}$ , 15 eq.) followed by pyridine (4.9  $\mu\text{L}$ , 60.3  $\mu\text{mol}$ , 8.0 eq.). The

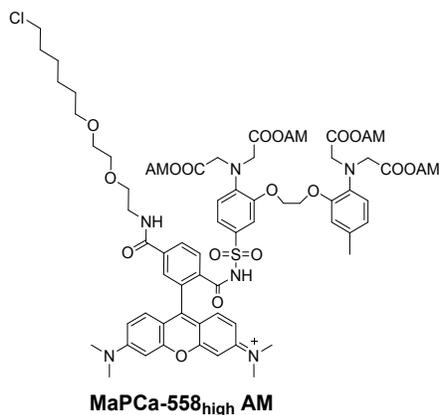
solution was stirred for 30 minutes. The mixture was heated to 60°C until almost no solvent was left. Then, the vial was closed and the crude dried under high vacuum. After 1 h, a solution of freshly prepared BAPTA-AM-sulfonamide **05** (7.8 mg, 9.05 μmol, 1.2 eq.), DIPEA (37 μL, 226 μmol, 30 eq.) and 4-dimethylaminopyridine (1.8 mg, 15.1 μmol, 2.0 eq.) in anhydrous MeCN (2.4 mL) was added under argon atmosphere. The solution was heated to 60°C and stirred for 1 h. The solvents were evaporated and the crude mixture purified by HPLC (8 mL/min; A/B 30-90%; 60 min) to obtain **MaPCa-619<sub>high</sub> AM** as blue powder (3.0 mg, 26%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ = 8.68 (t, *J* = 5.6 Hz, 1H), 7.90 (s, 2H), 7.13 (s, 1H), 7.02 (s, 1H), 6.97 – 6.94 (m, 2H), 6.87 (dtd, *J* = 16.8, 7.4, 1.7 Hz, 2H), 6.80 (s, 1H), 6.73 (dd, *J* = 7.7, 1.9 Hz, 1H), 6.53 (dd, *J* = 9.0, 2.5 Hz, 2H), 6.41 (d, *J* = 8.8 Hz, 2H), 5.54 (d, *J* = 2.3 Hz, 8H), 4.25 (dd, *J* = 15.6, 5.0 Hz, 4H), 4.11 (d, *J* = 15.4 Hz, 8H), 3.57 (t, *J* = 6.6 Hz, 2H), 3.47 – 3.35 (m, 8H), 3.28 (t, *J* = 6.5 Hz, 2H), 2.95 (s, 12H), 2.04 (s, 3H), 1.98 (d, *J* = 5.3 Hz, 12H), 1.85 (d, *J* = 5.7 Hz, 6H), 1.70 – 1.61 (m, 2H), 1.40 (p, *J* = 6.7 Hz, 2H), 1.36 – 1.27 (m, 2H), 1.31 – 1.17 (m, 2H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ = 169.8, 169.2, 166.3, 164.9, 158.3, 158.0, 155.5, 153.3, 149.6, 149.5, 145.0, 140.4, 138.3, 135.4, 133.9, 129.0, 128.3, 127.9, 121.3, 118.5, 115.6, 113.9, 112.3, 79.0, 71.9, 70.1, 69.5, 69.4, 68.6, 53.0, 52.6, 45.3, 40.3, 37.6, 35.6, 32.7, 32.0, 29.0, 26.1, 24.9, 20.4, 19.5. HRMS (ESI<sup>+</sup>) *m/z* calcd. for C<sub>73</sub>H<sub>89</sub>ClN<sub>6</sub>O<sub>24</sub>S [M]<sup>2+</sup>, 751.2741; found 751.2738.



**MaPCa-656<sub>high</sub> AM**

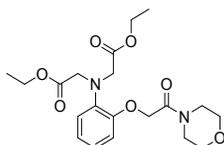
To a solution of SiR-CA (5.0 mg, 7.36 μmol, 1.0 eq.) in anhydrous DCM (1.4 mL) was added thionyl chloride (8.00 μL, 110 μmol, 15 eq.) followed by pyridine (4.8 μL, 58.9 μmol, 8.0 eq.). The solution was stirred for 30 minutes. In the following, the mixture was heated to 60°C until almost no solvent was left. Then, the vial was closed and the crude dried under high vacuum. After 1 h, a solution of freshly prepared BAPTA-AM-sulfonamide **05** (7.6 mg, 8.83 μmol, 1.2 eq.), DIPEA (36 μL, 221 μmol, 30 eq.) and 4-dimethylaminopyridine (1.8 mg, 14.7 μmol, 2.0 eq.) in anhydrous MeCN (3.1 mL) was added under argon atmosphere. The solution was heated to 60°C and stirred for 1 h. The solvents were evaporated and the crude mixture purified by HPLC (8 mL/min; A/B 30-

