Concerted, rapid, quantitative and site-specific dual labeling of proteins

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Supplementary Methods

Plasmid Construction

The pRSF ribo-Q1 O-*gst-cam* plasmids contain both the evolved quadruplet-decoding orthogonal ribosome (ribo-Q1) and O-*gst-cam* (a gene of *glutathione-S-transferase* and *calmodulin* (*gst-cam*) which is downstream of orthogonal ribosomal binding site). The O-*gst-cam* gene was amplified by PCR using primers GstCaMgeneF_KpnI and GstCaMgeneR_KpnI (**Supplementary Table 1**) from a pO-*gst-cam* plasmid[1] and cloned into the KpnI site of the pRSF ribo-Q1 plasmid (expressing ribo-Q1 rRNA from an IPTG inducible promoter)[1] to make the pRSF ribo-Q1 O-*gst-cam*. The 1st ATG Met codon of *cam* was mutated to amber codon (TAG) by Quickchange mutagenesis using primers CaM1TAGf and CaM1TAGr (**Supplementary Table 1**) to make plasmid pRSF ribo-Q1 O-*gst-cam*$_{1TAG}$. The 40th CTT Leu codon of *cam* was mutated to amber codon (TAG) by Quickchange mutagenesis using primers CaM40TAGf and CaM40TAGr (**Supplementary Table 1**) to make plasmid plasmid pRSF ribo-Q1 O-*gst-cam*$_{40TAG}$.

In a second round of mutagenesis, the quadruplet codon AGTA was introduced to replace either the 40th CTT Leu codon of O-*gst-cam*$_{1TAG}$ in pRSF ribo-Q1 O-*gst-cam*$_{1TAG}$ using primers CaM40AGTAf and CaM40AGTAr (**Supplementary Table 1**) to make plasmid pRSF ribo-Q1 O-*gst-cam*$_{1TAG\text{-}40AGTA}$, or the 149th AAG Lys codon of O-*gst-cam*$_{1TAG}$ using primers CaM149AGTAf and CaM149AGTAr (**Supplementary Table 1**) to make plasmid pRSF ribo-Q1 O-*gst-cam*$_{1TAG\text{-}149AGTA}$.

pCDF PylRS/tRNA$_{CUA}$ plasmid used in this work was generated by introduction of two additional copies of the *Methanosarcina barkeri* (*Mb*) PylRS gene into the pCDF plasmid expressing *Mb* PylRS and PyltRNA$_{CUA}$ that contains a single copy of *Mb* PylRS gene.[1] This was achieved by amplifying *Methanosarcina barkeri* (*Mb*) PylRS gene by PCR using primers pCDF1BamHIf and pCDF1743BglIIr (**Supplementary Table 1**) and subsequently cloning the amplified *Mb* PylRS gene in the same orientation into the BamHI site of the previously described single copy *Mb* PylRS pCDF plasmid expressing *Mb* PylRS and PyltRNA$_{CUA}$. To construct the pCDF PylRS/tRNA$_{UACU}$ plasmid, the anticodon stem-loop of the PyltRNA$_{CUA}$ in the pCDF PylRS/tRNA$_{CUA}$ was changed by enzymatic inverse PCR using primers PylTeAGTAf and PylTeAGTAr (**Supplementary Table 1**). To make the pSUP *Mj*PrpRS/tRNA$_{CUA}$, *Mj*

AzPheRS between NdeI and PstI sites in the pSUP *Mj*AzPheRS/tRNA$_{CUA}$ plasmid[2] was replaced with PrpRS[3] which recognizes para-propargyloxyphenylalanine.

Overexpression of GST-CaM1$_1$, GST-CaM2$_{40}$, GST-CaM1$_1$2$_{40}$ and GST-CaM1$_1$2$_{149}$ recombinant proteins

For overexpression of GST-CaM**1**$_1$, MDS42 recA *E. coli* cells were transformed by electroporation with pRSF ribo-Q1 O-*gst-cam$_{1UAG}$* and pSUP *Mj*PrpRS/tRNA$_{CUA}$, and recovered in 1 ml SOB medium for one hour at 37°C prior to aliquoting to 100 ml LB-KC (LB media with 25 μg ml^{-1} kanamycin, and 17.5 μg ml^{-1} chloramphenicol) and incubated overnight (37°C, 250 rpm, 16 h). The overnight culture was diluted to OD_{600}=0.1 in LB-KC. At OD_{600}=0.5, IPTG (1 mM final concentration) supplemented with **1** (2 mM) was added to the culture. The culture was incubated (37°C, 250 rpm) for 4 h, pelleted (3,000g, 10 min, 4°C) and washed twice with 1 ml PBS.

For overexpression of GST-CaM**2**$_{40}$, MDS42 recA *E. coli* cells were transformed by electroporation with pRSF ribo-Q1 O-*gst-cam$_{40UAG}$* and pCDF PylRS/tRNA$_{CUA}$, recovered in 1 ml SOB medium for one hour at 37°, and used to inoculate 100 ml LB-KS (LB media with 25 μg ml^{-1} kanamycin, and 37.5 μg ml^{-1} spectinomycin). The culture was incubated overnight (37°C, 250 rpm, 16 h), then diluted to OD_{600}=0.1 in LB-KS and incubated (37°C, 250 rpm) until OD_{600} reached 0.5. IPTG (1 mM final concentration) supplemented with **2** (1 mM) was added to the culture. The culture was incubated (37°C, 250 rpm) for 4 h, pelleted (3,000g, 10 min, 4°C) and washed twice with 1 ml PBS.

For overexpression of GST-CaM**1**$_1$**2**$_{40}$ or GST-CaM**1**$_1$**2**$_{149}$, MDS42 recA *E. coli* cells bearing pRSF ribo-Q1 O-*gst-cam$_{1TAG-40AGTA}$* or pRSF ribo-Q1 O-*gst-cam$_{1TAG-149AGTA}$* were co-transformed by electroporation with pSUP *Mj*PrpRS/tRNA$_{CUA}$ and pCDF PylRS/tRNA$_{UACU}$. Transformations were recovered in 1 ml SOB medium for one hour at 37°C, and used to inoculate 100 ml LB-KSC (LB media with 12.5 μg ml^{-1} kanamycin, 18.8 μg ml^{-1} spectinomycin, and 8.8 μg ml^{-1} chloramphenicol). The culture was incubated overnight (37°C, 250 rpm, 16 h), then diluted to OD_{600}=0.1 in LB-KSC and incubated (37°C, 250 rpm) until OD_{600} reached 0.5. IPTG (1 mM final concentration) without or with unnatural amino acids **1** (2 mM)

and **2** (1 mM) was added to the culture. The culture was incubated (37°C, 250 rpm) for 4 h, and 100 ml of the culture without or with supplemented unnatural amino acids was pelleted (3,000g, 10 min, 4°C) and washed twice with 1 ml PBS.

Purification of GST-CaM1$_1$2$_{40}$

To purify overexpressed proteins via the GST tag, cell pellets were resuspended in 1 ml of Novagen BugBuster Protein Extraction Reagent (supplemented with 1× Roche protease inhibitor cocktail tablet, 1 mg ml^{-1} Sigma lysozyme, 1 mg ml^{-1} Sigma DNase I) and lysed (25°C, 250 rpm, 1 h). The lysate was clarified by centrifugation (25,000g, 30 min, 4°C). GST containing proteins from the lysate were bound in batch (1 h, 4°C) to 70 µl of glutathione sepharose beads (GE Healthcare). Beads were washed 4 times with 1 ml PBS prior to elution by heating in 1× Invitrogen NuPAGE LDS sample buffer (95°C, 5 min) supplemented with 100 mM DTT. All samples were analysed on 4-12% Bis-Tris gels (Invitrogen) with BIO-RAD Low Range Molecular Weight Standard as marker. The gels were subsequently stained with Coomassie Blue (InstantBlue, Expedeon).