90%; 60 mins) to obtain **MaPCa-656<sub>high</sub> AM** as pale green-blue powder (4.3 mg, 38%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ = 7.94 (d, J = 8.0 Hz, 1H), 7.73 (dd, J = 8.0, 1.4 Hz, 1H), 7.46 – 7.41 (m, 2H), 7.11 (d, J = 1.2 Hz, 1H), 7.04 (dd, J = 9.0, 2.8 Hz, 2H), 7.00 – 6.86 (m, 4H), 6.82 (d, J = 9.0 Hz, 2H), 6.65 (t, J = 5.3 Hz, 1H), 6.58 (s, 1H), 5.61 (d, J = 12.3 Hz, 8H), 4.33 (s, 4H), 4.14 (d, J = 12.9 Hz, 8H), 3.62 – 3.47 (m, 10H), 3.41 (t, J = 6.7 Hz, 2H), 3.12 (s, 12H), 2.04 (d, J = 10.0 Hz, 12H), 1.99 (s, 3H), 1.74 (dt, J = 14.6, 6.7 Hz, 2H), 1.54 (p, J = 6.8 Hz, 2H), 1.48 – 1.38 (m, 2H), 1.37 – 1.27 (m, 2H), 0.64 (d, J = 15.8 Hz, 6H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ = 170.3, 169.9, 169.8, 169.7, 166.7, 166.1, 155.1, 154.4, 150.6, 145.0, 141.0, 138.7, 136.4, 136.1, 130.8, 129.3, 127.0, 125.1, 123.6, 122.2, 121.4, 120.3, 119.4, 115.6, 114.0, 79.5, 79.5, 74.7, 71.4, 70.3, 70.0, 69.6, 67.4, 67.0, 53.7, 53.5, 45.2, 43.9, 40.1, 32.6, 29.5, 26.8, 25.5, 20.8, 20.8, 20.2, 1.2, -0.1, -0.8. HRMS (ESI<sup>+</sup>) m/z calcd. for C<sub>72</sub>H<sub>89</sub>ClN<sub>6</sub>O<sub>24</sub>SSi [M]<sup>2+</sup>, 759.2626; found 759.2618.



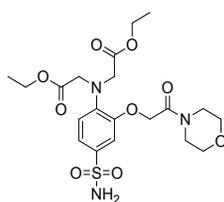
To a solution of TMR-CA (3.5 mg, 5.50 μmol, 1.0 eq.) in anhydrous DCM (1.0 mL) was added thionyl chloride (6.0 μL, 83.0 μmol, 15 eq.) followed by pyridine (3.6 μL, 44.0 μmol, 8.0 eq.). The solution was stirred for 30 minutes. Upon complete activation, the mixture was heated to 60°C until almost no solvent was left. Then, the vial was closed and the crude dried under high vacuum. After 1 h, a solution of freshly prepared BAPTA-AM-sulfonamide **05** (5.0 mg, 5.80 μmol, 1.1 eq.), DIPEA (27 μL 165 μmol, 30 eq.) and 4-dimethylaminopyridine (1.3 mg, 11.0 μmol, 2.0 eq.) in anhydrous MeCN (2.3 mL) was added under argon atmosphere. The solution was heated to 60°C and stirred for 1 h. The solvents were evaporated and the crude mixture purified by HPLC (8 mL/min; A/B 30-80%; 60 min) to obtain **MaPCa-558<sub>high</sub> AM** as red powder (2.3 mg, 28%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ = 8.70 (t, J = 5.5 Hz, 1H), 8.04 (d, J = 8.1 Hz, 1H), 7.91 (d, J = 8.0 Hz, 1H), 7.44 (s, 1H), 7.02 – 6.93 (m, 2H), 6.93 – 6.80 (m, 3H), 6.74 (dd, J = 7.7, 1.9 Hz, 1H), 6.43 (d, J = 16.6 Hz, 6H), 5.53 (s, 8H), 4.26 (h, J = 3.5 Hz, 4H), 4.10 (d, J = 20.7 Hz, 8H), 3.58 (t, J = 6.6 Hz, 2H), 3.49 – 3.38 (m, 4H), 3.29 (t, J = 6.5 Hz, 2H), 2.95 (s, 12H), 2.17 (s, 3H), 1.98 (s, 12H), 1.71 – 1.60 (m, 2H), 1.46 – 1.36 (m, 2H), 1.36 – 1.28 (m, 2H), 1.30 – 1.17 (m, 2H). <sup>13</sup>C NMR

(101 MHz, DMSO- $d_6$ )  $\delta$  = 169.7, 169.2, 169.2, 169.1, 164.6, 158.3, 157.9, 153.6, 151.3, 149.7, 138.3, 128.6, 128.3, 121.9, 121.4, 118.6, 115.7, 114.0, 98.3, 79.0, 78.9, 70.1, 69.5, 69.4, 68.6, 53.0, 52.6, 45.4, 32.0, 29.0, 26.1, 24.9, 24.9, 20.4, 20.4, 19.5. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for  $C_{70}H_{83}ClN_6O_{25}S [M]^{2+}$ , 738.2481; found 738.2479. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for molecule attached to the HaloTag-protein, 36904.4347; found 36904.4396.



**02**

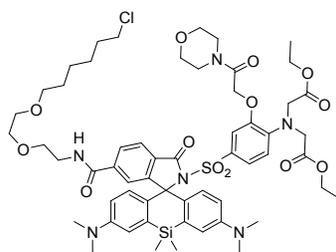
2-(2-morpholin-4-yl-2-oxoethoxy)aniline **S01** (300 mg, 1.27 mmol, 1.0 eq.) was dissolved in acetonitrile (12 mL). Then sodium iodide (952 mg, 6.35 mmol, 5.0 eq.), DIPEA (1.05 mL, 6.35 mmol, 5.0 eq.) and ethyl bromoacetate (704  $\mu$ L, 6.35 mmol, 5.0 eq.) were added. The reaction mixture was stirred at 100°C for 20 h and then quenched by addition of water. The mixture was extracted with DCM (3x), combined organic phases were dried over magnesium sulfate and the solvent was removed *in vacuo*. The crude was purified by flash column chromatography (25 g  $SiO_2$ , 20 - 60% EtOAc in hexane) and the pure product **02** was obtained as a beige solid (511 mg, 98%). <sup>1</sup>H NMR (400 MHz,  $CDCl_3$ ):  $\delta$  = 6.93 (s, 4H), 4.74 (s, 2H), 4.15 (q,  $J$  = 7.1 Hz, 4H), 4.13 (s, 4H), 3.64 – 3.62 (m, 4H), 3.58 – 3.59 (m, 4H), 1.25 (t,  $J$  = 7.1 Hz, 6H); <sup>13</sup>C NMR (101 MHz,  $CDCl_3$ ):  $\delta$  171.3, 166.9, 150.2, 140.2, 139.7, 123.0, 122.8, 120.4, 115.5, 68.6, 67.0, 60.8, 53.8, 14.4; HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for  $C_{20}H_{28}N_2O_7 [M+H]^+$  409.1969, found 409.1967.



**04**

MOBHA-Et **02** (511 mg, 1.25 mmol, 1.0 eq.) was dissolved in dichloromethane (5.8 mL) and added to pre-cooled (0°C) chlorosulfonic acid (5.83 mL, 87.5 mmol, 70 eq.). The reaction mixture was stirred at 0°C for 30 min. The cooling bath was removed and the reaction mixture was stirred at rt for further 18.5 h. The mixture was diluted with EtOAc (20 mL) and cooled to 0°C. Then the mixture was added slowly to a solution of ammonia (25%, 22 mL) in EtOAc (20 mL) at 0°C (pH  $\approx$  9). The cooling bath was removed and the mixture was stirred vigorously at rt for 23 h. The mixture was diluted with water and EtOAc and extracted with EtOAc against brine (5x). The combined

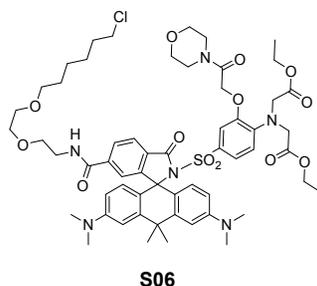
organic phases were dried over magnesium sulfate and the solvent was removed *in vacuo*. The crude product was purified by preparative HPLC (C18, 5  $\mu$ m, 50 mL/min, 30 – 60% MeCN (0.1% FA)/H<sub>2</sub>O (0.1% FA), R<sub>t</sub> = 25.7 min). After lyophilization the pure product **04** was obtained as a colorless solid (264 mg, 44%). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>CN):  $\delta$  = 7.35 (dd, *J* = 8.5, 2.3 Hz, 1H), 7.28 (d, *J* = 2.3 Hz, 1H), 6.91 (d, *J* = 8.6 Hz, 1H), 5.55 (s, 2H), 4.86 (s, 2H), 4.19 (s, 4H), 4.13 (q, *J* = 7.1 Hz, 4H), 3.63 (dt, *J* = 9.9, 4.8 Hz, 4H), 3.47 (dt, *J* = 29.0, 4.8 Hz, 4H), 1.21 (t, *J* = 7.1 Hz, 6H). <sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>CN):  $\delta$  171.7, 166.4, 153.4, 140.0, 136.7, 120.6, 117.5, 113.6, 67.2, 67.1, 66.8, 61.5, 54.2, 45.8, 42.7, 14.5. HRMS (ESI<sup>+</sup>) *m/z* calcd. for C<sub>20</sub>H<sub>29</sub>N<sub>3</sub>O<sub>9</sub>S [M+Na]<sup>+</sup> 510.1517, found 510.1516.



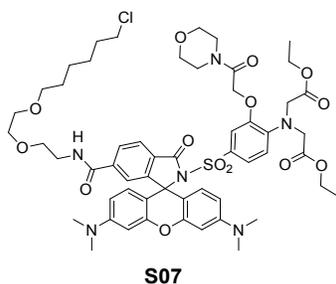
**S05**

SiR-CA (4.3 mg, 6.33  $\mu$ mol, 1.0 eq.) was dissolved in anhydrous dichloromethane (1.3 mL). Freshly distilled thionyl chloride (6.9  $\mu$ L, 94.9  $\mu$ mol, 15 eq.) and anhydrous pyridine (4.3  $\mu$ L, 53.2  $\mu$ mol, 8.4 eq.) were added. The reaction mixture was stirred at rt for 30 min before the solvent was evaporated at 50°C until almost anhydrous. In a separate vessel MOBHA-sulfonamide **04** (4.3 mg, 8.82  $\mu$ mol, 1.4 eq.) was dissolved in anhydrous acetonitrile (2.0 mL), and DIPEA (31  $\mu$ L, 190  $\mu$ mol, 30 eq.) and DMAP (387  $\mu$ g, 3.17  $\mu$ mol, 0.5 eq.) were added. The solution of **04** was heated to 50°C and added to the previously concentrated reaction mixture. After the reaction was stirred at 60°C for 2.5 h the solvent was removed *in vacuo*. The residue was purified by preparative HPLC (8 mL/min, 30 – 80% MeCN/H<sub>2</sub>O (0.1% TFA), R<sub>t</sub> = 39.3 min) to yield the pure product **S05** as a turquoise solid (1.0 mg, 14%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  = 9.04 (s, 1H), 8.65 (t, *J* = 5.6 Hz, 1H), 7.89 (s, 2H), 7.06 – 7.01 (m, 2H), 6.91 (d, *J* = 2.9 Hz, 2H), 6.85 (d, *J* = 8.9 Hz, 1H), 6.65 (d, *J* = 2.2 Hz, 1H), 6.50 (dd, *J* = 9.2, 2.8 Hz, 1H), 6.34 (d, *J* = 9.1 Hz, 2H), 4.92 (s, 2H), 4.05 (q, *J* = 7.1 Hz, 4H), 4.00 (s, 4H), 3.61 – 3.55 (m, 4H), 3.57 (t, *J* = 6.6 Hz, 2H), 3.45 – 3.40 (m, 2H), 3.40 – 3.37 (m, 2H), 3.27 (t, *J* = 6.5 Hz, 4H), 3.10 (q, *J* = 7.3 Hz, 2H), 3.09 (q, *J* = 7.3 Hz, 2H), 2.93 (s, 12H), 1.64 (p, *J* = 6.7 Hz, 2H), 1.39 (p, *J* = 7.0 Hz, 2H), 1.34 – 1.27 (m, 2H), 1.26 – 1.21 (m, 2H), 1.15 (t, *J* = 7.1 Hz, 6H), 0.58 (s, 1H), 0.52 (s, 1H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>):  $\delta$  170.2, 166.0, 165.2, 164.8, 157.9, 157.6, 155.9, 153.0, 148.2, 140.4, 137.9, 135.5, 134.7, 130.8, 129.9, 129.1, 128.3, 127.0, 122.3, 115.0, 114.3, 78.3, 75.0, 70.1, 69.5, 69.3, 68.6, 66.0, 65.9,