Thrombin cleavage and purification of CaM1$_1$, CaM2$_{40}$, CaM1$_1$2$_{40}$ and CaM1$_1$2$_{149}$ recombinant proteins

To purify overexpressed proteins without the GST tag, cell pellets were resuspended in 1 ml of Novagen BugBuster Protein Extraction Reagent (supplemented with 1× Roche protease inhibitor cocktail tablet, 1 mg ml^{-1} Sigma lysozyme, 1 mg ml^{-1} Sigma DNase I), washed with PBS, and lysed (25°C, 250 rpm, 1 h). The lysate was clarified by centrifugation (25,000g, 30 min, 4°C). GST-CaM containing proteins from the lysate were bound in batch (1 h, 4°C) to 70 µl of glutathione sepharose beads (GE Healthcare). Beads were washed 4 times with 1 ml PBS prior to adding 300 µl PBS with 1 unit of thrombin (Invitrogen), which recognizes the thrombin cleavage site between GST and CaM in GST-CaM. The CaM was cleaved from the beads overnight (16 h, 25°C, 20 rpm). 300 µl supernatant, after the overnight cleavage, was

transferred to a fresh tube and the glutathione sepharose beads were washed 2 times with 300 µl PBS. All washing fractions were pooled together with the original 300 µl supernatant to reach a total volume of 900µl before adding 100 µl of Ni NTA Agarose beads (Qiagen) and binding for 1 h (4°C, 20 rpm). The cleaved CaM was purified by its C-terminal His×6 tag. Beads were washed 4 times with 1 ml PBS supplemented with 10 mM imidazole and eluted with 4 fractions of 60 µl PBS supplemented with 250 mM imidazole. The four fractions of eluted CaM were pooled together to a total volume of 240 µl. Salt and imidazole was removed using 7K MWCO Zeba spin desalting columns (Thermo Scientific). These samples were then analyzed by SDS-PAGE and mass spectrometry, and subsequently used for labeling experiments.

Procedure for labeling of CaM$\mathbf{1}_1$ with 3 via Cu(I)-catalyzed click reaction

Labeling of CaM$\mathbf{1}_1$ with **3** was performed in 20 µl reaction volume. 5 µl of 20 µM purified CaM$\mathbf{1}_1$ in water (~100 pmol), 1 µl of 2 mM stock of **3** (2 nmole) in DMF, 2 µl of 10x PBS buffer (pH 7.4), and 0.2 µl of click mix (100x) were incubated at 25°C for 30 min. The click mix was prepared by mixing 1 µl of 100 mM $CuSO_4$ with 2.5 µl of 100 mM sodium ascorbate, and subsequent addition of 5 µl of 100 mM THPTA and 1.5 µl of water. To demonstrate that cyclopropene-containing protein does not undergo reaction with azide **3** in presence of Cu(I), CaM$\mathbf{2}_{40}$ was incubated with **3** under same reaction conditions as a control. After 30 min, the labeling reactions were diluted to 100 µl and desalted using 7K MWCO Zeba spin desalting columns (Thermo Scientific) to remove excess dye and other salts, and analyzed by SDS-PAGE and electrospray ionization mass spectrometry. For analysis using SDS-PAGE, 20 µl of these samples were mixed with 8 µl of NuPAGE LDS sample buffer and DTT (100 mM final concentration), heated at 95ºC for 5 min, and loaded onto 4-12% Bis-Tris gels (Invitrogen) with BIO-RAD Low Range Molecular Weight Standard as marker. The fluorescent bands on the gel were imaged using Typhoon Trio phosphoimager (GE Life Sciences) with excitation filter set to 532 nm and emission filter set to 580 nm. Subsequently, the same gel was stained with Coomassie Blue (InstantBlue, Expedeon). The labeling resulted in a migration shift on the SDS-PAGE. For analysis of labeling using mass spectrometry, labeled

CaM**1-3**$_1$ was desalted using 7K MWCO Zeba spin desalting columns (Thermo Scientific) to remove excess dye and other salts. Samples were analyzed using electrospray ionization mass spectrometry.

Procedure for labeling of CaM2$_{40}$ with 4 via inverse electron-demand Diels-Alder reaction

Labeling of CaM**2**$_{40}$ with **4** was performed in 20 µl reaction volume. 5 µl of 20 µM purified CaM**2**$_{40}$ in water (~100 pmol), 1 µl of 2 mM stock of **4** (2 nmole) in DMF and 2 µl of 10x PBS buffer (pH 7.4) were incubated at 25^{o}C for 30 min. To demonstrate that alkyne-containing protein does not undergo reaction with tetrazine **4**, CaM**1**$_1$ was incubated with **4** under same reaction conditions as a control. After 30 min, the labeling reactions were diluted to 100 µl and desalted using 7K MWCO Zeba spin desalting columns (Thermo Scientific) to remove excess dye and other salts, and analyzed by SDS-PAGE and electrospray ionization mass spectrometry. For analysis using SDS-PAGE, these samples were mixed with 8 µl of NuPAGE LDS sample buffer and DTT (100 mM final concentration), heated at 95ºC for 5 min, and loaded onto 4-12% Bis-Tris gels (Invitrogen) with BIO-RAD Low Range Molecular Weight Standard as marker. The fluorescent bands on the gel were imaged using Typhoon Trio phosphoimager (GE Life Sciences) with excitation filter set to 488 nm and emission filter set to 526 nm. Subsequently, the same gel was stained with Coomassie Blue (InstantBlue, Expedeon). The labeling resulted in a migration shift on the SDS-PAGE. For analysis of labeling using mass spectrometry, labeled CaM**2-4**$_{40}$ was desalted using 7K MWCO Zeba spin desalting columns (Thermo Scientific) to remove excess dye and other salts. Samples were analyzed using electrospray ionization mass spectrometry.

Labeling time course for the Cu(I)-catalyzed reaction of CaM1$_1$2$_{40}$ or CaM1$_1$2$_{149}$ with 3

Labeling of CaM**1**$_1$**2**$_{40}$ or CaM**1**$_1$**2**$_{149}$ with **3** was performed in 100 µl reaction volume. 50 µl of 20 µM purified CaM**1**$_1$**2**$_{40}$ or CaM**1**$_1$**2**$_{149}$ in water (~1 nmol), 10 µl of 2 mM stock of **3** (20 nmole) in DMF, 10 µl of 10x PBS buffer (pH 7.4), and 1 µl of click mix (100x) were incubated at 25^{o}C for 0 to 2 h. The click mix was prepared by mixing 1 µl of 100 mM $CuSO_4$ with 2.5 µl of 100 mM sodium ascorbate, and subsequent addition of 5 µl of 100 mM THPTA and 1.5 µl of water.

At each time point, 10 µl aliquots of the labeling reaction were quenched by adding 1 µl of 100 mM EDTA and 1 µl of 50 mM Bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN-OH, exo/endo mixture ~2/1, SynAffix, Netherlands) in a separate tube. EDTA was employed to sequester Cu(I) and BCN-OH, a stained alkyne reacts with azide fluorophore **3,** thus deactivating **3**. For analysis using SDS-PAGE, these samples were mixed with 8 µl of NuPAGE LDS sample buffer and DTT (100 mM final concentration), heated at 95ºC for 5 min, and loaded onto either 4-12% Bis-Tris gels or 12% Bis-Tris gels (Invitrogen) with BIO-RAD Low Range Molecular Weight Standard as marker. The fluorescent bands on the gel were imaged using Typhoon Trio phosphoimager (GE Life Sciences) with excitation filter set to 532 nm and emission filter set to 580 nm. Subsequently, the same gel was stained with Coomassie Blue (InstantBlue, Expedeon). The labeling resulted in a migration shift on the SDS-PAGE.

Time course for the inverse electron-demand Diels-Alder reaction of CaM$\mathbf{1}_1\mathbf{2}_{40}$ or CaM$\mathbf{1}_1\mathbf{2}_{149}$ with 4 or 5

Labeling of CaM$\mathbf{1}_1\mathbf{2}_{40}$ or CaM$\mathbf{1}_1\mathbf{2}_{149}$ with **4** or **5** was performed in 100 µl reaction volume. 50 µl of 20 µM purified CaM$\mathbf{1}_1\mathbf{2}_{40}$ or CaM$\mathbf{1}_1\mathbf{2}_{149}$ in water (~1 nmol), 10 µl of 2 mM stock of **4** or **5** (20 nmole) in DMF and 10 µl of 10x PBS buffer (pH 7.4) were incubated at 25°C for 0 to 2 h. At each time point, 10 µl aliquots of the labeling reaction were quenched by adding 1 µl of 50 mM Bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN-OH, exo/endo mixture ~2/1, SynAffix, Netherlands) in a separate tube. BCN-OH reacts with tetrazine fluorophore **4** or **5**. The rate constant for this reaction is 437±13 M^{-1} s^{-1} ,[4] approximately 20× that of the reaction between **2** and **4/5**.[5] For analysis using SDS-PAGE, these samples were mixed with 8 µl of NuPAGE LDS sample buffer and DTT (100 mM final concentration), heated at 95ºC for 5 min, and loaded onto 4-12% Bis-Tris gels or 12% Bis-Tris gels (Invitrogen) with BIO-RAD Low Range Molecular Weight Standard as marker. The fluorescent bands on the gel were imaged using Typhoon Trio phosphoimager (GE Life Sciences) with excitation filter set to 488 nm and emission filter set to 526 nm. Subsequently, the same gel was stained with Coomassie Blue (InstantBlue, Expedeon). The labeling resulted in a migration shift on the SDS-PAGE.

Procedure for sequential and one-pot labeling of CaM$\mathbf{1}_1\mathbf{2}_{40}$ or CaM$\mathbf{1}_1\mathbf{2}_{149}$ with 3 and 4/5 via Cu (I)-catalyzed click reaction and inverse electron-demand Diels-Alder reactions respectively

Labeling of CaM$\mathbf{1}_1\mathbf{2}_{40}$ with **3** and **4** was performed either in two steps (**Figure 3a**, lane 4) or in one-pot (**Figure 3a**, lane 5 and 6). All labeling reactions were performed in 20 µl reaction volume. For labeling in two steps (**Figure 3a**, lane 4), 5 µl of 20 µM purified CaM$\mathbf{1}_1\mathbf{2}_{40}$ in water (~100 pmol) was incubated with 1 µl of 2 mM stock of **4** (2 nmole) in DMF and 2 µl of 10x PBS buffer (pH 7.4) at 25^{o}C for 30 min. The sample was diluted to 100 µl. Excess **4** and salts were removed by running the sample through 7K MWCO Zeba spin desalting columns (Thermo Scientific) (pre-equilibrated with water) three times. The resulting singly labeled CaM$\mathbf{1}_1$-**2-4**$_{40}$ was concentrated to 5 µl using SpeedVac. This sample was then used for second labeling with **3**. 5 µl of purified CaM$\mathbf{1}_1$-**2-4**$_{40}$ in water (~100 pmol), 1 µl of 2 mM stock of **3** (2 nmole) in DMF, 2 µl of 10x PBS buffer (pH 7.4), and 0.2 µl of click mix (100x) were incubated at 25^{o}C for 15 min. The click mix was prepared by mixing 1 µl of 100 mM $CuSO_4$ with 2.5 µl of 100 mM sodium ascorbate, and subsequent addition of 5 µl of 100 mM THPTA and 1.5 µl of water. Resulting doubly labeled CaM**1-3**$_1$-**2-4**$_{40}$ was diluted to 100 µl. Excess **3** and salts were removed by running this sample through 7K MWCO Zeba spin desalting columns (Thermo Scientific) (pre-equilibrated with water) three times. The resulting doubly labeled CaM**1-3**$_1$-**2-4**$_{40}$ was concentrated to 20 µl using SpeedVac.

One-pot labeling of CaM$\mathbf{1}_1\mathbf{2}_{40}$ with **3** and **4** was performed either in a sequential manner (**Figure 3a**, lane 5) or in a single step (**Figure 3a**, lane 6). All labeling reactions were performed in 20 µl reaction volume. For sequential one-pot labeling (**Figure 3a**, lane 5), 5 µl of 20 µM purified CaM$\mathbf{1}_1\mathbf{2}_{40}$ in water (~100 pmol) was incubated with 1 µl of 2 mM stock of **4** (2 nmole) in DMF and 2 µl of 10x PBS buffer (pH 7.4) at 25^{o}C for 30 min. After 30 min, 1 µl of 2 mM stock of **3** (2 nmole) in DMF, and 0.2 µl of click mix (100x) were added to this reaction mixture and incubated at 25^{o}C for another 30 min. The click mix (100x) was prepared by mixing 1 µl of 100 mM $CuSO_4$ with 2.5 µl of 100 mM sodium ascorbate, and subsequent addition of 5 µl of 100 mM THPTA and 1.5 µl of water. The resulting doubly labeled CaM**1-3**$_1$-**2-4**$_{40}$ was diluted to 100 µl, excess **3, 4** and salts were removed by running the sample through 7K

MWCO Zeba spin desalting columns (Thermo Scientific) (pre-equilibrated with water) three times, and the sample was concentrated to 20 µl using SpeedVac.

For concerted one-pot labeling of CaM**1**$_1$**2**$_{40}$ with **3** and **4** in a single step (**Figure 3a**, lane 6), 5 µl of 20 µM purified CaM**1**$_1$**2**$_{40}$ in water (~100 pmol) was incubated with 1 µl of 2 mM stock of **4** (2 nmole) in DMF, 1 µl of 2 mM stock of **3** (2 nmole) in DMF, and 0.2 µl of click mix (100x) and 2 µl of 10x PBS buffer (pH 7.4). The total reaction volume was 20 µl and the reaction was incubated at 25^{o}C for 30 min. The resulting doubly labeled CaM**1-3**$_1$-**2-4**$_{40}$ was diluted to 100 µl. Excess **3, 4** and salts were removed by running this sample through 7K MWCO Zeba spin desalting columns (Thermo Scientific) (pre-equilibrated with water) three times. The resulting doubly labeled CaM**1-3**$_1$-**2-4**$_{40}$ was concentrated to 20 µl using SpeedVac.

For concerted one-pot labeling of CaM**1**$_1$**2**$_{40}$/ CaM**1**$_1$**2**$_{149}$ with **3** and **5** in a single step (**Supplementary Figure 4b and Supplementary Figure 5d**), 5 µl of 20 µM purified CaM**1**$_1$**2**$_{40}$ or CaM**1**$_1$**2**$_{149}$ in water (~100 pmol) was incubated with 1 µl of 2 mM stock of **5** (2 nmole) in DMF, 1 µl of 2 mM stock of **3** (2 nmole) in DMF, and 0.2 µl of click mix (100x) and 2 µl of 10x PBS buffer (pH 7.4). The total reaction volume was 20 µl and the reaction was incubated at 25^{o}C for 30 min. The resulting doubly labeled CaM**1-3**$_1$-**2-5**$_{40}$ or CaM**1-3**$_1$-**2-5**$_{149}$ was diluted to 100 µl. Excess **3, 5** and salts were removed by running this sample through 7K MWCO Zeba spin desalting columns (Thermo Scientific) (pre-equilibrated with water) three times. The resulting doubly labeled CaM**1-3**$_1$-**2-5**$_{40}$ and CaM**1-3**$_1$-**2-5**$_{149}$ were concentrated to 20 µl using SpeedVac.