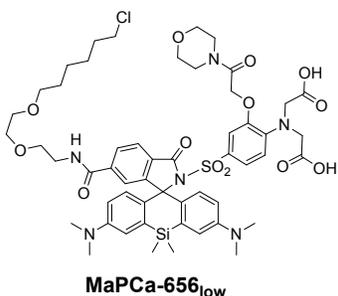
65.7, 60.1, 52.8, 45.7, 45.3, 32.0, 29.0, 26.0, 24.8, 14.1, 8.6, -0.1, -0.7. HRMS (ESI<sup>+</sup>) m/z calcd. for C<sub>57</sub>H<sub>75</sub>ClN<sub>6</sub>O<sub>13</sub>SSi [M+H]<sup>+</sup> 1147.4643, found 1147.4645.



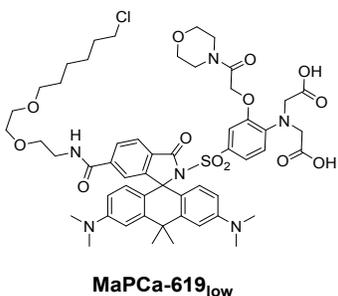
CPY-CA (5.0 mg, 7.55  $\mu$ mol, 1.0 eq.) was dissolved in anhydrous dichloromethane (1.6 mL). Freshly distilled thionyl chloride (8.2  $\mu$ L, 113  $\mu$ mol, 15 eq.) and anhydrous pyridine (4.9  $\mu$ L, 60.4  $\mu$ mol, 8.0 eq.) were added. The reaction mixture was stirred at rt for 30 min before the solvent was evaporated at 50°C until almost anhydrous. In a separate vessel MOBHA-sulfonamide **04** (5.0 mg, 10.3  $\mu$ mol, 1.4 eq.) was dissolved in anhydrous acetonitrile (2.0 mL), and DIPEA (31  $\mu$ L, 190  $\mu$ mol, 30 eq.) and DMAP (387  $\mu$ g, 3.17  $\mu$ mol, 0.5 eq.) were added. The solution of **04** was heated to 50°C and added to the previously evaporated reaction mixture. After the reaction was stirred at 60°C for 3.5 h the solvent was removed *in vacuo*. The residue was purified by preparative HPLC (8 mL/min, 30 – 80% MeCN/H<sub>2</sub>O (0.1% TFA), R<sub>t</sub> = 33.6 min) to yield the pure product **S06** as a blue-turquoise solid (2.3 mg, 27%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  = 9.07 (s, 1H), 8.67 (t, *J* = 5.6 Hz, 1H), 7.89 (s, 2H), 7.14 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.04 – 7.03 (m, 1H), 6.89 (d, *J* = 9.2 Hz, 1H), 6.87 (d, *J* = 2.8 Hz, 1H), 6.64 (d, *J* = 2.2 Hz, 1H), 6.42 (dd, *J* = 8.9, 2.5 Hz, 2H), 6.28 (d, *J* = 8.9 Hz, 2H), 4.94 (s, 2H), 4.03 (q, *J* = 7.1 Hz, 4H), 4.01 (s, 4H), 3.64 – 3.52 (m, 4H), 3.61 – 3.52 (m, 2H), 3.44 – 3.40 (m, 4H), 3.40 – 3.36 (m, 2H), 3.27 (t, *J* = 6.4 Hz, 4H), 3.10 (qd, *J* = 7.3, 4.7 Hz, 4H), 2.95 (s, 12H), 1.85 (d, *J* = 2.2 Hz, 4H), 1.70 – 1.59 (m, 2H), 1.43 – 1.35 (m, 2H), 1.34 – 1.27 (m, 2H), 1.26 – 1.21 (m, 2H), 1.14 (t, *J* = 7.1 Hz, 6H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>):  $\delta$  170.2, 165.9, 165.2, 164.8, 157.9, 157.6, 155.4, 153.0, 149.6, 145.2, 140.5, 137.9, 130.9, 128.4, 127.8, 127.4, 121.9, 118.0, 116.9, 112.0, 111.7, 109.6, 71.7, 70.1, 69.5, 69.3, 68.6, 66.0, 65.7, 60.1, 52.8, 45.7, 45.3, 44.5, 41.6, 37.6, 35.6, 32.7, 32.0, 29.0, 26.1, 24.9, 21.1, 14.1, 8.6. HRMS (ESI<sup>+</sup>) m/z calcd. for C<sub>58</sub>H<sub>75</sub>ClN<sub>6</sub>O<sub>13</sub>S [M+H]<sup>+</sup> 1131.4874, found 1131.4880.