All these samples were analyzed by SDS-PAGE. Samples were mixed with NuPAGE LDS sample buffer supplemented with DTT (100 mM final concentration), heated for 5 min at 95^{o}C and loaded onto 12% Bis-Tris gels (Invitrogen) with BIO-RAD Low Range Molecular Weight Standard as marker. The fluorescent bands on the gel were imaged using Typhoon Trio phosphoimager (GE Life Sciences). Subsequently, the same gel was stained with Coomassie Blue (InstantBlue, Expedeon). The labeling resulted in a migration shift on SDS-PAGE. Double-labeled samples CaM**1-3**$_1$-**2-4**$_{40,}$ CaM**1-3**$_1$-**2-5**$_{40}$ and CaM**1-3**$_1$-**2-5**$_{149}$ were also analyzed by electrospray ionization mass spectrometry.

Fluorescence measurements

All fluorescence spectra were measured on Luminescence Spectrometer (Perkin Elmer LS55). In order to measure FRET between the BODIPY-FL and BODIPY-TMRX labeled at position 40/149 and 1 in CaM**1-3**$_1$-**2-4**$_{40}$ or CaM**1-3**$_1$-**2-4**$_{149}$, the emission spectra were acquired between 505 nm and 650 nm using an excitation wavelength of 485 nm. The excitation and emission bandwidth were set to 10 nm for all measurements. Fluorescence spectra of doubly labeled CaM**1-3**$_1$-**2-5**$_{40}$ or CaM**1-3**$_1$-**2-5**$_{149}$ and donor only CaM**1**$_1$-**2-5**$_{40}$ or CaM**1**$_1$-**2-5**$_{149}$ were normalised by total emission (the total area below the spectra curve). The spectra of acceptor only CaM**1-3**$_1$-**2**$_{40}$ or CaM**1-3**$_1$-**2**$_{149}$ were normalised by matching the acceptor emission at 570 nm when excited at 535 nm to that of the doubly labeled CaM**1-3**$_1$-**2-5**$_{40}$ or CaM**1-3**$_1$-**2-5**$_{149}$ measured after excitation at 535 nm.

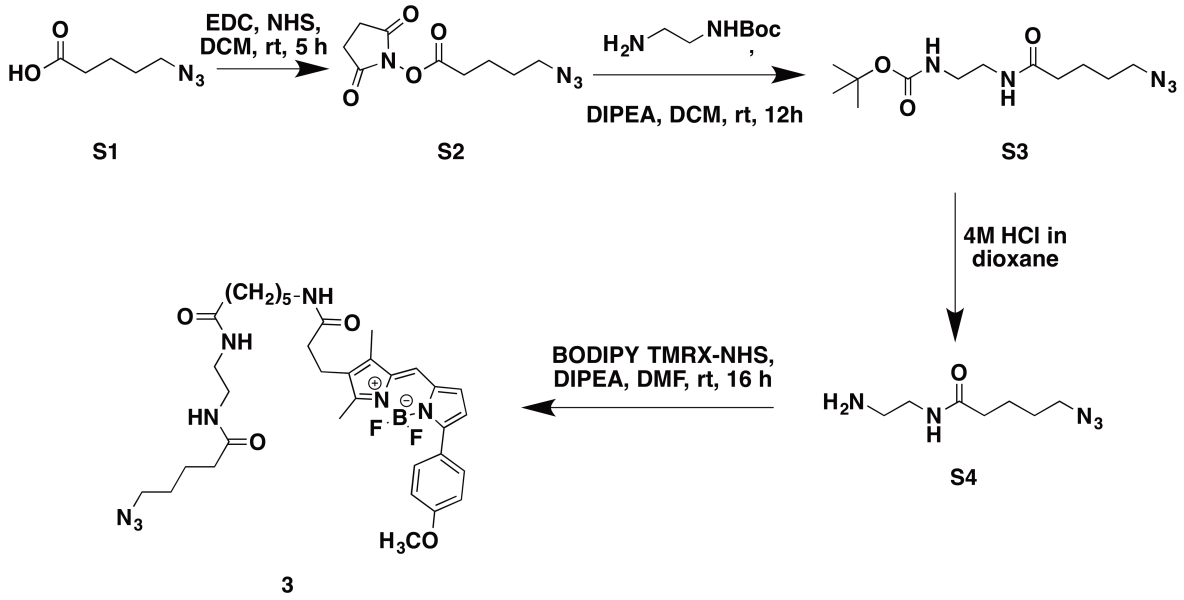
Chemical synthesis

General Methods

Analytical thin-layer chromatography (TLC) was carried out on silica 60F-254 plates and flash chromatography was carried out on silica gel 60 (230-400 mesh). The spots on the TLC were visualized by UV light and /or ninhydrin and/or Vanillin staining. ESI-MS was acquired using an Agilent 1200 LC-MS system with a 6130 Quadrupole spectrometer. The solvent system used for the LC-MS was 0.2% formic acid in water as buffer A and 0.2% formic acid in acetonitrile (MeCN) as buffer B. For analysing small molecules, Phenomenex Jupiter C18 column (150 x 2 mm, 5 μm) was used for liquid chromatography and mass spectra were acquired in both positive and negative modes. Semi-preparative HPLC purification was carried out using a Varian PrepStar/ProStar HPLC system with an automated fraction collector. Phenomenex C18 column (250 x 30 mm, 5 μm) column was used to separation and compounds were identified by UV absorbance at 191 nm. All solvents and chemical reagents were purchased from commercial suppliers and used without further purification. The ligand used in Cu(I)-

catalyzed click reaction, Tris-(hydroxypropyltriazolylmethyl)amine (THPTA) was synthesized as previously described.[6] Unnatural amino acids **1** and **2** were synthesized as previously described.[5,7]

Synthesis of Azide-BODIPY-TMRX (3)



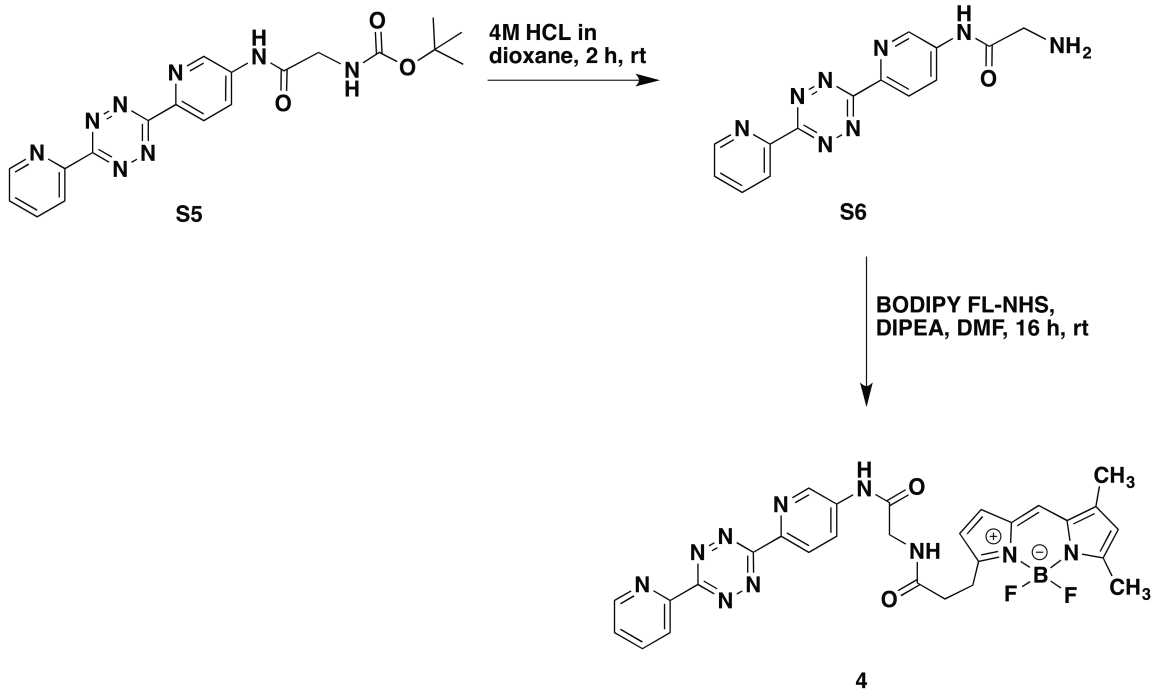
NHS-activated azide **S2** was synthesized by adding 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC (190 mg, 1.2 mmol) and *N*-Hydroxysuccinimide, NHS (170 mg, 1.5 mmol) sequentially to the stirring solution of 5-azidopentanoic acid **S1** (150 mg, 1 mmol) in DCM (25 ml). The reaction was stirred at room temperature for 5 h, subsequently diluted with DCM (50 ml), washed twice with 2% HCL (50 ml) and then twice with water (50 ml). The organic layer was dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure to yield. This compound was used directly in the next step without any further purification.

The azide-succinimidyl ester **S2** from previous step was dissolved in dry DCM (20 ml). To this solution, tert-butyl(2-aminoethyl)carbmate hydrochloride (300 mg, 1.5 mol) and N,N-Diisopropylethylamine, DIPEA (0.7 ml, 3.85 mmol) were added and stirred for 2 h. This reaction mixture was then diluted with DCM (50 ml), washed twice with 2M Na_2CO_3 (50 ml), and then twice with water (50 ml). The organic layer was dried over anhydrous $MgSO_4$, solvent removed under reduced pressure and the crude was purified using column chromatography on SiO_2 (0-8% methanol in DCM) to yield purified compound **S3**.

Boc-deprotection of compound **S3** was carried out by adding 4M HCl in dioxane (500 µL, 2.0 mmol) to the solution of Boc-protected compound **S3** (3 mg, 0.01 mmol) in DCM (500 µL). The reaction mixture was stirred for 2 h at room temperature. Subsequently the solvent was removed under reduced pressure to yield primary amine hydrochloride **S4**. This compound was directly used in the next step without any further purification.

BODIPY-TMRX succinimidyl ester (5mg, 0.008 mmol, Life Technologies) and N,N-Diisopropylethylamine, DIPEA (50 µl, 2.8 mmol) were added to the solution of azide-amine **S4** in dry DMF (1 mL). The reaction mixture was stirred at room temperature for 16 h and subsequently, diluted with 4 mL of water. The product was purified by semi-preparative reverse phase HPLC using a gradient from 10% to 90% of buffer B in buffer A (buffer A: H_2O; buffer B: acetonitrile). The identity and purity of the Azide-BODIPY-TMRX conjugate **3** was confirmed by LC-MS. ESI-MS: $[M-H]^-$, calcd. 677.6, found 677.3.

Synthesis of Tet1-BODIPY FL (4)

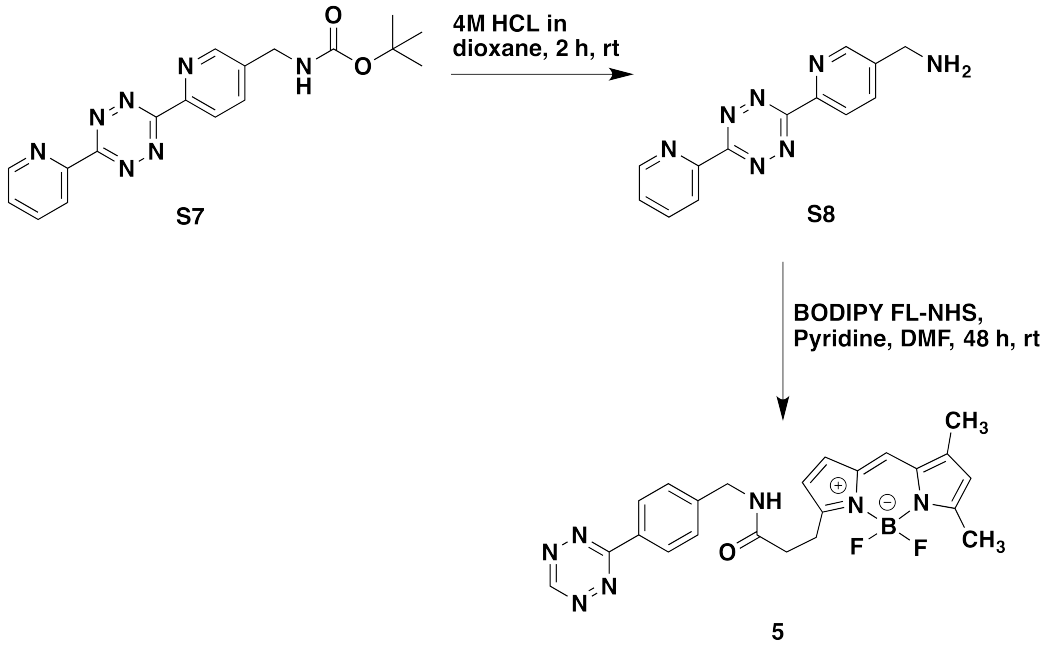


Boc-protected Tetrazine **S5** was synthesized and characterized as previously described.[8] Boc-deprotection was carried out by adding 4M HCl in dioxane (500 µL, 2.0 mmol) to the solution of Tetrazine **S5** (8 mg,

0.02 mmol) in DCM (500 µL) and stirring for 2 h at room temperature. Subsequently the solvent was removed under reduced pressure to yield primary amine hydrochloride **S6** as a pink solid (6mg, 0.02 mmol, 100%). The compound was directly used in the next step without any further purification.

BODIPY FL succinimidyl ester (5mg, 0.013 mmol, Life Technologies) and N,N-Diisopropylethylamine, DIPEA (50 µl, 0.28 mmol) were added to the solution of Tetrazine-amine **S6** (6mg, 0.02 mmol) in dry DMF (1 mL). The reaction mixture was stirred at room temperature for 16 h and subsequently, diluted with 4 mL of water. The product was purified by semi-preparative reverse phase HPLC using a gradient from 10% to 90% of buffer B in buffer A (buffer A: H_2O; buffer B: acetonitrile). The identity and purity of the Tet1-BODIPY FL conjugate **4** was confirmed by LC-MS. ESI-MS: $[M-H]^-$, calcd. 581.4, found 581.2.

Synthesis of TetD-BODIPY FL (5)



Boc-deprotection of the Tetrazine **S7** was carried out by adding 4M HCl in dioxane (500 µL, 2.0 mmol) to the solution of **S7** (7.5 mg, 0.02 mmol, Sigma) in DCM (500 µL) and stirring for 2 h at room temperature. Subsequently the solvent was removed under reduced pressure to yield primary amine hydrochloride **S8.** This compound was directly used in the next step without any further purification.