TMR-CA (5.4 mg, 8.47  $\mu\text{mol}$ , 1.0 eq.) and MOBHA-sulfonamide **04** (5.0 mg, 10.2  $\mu\text{mol}$ , 1.2 eq.) were dissolved in anhydrous dichloromethane (1.7 mL) in a crimp-top vial. Then DMAP (7.8 mg, 64.0  $\mu\text{mol}$ , 8.0 eq.) and EDC-HCl (12.3 mg, 64.0  $\mu\text{mol}$ , 8.0 eq.) were added. The reaction mixture was then stirred at 65°C for 22 h. After evaporating the solvent *in vacuo*, the residue was purified by preparative HPLC (8 mL/min, 30 – 80% MeCN/H<sub>2</sub>O (0.1% TFA),  $R_t$  = 34.1 min) to yield the pure product **S07** as a purple solid (3.0 mg, 32%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  = 8.69 (t,  $J$  = 5.6 Hz, 1H), 8.02 (dd,  $J$  = 8.1, 1.4 Hz, 1H), 7.90 (d,  $J$  = 8.0 Hz, 1H), 7.45 – 7.42 (m, 1H), 6.95 (dd,  $J$  = 8.7, 2.2 Hz, 1H), 6.86 (d,  $J$  = 8.8 Hz, 1H), 6.79 (d,  $J$  = 2.2 Hz, 1H), 6.47 (d,  $J$  = 2.0 Hz, 2H), 6.41 – 6.30 (m, 4H), 4.94 (s, 2H), 4.07 (s, 4H), 4.03 (q,  $J$  = 7.1 Hz, 4H), 3.63 – 3.52 (m, 8H), 3.58 (t,  $J$  = 6.6 Hz, 2H), 3.44 – 3.43 (m, 2H), 3.28 (t,  $J$  = 6.5 Hz, 4H), 3.15 – 3.05 (m, 2H), 2.96 (s, 12H), 1.65 (p,  $J$  = 6.8 Hz, 2H), 1.39 (p,  $J$  = 7.0 Hz, 2H), 1.35 – 1.28 (m, 2H), 1.27 – 1.20 (m, 2H), 1.14 (t,  $J$  = 7.1 Hz, 6H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>):  $\delta$  170.2, 165.2, 164.8, 164.6, 158.0, 157.7, 153.3, 152.4, 151.2, 140.4, 138.0, 130.8, 129.3, 128.3, 123.7, 122.8, 122.2, 117.3, 112.0, 108.4, 106.2, 98.2, 70.1, 69.5, 69.3, 68.6, 66.0, 65.8, 60.2, 52.9, 45.8, 45.4, 44.5, 41.6, 32.0, 29.0, 26.1, 24.9, 14.1, 8.6. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for C<sub>55</sub>H<sub>69</sub>ClN<sub>6</sub>O<sub>14</sub>S [M+H]<sup>+</sup> 1105.4354, found 1105.4352;  $m/z$  calcd. for C<sub>55</sub>H<sub>69</sub>ClN<sub>6</sub>O<sub>14</sub>S [M+2H]<sup>2+</sup> 553.2213, found 553.2207.

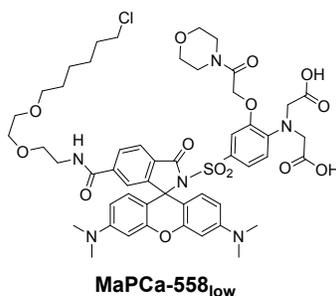


SiR-CA-MOBHA-COOEt **S05** (2.0 mg, 1.74  $\mu\text{mol}$ , 1.0 eq.) was dissolved in THF (1.0 mL). Then methanol (1.0 mL) and potassium hydroxide solution (0.1M, 1.3 mL) were added. The reaction mixture was stirred at rt for 6 h. The reaction was neutralized by addition of hydrochloric acid (0.1M, 0.2 mL) and acetic acid ( $\geq 99\%$ , 5.0  $\mu\text{L}$ ) before the solvent was evaporated *in vacuo*. The residue was purified by preparative HPLC (8 mL/min, 30 – 70% MeCN/H<sub>2</sub>O (0.1% TFA),  $R_t = 30.6$  min) to yield the pure product **MaPCa-656<sub>low</sub>** as a light green solid (1.1 mg, 58%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta = 12.42$  (bs, 2H), 9.04 (s, 1H), 8.65 (t,  $J = 5.6$  Hz, 1H), 7.94 – 7.84 (m, 2H), 7.04 (s, 1H), 6.96 (dd,  $J = 8.7, 2.2$  Hz, 1H), 6.91 (d,  $J = 2.9$  Hz, 1H), 6.82 (d,  $J = 8.9$  Hz, 1H), 6.75 (d,  $J = 2.2$  Hz, 1H), 6.50 (dd,  $J = 9.1, 2.8$  Hz, 2H), 6.34 (d,  $J = 9.0$  Hz, 2H), 4.92 (s, 2H), 3.93 (s, 4H), 3.57 (t,  $J = 6.6$  Hz, 2H), 3.41 – 3.37 (m, 2H), 3.27 (t,  $J = 6.5$  Hz, 4H), 3.10 (qd,  $J = 7.3, 4.7$  Hz, 4H), 2.92 (s, 12H), 1.65 (p,  $J = 6.8$  Hz, 2H), 1.39 (p,  $J = 6.8$  Hz, 2H), 1.34 – 1.27 (m, 2H), 1.27 – 1.20 (m, 2H), 0.58 (s, 1H), 0.54 (s, 1H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>):  $\delta = 171.7, 166.1, 165.3, 164.8, 162.5, 158.1, 157.7, 155.9, 153.0, 148.1, 147.0, 140.4, 139.6, 130.8, 129.2, 128.4, 127.0, 126.7, 121.5, 115.1, 114.3, 113.0, 75.1, 70.1, 69.5, 69.3, 68.6, 65.9, 65.7, 52.5, 45.7, 45.3, 32.0, 29.0, 26.1, 24.9, 8.6, -0.1, -0.7$ . HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for C<sub>53</sub>H<sub>67</sub>ClN<sub>6</sub>O<sub>13</sub>SSi [M+2H]<sup>2+</sup> 546.2045, found 546.2040.

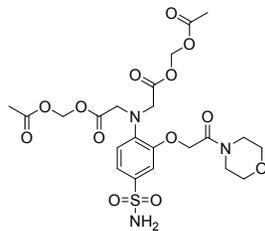


CPY-CA-MOBHA-COOEt **S06** (5.4 mg, 4.77  $\mu\text{mol}$ , 1.0 eq.) was dissolved in THF (1.0 mL). Then methanol (1.0 mL) and potassium hydroxide solution (0.1 M, 1.3 mL) were added. The reaction mixture was stirred at rt for 4 h. The reaction was neutralized by addition of hydrochloric acid (0.1M, 0.2 mL) and acetic acid ( $\geq 99\%$ , 6.0  $\mu\text{L}$ ) before the solvent was evaporated *in vacuo*. The residue was purified by preparative HPLC (8 mL/min, 30 – 70% MeCN/H<sub>2</sub>O (0.1% TFA),  $R_t = 33.0$  min) to

yield the pure product **MaPCa-619<sub>low</sub>** as a light blue solid (1.2 mg, 23%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ = 12.41 (s, 2H), 8.67 (t, *J* = 5.7 Hz, 1H), 7.90 (s, 2H), 7.07 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.03 (s, 1H), 6.89 (d, *J* = 2.6 Hz, 1H), 6.86 (d, *J* = 8.9 Hz, 1H), 6.75 (d, *J* = 2.3 Hz, 1H), 6.45 (dd, *J* = 9.0, 2.6 Hz, 2H), 6.30 (d, *J* = 8.8 Hz, 2H), 4.94 (s, 2H), 3.95 (s, 4H), 3.57 (t, *J* = 6.6 Hz, 2H), 3.27 (t, *J* = 6.5 Hz, 2H), 3.10 (qd, *J* = 7.3, 4.8 Hz, 2H), 2.95 (s, 12H), 1.85 (d, *J* = 4.4 Hz, 6H), 1.65 (p, *J* = 6.7 Hz, 2H), 1.39 (p, *J* = 7.0 Hz, 2H), 1.34 – 1.27 (m, 2H), 1.26 – 1.20 (m, 2H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 171.8, 165.9, 165.3, 164.9, 158.6, 158.1, 153.0, 149.5, 145.2, 140.4, 138.4, 130.9, 128.4, 127.8, 127.3, 123.8, 117.2, 112.0, 111.9, 71.7, 70.1, 69.5, 69.4, 68.6, 66.0, 52.4, 45.8, 45.4, 44.6, 41.6, 37.6, 35.7, 32.8, 32.0, 29.0, 26.1, 24.9, 8.6. HRMS (ESI<sup>+</sup>) *m/z* calcd. for C<sub>54</sub>H<sub>67</sub>ClN<sub>6</sub>O<sub>13</sub>S [M+2H]<sup>2+</sup> 538.2160, found 538.2157.



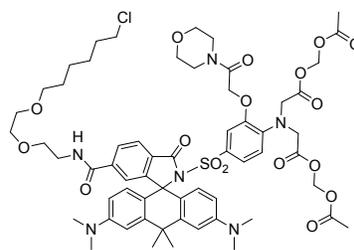
TMR-CA-MOBHA-COOEt **S07** (4.4 mg, 3.98 μmol, 1.0 eq.) was dissolved in THF (1.0 mL). Then methanol (1.0 mL) and potassium hydroxide solution (0.1M, 1.3 mL) were added. The reaction mixture was stirred at rt for 5 h. The reaction was neutralized by addition of hydrochloric acid (0.1M, 0.2 mL) and acetic acid (≥ 99%, 5.0 μL) before the solvent was evaporated *in vacuo*. The residue was purified by preparative HPLC (8 mL/min, 30 – 70% MeCN/H<sub>2</sub>O (0.1% TFA), *R<sub>t</sub>* = 30.3 min) to yield the pure product **MaPCa-558<sub>low</sub>** as a dark purple solid (0.9 mg, 22%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ = 12.44 (bs, 2H), 8.69 (t, *J* = 5.6 Hz, 1H), 8.02 (d, *J* = 8.1 Hz, 1H), 7.91 (d, *J* = 8.0 Hz, 1H), 7.42 (s, 1H), 7.02 (dd, *J* = 8.7, 2.2 Hz, 1H), 6.86 (d, *J* = 8.9 Hz, 1H), 6.74 (d, *J* = 2.3 Hz, 1H), 6.46 (d, *J* = 2.2 Hz, 2H), 6.41 – 6.28 (m, 4H), 4.94 (s, 2H), 3.97 (s, 4H), 3.58 (t, *J* = 6.6 Hz, 2H), 3.31 – 3.25 (m, 2H), 3.14 – 3.06 (m, 2H), 2.95 (s, 12H), 1.65 (p, *J* = 6.7 Hz, 2H), 1.40 (p, *J* = 7.0 Hz, 2H), 1.35 – 1.27 (m, 2H), 1.27 – 1.19 (m, 2H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 171.7, 165.3, 164.8, 164.6, 157.8, 153.3, 153.2, 152.3, 151.2, 140.3, 138.4, 130.7, 129.3, 128.4, 117.1, 114.5, 110.9, 108.4, 106.2, 98.2, 70.1, 69.5, 69.3, 68.6, 66.0, 65.7, 52.5, 45.4, 32.0, 29.0, 26.1, 24.9, 5.4. HRMS (ESI<sup>+</sup>) *m/z* calcd. for C<sub>51</sub>H<sub>61</sub>ClN<sub>6</sub>O<sub>14</sub>S [M+H]<sup>+</sup> 1049.3728, found 1049.3729; *m/z* calcd. for C<sub>51</sub>H<sub>61</sub>ClN<sub>6</sub>O<sub>14</sub>S [M+2H]<sup>2+</sup> 525.1900, found 525.1896. HRMS (ESI<sup>+</sup>) *m/z* calcd. for molecule attached to the HaloTag-protein, 36478.3167; found 36478.2970.