BODIPY FL succinimidyl ester (5mg, 0.013 mmol, Life Technologies) and pyridine (10 µl, 0.12 mmol) were added to the solution of Tetrazine-amine **S8** (5.3 mg, 0.02 mmol) in dry DMF (1 mL). The reaction mixture was stirred at room temperature for 48 h and subsequently, diluted with 4 mL of water. The product was purified by semi-preparative reverse phase HPLC using a gradient from 10% to 90% of buffer B in buffer A (buffer A: H_2O; buffer B: acetonitrile). The identity and purity of the TetD-BODIPY FL conjugate **5** was confirmed by LC-MS. ESI-MS: $[M-H]^-$, calcd. 460.28, found 460.3.

Electrospray ionization mass spectrometry

Mass spectra for protein samples were acquired on an Agilent 1200 LC-MS system that employs a 6130 Quadrupole spectrometer. The solvent system used for liquid chromatography (LC) was 0.2 % formic acid in H_2O as buffer A, and 0.2 % formic acid in acetonitrile (MeCN) as buffer B. Samples were injected into Phenomenex Jupiter C4 column (150 x 2 mm, 5 µm) and subsequently into the mass spectrometer using a fully automated system. Spectra were acquired in the positive mode and analyzed using the MS Chemstation software (Agilent Technologies). The deconvolution program provided in the software was used to obtain the mass spectra. Theoretical average molecular weight of proteins with unnatural amino acids was calculated by first computing the theoretical molecular weight of wild-type protein using an online tool (http://www.peptidesynthetics.co.uk/tools/), and then manually correcting for the theoretical molecular weight of unnatural amino acids.

Supplementary Figure Legends

Supplementary Figure 1. Efficient and specific incorporation unnatural amino acids **1** and **2** in GST-CaM**1**$_1$-**2**$_{40}$ is dependent on the addition of both unnatural amino acids**.** Cells containing O-*gst-cam*$_{1TAG+40AGTA}$ and ribo-Q1 rRNA, expressed from an RSF plasmid, the pSUP *Mj*PrpRS/tRNA$_{CUA}$ plasmid, and the pCDF PylRS/tRNA plasmid were grown in the presence of the indicated unnatural amino acids.

Supplementary Figure 2. **a) Top panel**: Raw ESI MS spectra for each of the de-convoluted ESI MS shown in Figures 1 and 3 in the main text and Supplementary Figure 4. **Bottom panel**: Simulated ESI MS spectra that were used to generate the de-convoluted spectra shown in Figures 1 and 3 in the main text and Supplementary Figure 4. CaM**1**$_1$ is CaM bearing **1** at position 1. CaM**1-3**$_1$ is CaM bearing **1** at position 1 labeled with **3**. CaM**2**$_{40}$ is CaM bearing **2** at position 40. CaM**2-4**$_{40}$ is CaM bearing **2** at position 40 labeled with **4**. CaM**1**$_1$**2**$_{40}$ is CaM bearing **1** at position 1 and **2** at position 40. CaM**1-3**$_1$**-2-4**$_{40}$ is CaM bearing **1** at position 1 labeled with **3** and **2** at position 40 labeled with **4**. CaM**1-3**$_1$**-2-5**$_{40}$ is CaM bearing **1** at position 1 labeled with **3** and **2** at position 40 labeled with **5. b) Top panel**: Raw ESI MS spectra for each of the de-convoluted ESI MS shown in Supplementary Figure 5. **Bottom panel**: Simulated ESI MS spectra that were used to generate the de-convoluted spectra shown in Supplementary Figure 5. CaM**1**$_1$**2**$_{149}$ is CaM bearing **1** at position 1 and **2** at position 149. CaM**1-3**$_1$**-2-5**$_{149}$ is CaM bearing **1** at position 1 labeled with **3** and **2** at position 149 labeled with **5.**

Supplementary Figure 3. Calmodulin bearing amino acids **1** and **2** is specifically labeled with probes **3** and **4** respectively. However, no labeling reaction was observed at any site in the wild-type CaM as demonstrated by in-gel fluorescence and migration of protein visualised by Coomassie staining.

Supplementary Figure 4. Site-specific double labeling of CaM1$_1$2$_{40}$ with a FRET pair. a) Labeling time course for reaction of CaM**1**$_1$**2**$_{40}$ with **5.** The reaction was followed for 2h by in gel fluorescence and

mobility shift, and is complete in less than 30 minutes. **b)** Quantitative and site-specific double labeling of CaM$\mathbf{1}_1\mathbf{2}_{40}$. Purified CaM$\mathbf{1}_1\mathbf{2}_{40}$ (**Lane 1**) was labeled with **3** yielding CaM**1-3**$_1$-**2**$_{40}$ (**Lane 2**) or **5** yielding CaM$\mathbf{1}_1$-**2-5**$_{40}$ (**Lane 3**). CaM$\mathbf{1}_1\mathbf{2}_{40}$ was labeled simultaneously with **3** and **5** in a single pot yielding CaM**1-3**$_1$-**2-5**$_{40}$ (**Lane 4**). Labeling was visualised by fluorescence imaging and led to a mobility shift. **c)** Labeling reaction was quantitative as judged by ESI MS. ESI MS of CaM$\mathbf{1}_1\mathbf{2}_{40}$ (black peak; Calculated molecular weight= 18000 Da, Observed molecular weight =18000 Da) and double labeled CaM**1-3**$_1$-**2-5**$_{40}$ (gold peak; Calculated molecular weight= 19112 Da, Observed molecular weight =19110 Da) obtained after one-pot double labeling reaction. Raw (before deconvolution) ESI-MS spectra in **Supplementary Figure 2a**. **d)** Fluorescence spectra of double labeled CaM**1-3**$_1$-**2-5**$_{40}$ (orange trace), single labeled CaM$\mathbf{1}_1$-**2-5**$_{40}$ (green trace, donor fluorophore only), and the single labeled CaM**1-3**$_1$-**2**$_{40}$ (red trace, acceptor fluorophore only) measured following excitation at 485 nm are consistent with FRET.

Supplementary Figure 5. Site-specific double labeling of CaM$\mathbf{1}_1\mathbf{2}_{149}$ with a FRET pair. a) Labeling time course for reaction of CaM$\mathbf{1}_1\mathbf{2}_{149}$ with **3. b)** Labeling time course for reaction of CaM$\mathbf{1}_1\mathbf{2}_{149}$ with **4. c)** Labeling time course for reaction of CaM$\mathbf{1}_1\mathbf{2}_{149}$ with **5.** All the labeling reactions were followed for 2h by in-gel fluorescence and mobility shift. Labeling of CaM$\mathbf{1}_1\mathbf{2}_{149}$ with **3** and **5** is complete in less than 30 minutes. However, the labeling with **4** is slower. **d)** Quantitative and site-specific double labeling of CaM$\mathbf{1}_1\mathbf{2}_{149}$. Purified CaM$\mathbf{1}_1\mathbf{2}_{149}$ (**Lane 1**) was labeled with **3** yielding CaM**1-3**$_1$-**2**$_{149}$ (**Lane 2**) or **5** yielding CaM$\mathbf{1}_1$-**2-5**$_{149}$ (**Lane 3**). CaM$\mathbf{1}_1\mathbf{2}_{149}$ was labeled simultaneously with **3** and **5** in a single pot yielding CaM**1-3**$_1$-**2-5**$_{149}$ (**Lane 4**). Labeling was visualised by fluorescence imaging and led to a mobility shift. **c)** Labeling reaction was quantitative as judged by ESI MS. ESI MS of CaM$\mathbf{1}_1\mathbf{2}_{149}$ (black peak; Calculated molecular weight= 17985. Da, Observed molecular weight =17983 Da) and double labeled CaM**1-3**$_1$-**2-5**$_{149}$ (gold peak; Calculated molecular weight= 19096 Da. With loss of fluorine from the fluorophore in the mass spectrometer the Calculated molecular weight= 19077 Da, Observed molecular weight =19074 Da) obtained after one-pot double labeling reaction. Raw (before deconvolution) ESI-MS spectra in