**06**

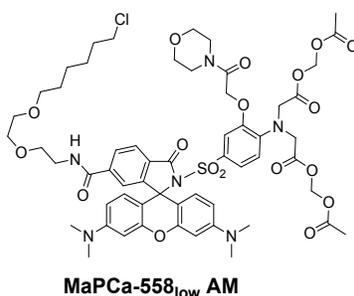
MOBHA-sulfonamide **05** (50 mg, 103  $\mu\text{mol}$ , 1.0 eq.) was dissolved in acetonitrile (9.0 mL). Then DMAP (6.3 mg, 51.3  $\mu\text{mol}$ , 0.5 eq.) and di-tert-butyl-carbonate (110  $\mu\text{L}$ , 513  $\mu\text{mol}$ , 5.0 eq.) in acetonitrile (1.0 mL) was added. The reaction was stirred at rt for 19 h followed by evaporation of the solvent *in vacuo*. di-tert-butyl-carbonate (110  $\mu\text{L}$ , 513  $\mu\text{mol}$ , 5.0 eq.) and DMAP (6.3 mg, 51.3  $\mu\text{mol}$ , 0.5 eq.) were dissolved in DCM (8.0 mL) and added to the residue. The reaction mixture was stirred at 35°C for 24 h. Afterwards the reaction was quenched by addition of water and extracted with DCM (3x). The combined organic phases were dried over magnesium sulfate and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography (12 g  $\text{SiO}_2$ , 0 – 5% MeOH/DCM) to yield the intermediate product as a beige-yellow solid (52.4 mg, 79%). It was directly re-dissolved in THF (2.0 mL). Then methanol (2.0 mL) and potassium hydroxide solution (0.1M, 2.8 mL) were added. The reaction mixture was stirred at rt for 5.5 h followed by neutralizing with hydrochloric acid (0.1M, 0.4 mL) and acetic acid ( $\geq 99\%$ , 2.8  $\mu\text{L}$ ) and evaporation of the solvents *in vacuo*. The residue was suspended in acetonitrile (2.0 mL). Bromomethyl acetate (73  $\mu\text{L}$ , 752  $\mu\text{mol}$ , 8.8 eq.) and DIPEA (355  $\mu\text{L}$ , 2.15 mmol, 25 eq.) were added. After stirring the reaction at rt for 21 h the solvent was evaporated *in vacuo* again. The residue was suspended in DCM (0.9 mL) and triisopropyl silane (135  $\mu\text{L}$ , 7.5% v/v) and TFA (0.9 mL) were added. The reaction was stirred at rt for 30 min and then neutralized by addition of a saturated  $\text{NaHCO}_3$ -solution (1.2 mL). Extraction with EtOAc (5x) against brine, drying of the combined organic phases over magnesium sulfate and evaporation of the solvent *in vacuo* led to the crude product. After purification *via* preparative HPLC (C18, 5  $\mu\text{m}$ , 50 mL/min, 30 – 50% MeCN (0.1% FA)/ $\text{H}_2\text{O}$  (0.1% FA),  $R_t = 22.9$  min) the pure product **06** was obtained as a colorless solid (22.4 mg, 36% over 5 steps).  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{CN}$ ):  $\delta = 7.38$  (dd,  $J = 8.6, 2.2$  Hz, 1H), 7.23 (d,  $J = 2.2$  Hz, 1H), 6.92 (d,  $J = 8.6$  Hz, 1H), 5.71 (s, 4H), 5.58 (s, 2H), 4.88 (s, 2H), 4.26 (s, 4H), 3.63 (dt,  $J = 12.7, 4.8$  Hz, 4H), 3.47 (dt,  $J = 29.6, 4.8$  Hz, 4H), 2.06 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  170.7, 170.7, 166.4, 153.4, 139.4, 136.8, 121.0, 117.4, 113.6, 80.2, 66.7, 53.9, 45.7, 42.7, 20.9. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for  $\text{C}_{22}\text{H}_{29}\text{N}_3\text{O}_{13}\text{S}$   $[\text{M}+\text{H}]^+$  576.1494, found 576.1495.





**MaPCa-619<sub>low</sub> AM**

CPY-CA (5.0 mg, 7.55  $\mu\text{mol}$ , 1.0 eq.) was dissolved in DCM (1.6 mL). Freshly distilled thionyl chloride (8.2  $\mu\text{L}$ , 112  $\mu\text{mol}$ , 15 eq.) and anhydrous pyridine (4.9  $\mu\text{L}$ , 60.4  $\mu\text{mol}$ , 8.0 eq.) were added. The reaction mixture was stirred at rt for 30 min before the solvent was evaporated at 50°C until almost dry. In a separate vessel MOBHA-AM-sulfonamide **06** (5.4 mg, 9.44  $\mu\text{mol}$ , 1.3 eq.) was dissolved in anhydrous acetonitrile (2.4 mL), and DIPEA (37.4  $\mu\text{L}$ , 227  $\mu\text{mol}$ , 30 eq.) and DMAP (461  $\mu\text{g}$ , 3.78  $\mu\text{mol}$ , 0.5 eq.) were added. The solution of **06** was heated to 50°C and added to the previously evaporated reaction mixture. After the reaction was stirred at 60°C for 2.5 h the solvent was removed *in vacuo*. The residue was purified by preparative HPLC (8 mL/min, 30 – 70% MeCN/H<sub>2</sub>O (0.1% TFA),  $R_t$  = 35.1 min) to yield the pure product **MaPCa-619<sub>low</sub> AM** as a dark blue solid (1.4 mg, 15%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  = 8.68 (t,  $J$  = 5.7 Hz, 1H), 7.94 – 7.87 (m, 2H), 7.08 (dd,  $J$  = 8.6, 2.2 Hz, 1H), 7.04 (s, 1H), 6.91 (s, 2H), 6.88 (s, 1H), 6.60 (d,  $J$  = 2.2 Hz, 1H), 6.42 (d,  $J$  = 8.9 Hz, 2H), 6.26 (d,  $J$  = 8.8 Hz, 2H), 5.68 (s, 4H), 4.94 (s, 2H), 4.10 (s, 4H), 3.57 (t,  $J$  = 6.6 Hz, 2H), 3.48 – 3.36 (m, 4H), 3.47 – 3.34 (m, 8H), 3.27 (t,  $J$  = 6.2 Hz, 4H), 2.95 (s, 12H), 2.04 (s, 6H), 1.85 (s, 6H), 1.65 (p,  $J$  = 6.8 Hz, 2H), 1.39 (p,  $J$  = 7.1 Hz, 2H), 1.35 – 1.27 (m, 2H), 1.27 – 1.19 (m, 2H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  169.3, 169.2, 166.0, 165.2, 164.8, 158.2, 157.9, 155.3, 152.9, 149.4, 145.2, 140.5, 137.4, 130.9, 128.4, 127.8, 127.4, 123.8, 122.4, 121.7, 116.5, 112.2, 111.9, 109.8, 79.1, 71.6, 70.1, 69.5, 69.3, 68.6, 66.0, 65.9, 65.6, 52.5, 45.3, 44.4, 41.6, 37.6, 35.6, 32.7, 32.0, 29.0, 26.1, 24.9, 20.5, 8.6. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for C<sub>60</sub>H<sub>75</sub>ClN<sub>6</sub>O<sub>17</sub>S [M+H]<sup>+</sup> 1219.4671, found 1219.4670;  $m/z$  calcd. for C<sub>60</sub>H<sub>75</sub>ClN<sub>6</sub>O<sub>17</sub>S [M+2H]<sup>2+</sup> 610.2372, found 610.2383.



TMR-CA (5.0 mg, 7.86  $\mu\text{mol}$ , 1.0 eq.) was dissolved in DCM (1.6 mL). Freshly distilled thionyl chloride (8.5  $\mu\text{L}$ , 118  $\mu\text{mol}$ , 15 eq.) and anhydrous pyridine (5.1  $\mu\text{L}$ , 62.9  $\mu\text{mol}$ , 8.0 eq.) were added. The reaction mixture was stirred at rt for 30 min before the solvent was evaporated at 50°C until almost dry. In a separate vessel MOBHA-AM-sulfonamide **06** (5.65 mg, 9.82  $\mu\text{mol}$ , 1.3 eq.) was dissolved in anhydrous acetonitrile (2.5 mL), and DIPEA (39.0  $\mu\text{L}$ , 236  $\mu\text{mol}$ , 30 eq.) and DMAP (480  $\mu\text{g}$ , 3.93  $\mu\text{mol}$ , 0.5 eq.) were added. The solution of **06** was heated to 50°C and added to the previously evaporated reaction mixture. After the reaction was stirred at 60°C for 2.5 h the solvent was removed *in vacuo*. The residue was purified by preparative HPLC (8 mL/min, 30 – 80% MeCN/H<sub>2</sub>O (0.1% TFA),  $R_t$  = 33.3 min) to yield the pure product **MaPCa-558<sub>low</sub> AM** as a purple solid (1.2 mg, 13%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  = 8.69 (t,  $J$  = 5.7 Hz, 1H), 8.02 (dd,  $J$  = 8.0, 1.4 Hz, 1H), 7.92 (d,  $J$  = 8.0 Hz, 1H), 7.43 (d,  $J$  = 1.4 Hz, 1H), 7.01 (dd,  $J$  = 8.7, 2.2 Hz, 1H), 6.89 (d,  $J$  = 8.9 Hz, 1H), 6.63 (d,  $J$  = 2.3 Hz, 1H), 6.46 (d,  $J$  = 2.4 Hz, 2H), 6.35 (dd,  $J$  = 9.0, 2.4 Hz, 2H), 6.31 (d,  $J$  = 8.8 Hz, 2H), 5.68 (s, 4H), 4.94 (s, 2H), 4.14 (s, 4H), 3.58 (t,  $J$  = 6.6 Hz, 2H), 3.47 – 3.37 (m, 4H), 3.32 – 3.25 (m, 4H), 2.96 (s, 12H), 2.05 (s, 6H), 1.65 (p,  $J$  = 6.7 Hz, 2H), 1.40 (p,  $J$  = 7.1 Hz, 2H), 1.35 – 1.28 (m, 2H), 1.27 – 1.19 (m, 2H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  169.3, 169.2, 165.2, 164.8, 164.5, 158.0, 157.7, 153.2, 153.1, 152.4, 151.2, 140.4, 137.4, 130.7, 129.3, 128.3, 123.7, 122.8, 122.2, 116.5, 112.1, 108.3, 106.1, 98.1, 79.0, 70.1, 69.5, 69.3, 68.6, 66.0, 65.9, 65.6, 52.5, 45.3, 44.5, 41.6, 32.0, 29.0, 26.1, 24.9, 20.5. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for C<sub>57</sub>H<sub>69</sub>ClN<sub>6</sub>O<sub>18</sub>S [M+H]<sup>+</sup> 1193.4150, found 1193.4145;  $m/z$  calcd. for C<sub>57</sub>H<sub>69</sub>ClN<sub>6</sub>O<sub>18</sub>S [M+2H]<sup>2+</sup> 597.2112, found 597.2104. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for molecule attached to the HaloTag-protein, 36904.4347; found 36904.4396. HRMS (ESI<sup>+</sup>)  $m/z$  calcd. for molecule attached to the HaloTag-protein, 36622.36076; found 36622.4003.

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