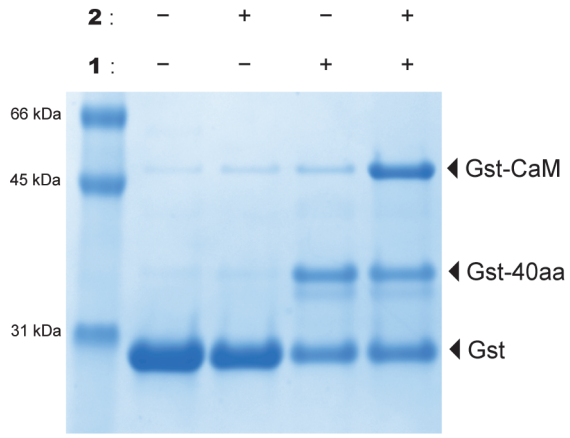
Supplementary Figure 2b. **d)** Fluorescence spectra of double labeled CaM**1-3**$_1$-**2-5**$_{149}$ (orange trace), single labeled CaM**1**$_1$-**2-5**$_{149}$ (green trace, donor fluorophore only), and the single labeled CaM**1-3**$_1$-**2**$_{149}$ (red trace, acceptor fluorophore only) measured following excitation at 485 nm.

Supplementary Table 1

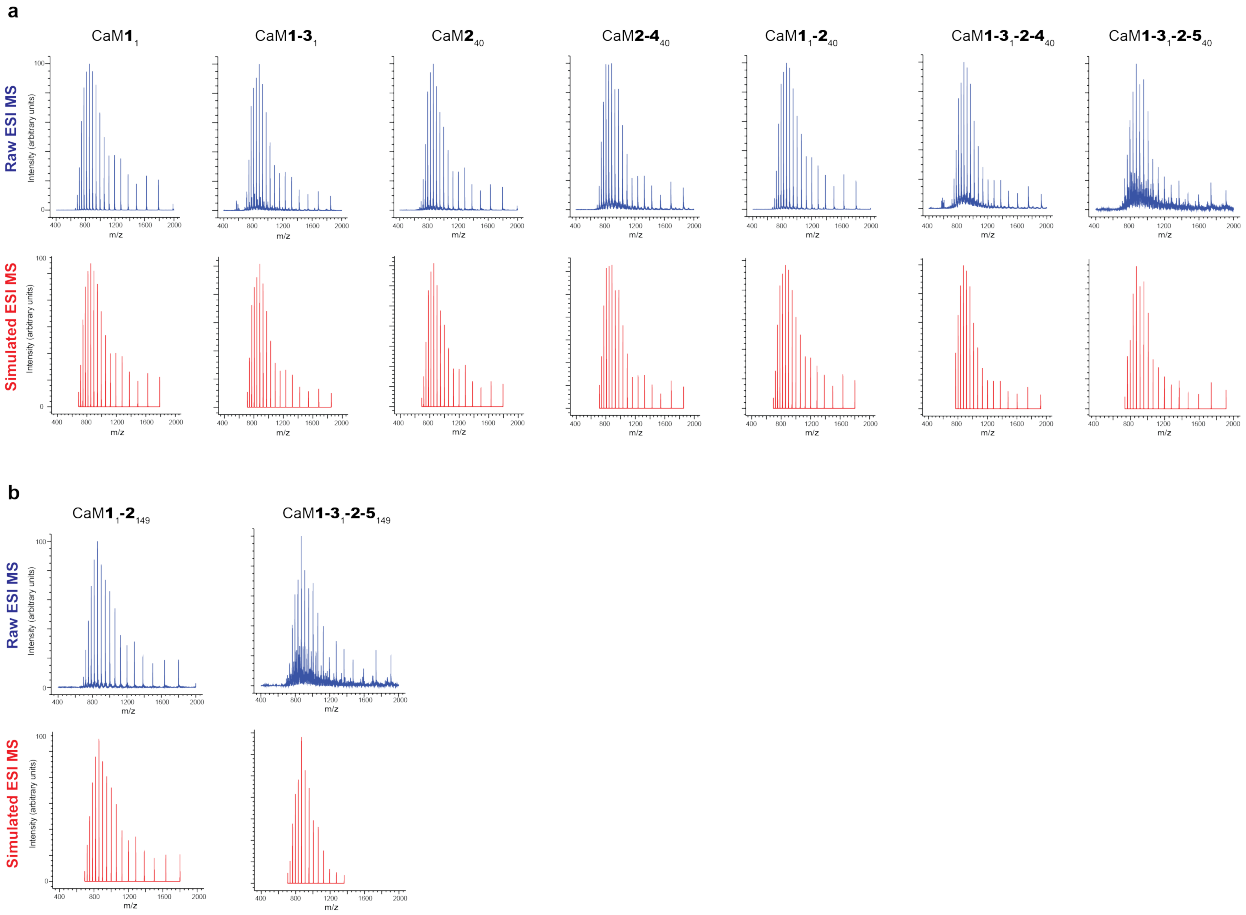
Primer Name	Primer Sequence (5' to 3')
GstCaMgeneF_KpnI	caGGTACCagcgcccaatacgcaaaccgcctctcc
GstCaMgeneR_KpnI	caGGTACCcaccgcatatggtgcactctcagtacaatc
CaM1TAGf	cgtggatccTAGgctgaccaactgacagaagagc
CaM1TAGr	gttggtcagcCTAggatccacgcggaaccagatcc
CaM40TAGf	atgaggtcgTAGggacaaaacccaacggaagcag
CaM4TAGr	gttttgtccCTAcgacctcataacggtgccaagttc
CaM40AGTAf	atgaggtcgAGTAggacaaaacccaacggaagcag
CaM40AGTAr	gttttgtccTACTcgacctcataacggtgccaagttc
CaM149AGTAf	tgatgacagcaAGTAcatcaccatcaccatcactaagcttaattagctg
CaM149AGTAr	gtgatggtgatgTACTtgctgtcatcatttgtacaaactcttcgtagt
pCDF1BamHIf	agccaggatccTCGGGagttgtcag
pCDF1743BglIIr	GAAagatctCACCATACCCACGCCGAAACAAG
PylTeAGTAf	CAGAAggtctcaGCTGAACaggatagtaagccaATTCGATCTACATGATCAGGTTCCCagatctAGC
PylTeAGTAr	CAGAAggtctctCAGCCGGGTTAGATTCCCGGGGTTTCCGCCAactag

Supplementary Figures

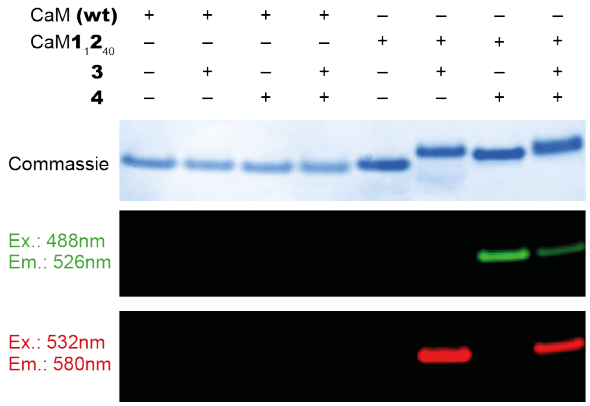
Supplementary Figure 1



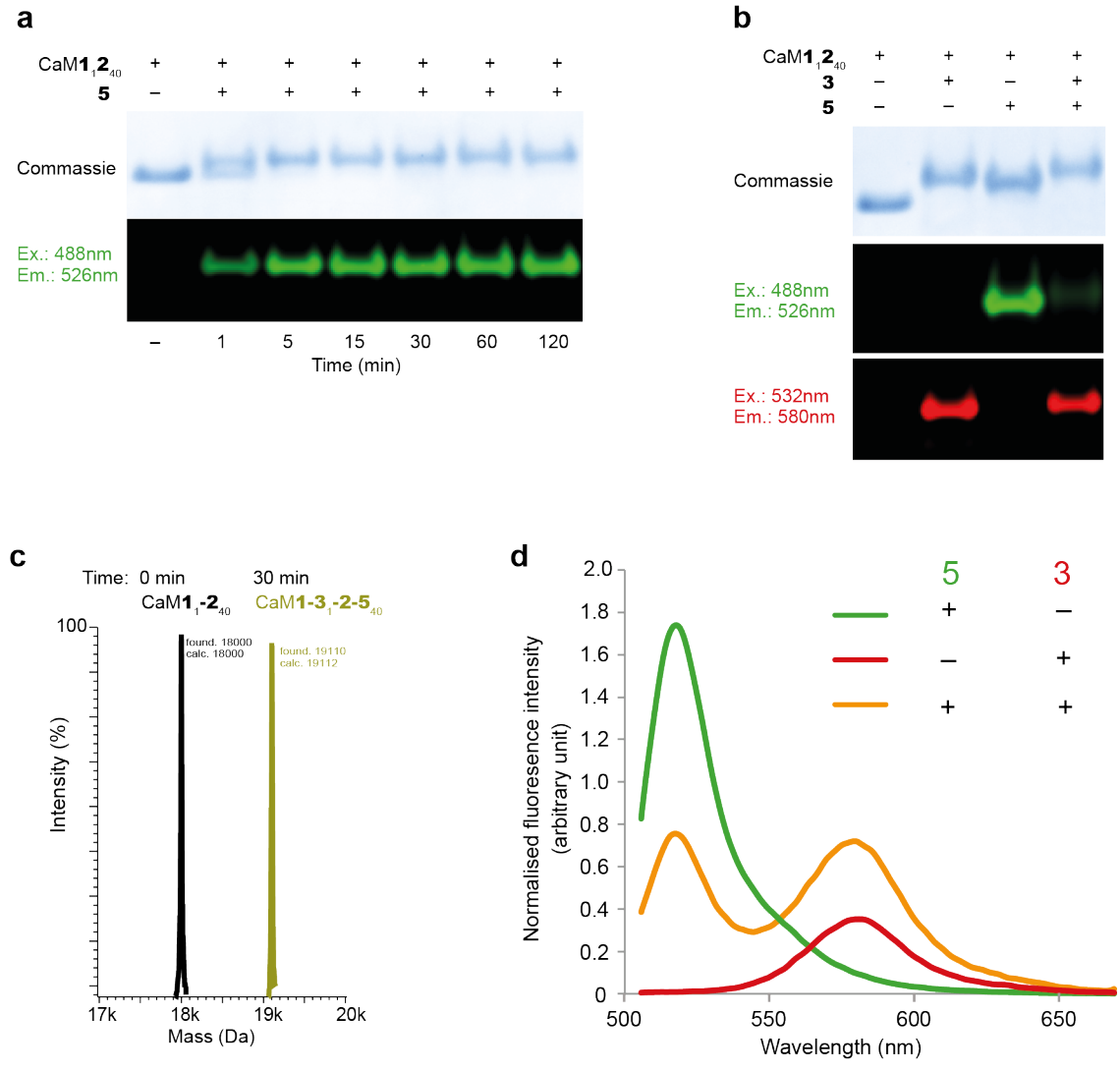
Supplementary Figure 2



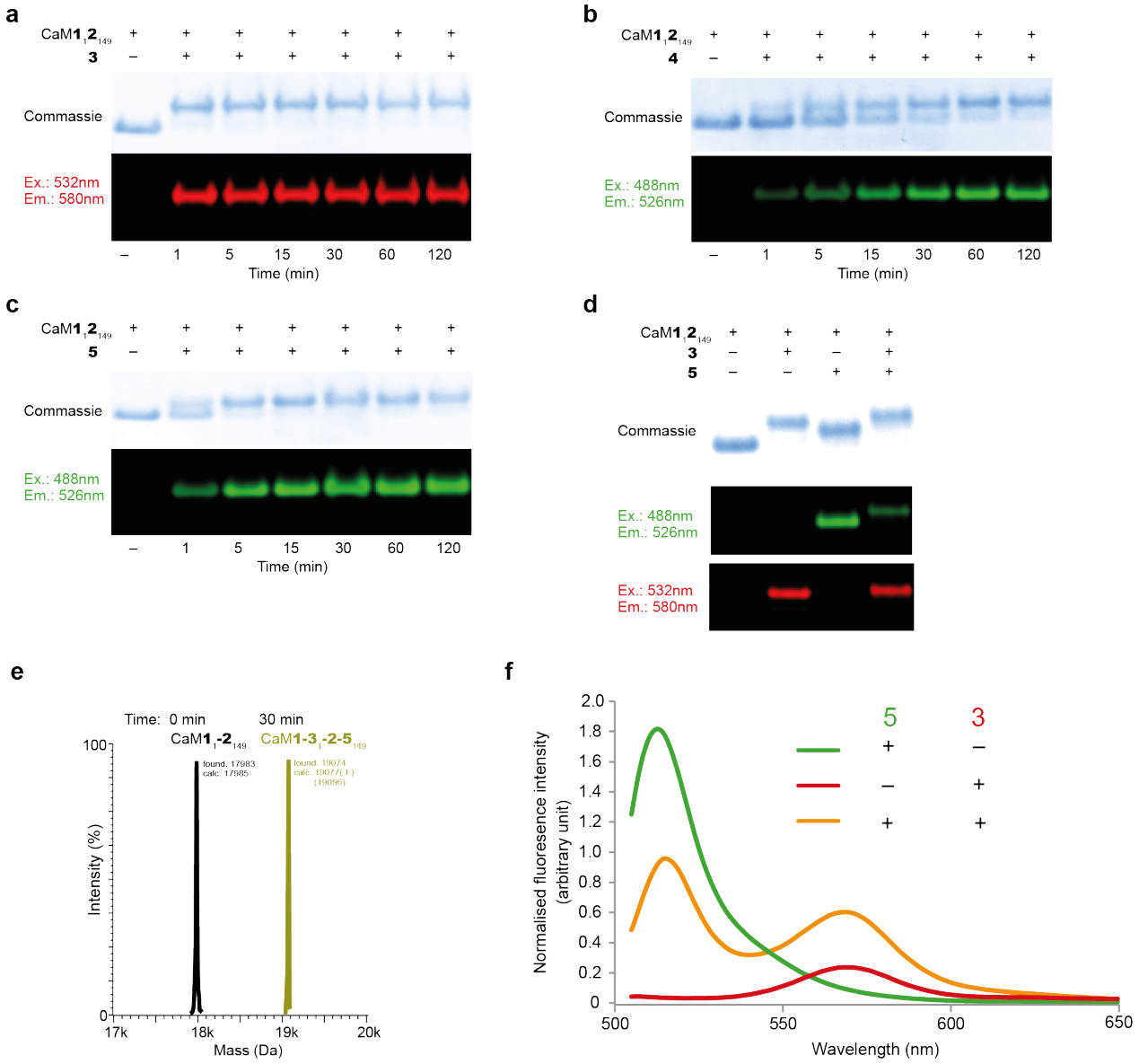
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary References

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