

Supplementary Materials for

Distinct beta-arrestin coupling and intracellular trafficking of metabotropic glutamate receptor homo- and heterodimers

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Figs. S1 to S7
NMR spectra
LCMS data

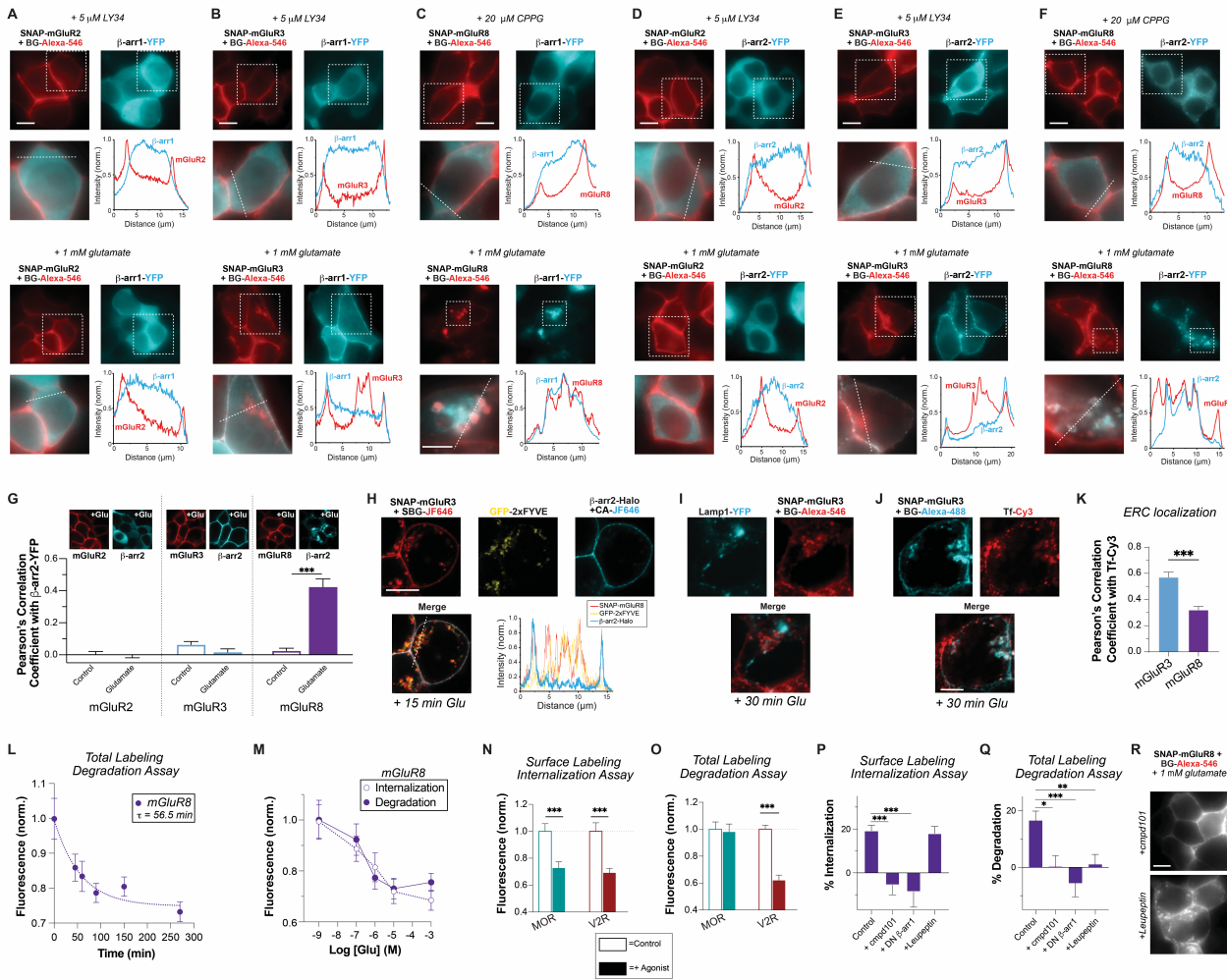


Fig. S1. Further analysis of mGluR internalization, trafficking, and degradation. (A to F) Representative live HEK 293T cell images showing SNAP-mGluR2 (A and D), SNAP-mGluR3 (B and E) or SNAP-mGluR8 (C and F) in red with β -arr1-YFP (A to C) or β -arr2-YFP (D to F) in green. Line scan profiles (from dotted lines) are shown as red for SNAP-tagged mGluRs and green for β -arr-YFP. All conditions are shown in the presence of antagonist or 15-30 min treatment with glutamate. (G) Pearson's correlation coefficient (PCC) analysis from confocal images comparing the top 10% of pixels between receptor and β -arr2-YFP for SNAP-mGluR2, -mGluR3, and -mGluR8. Representative scanning confocal images are shown. Scale bar is 10 μ m. (H) Scanning confocal images of live cells showing co-localization of SNAP-mGluR3 (red) with endosomal marker, GFP-2xFYVE (green), and β -arr2-Halo (cyan) in live cells. Line scan profiles are from dotted line in the merged image. (I) Scanning confocal images of fixed cells showing minimal co-localization of SNAP-mGluR3 (red) and Lamp1-YFP (green) in cells fixed after 30 min treatment with 1 mM Glu. (J) Scanning confocal images of fixed cells showing co-localization of SNAP-mGluR3 (green) and Tf-Cy3 (red) in cells fixed after 30 min treatment with 1 mM Glu. (K) PCC analysis comparing the top 10% of pixels between SNAP-mGluR3 or SNAP-mGluR8 with Tf-Cy3. (L) Quantification of SNAP-mGluR8 total labeling degradation assay at different time points. Tau of 56.5 min was found by fitting the data with a single exponential function. (M) Quantification of changes in fluorescence levels of SNAP-mGluR8 in surface and total labeling assays across glutamate concentration (N and O) Surface (N) or total (O) fluorescence levels from live cells expressing SNAP-tagged MOR or V2R treated with 60 min agonist (10 μ M DAMGO for MOR or

1 μ M Vasopressin for V2R) or control (antagonist; see Methods). Values are normalized to the fluorescence of a given receptor under control/antagonist conditions. **(P and Q)** Surface (P) or total (Q) fluorescence levels from live cells expressing SNAP-tagged mGluR8 with cmpd101, DN- β -arr1, or leupeptin. **(R)** Live cell fluorescence images of SNAP tagged mGluR8 with cmpd101 or leupeptin. Scale bars are 10 μ m. Data are represented as mean \pm SEM. Unpaired t-tests (G, K, N, O) or One-way ANOVA (P and Q), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

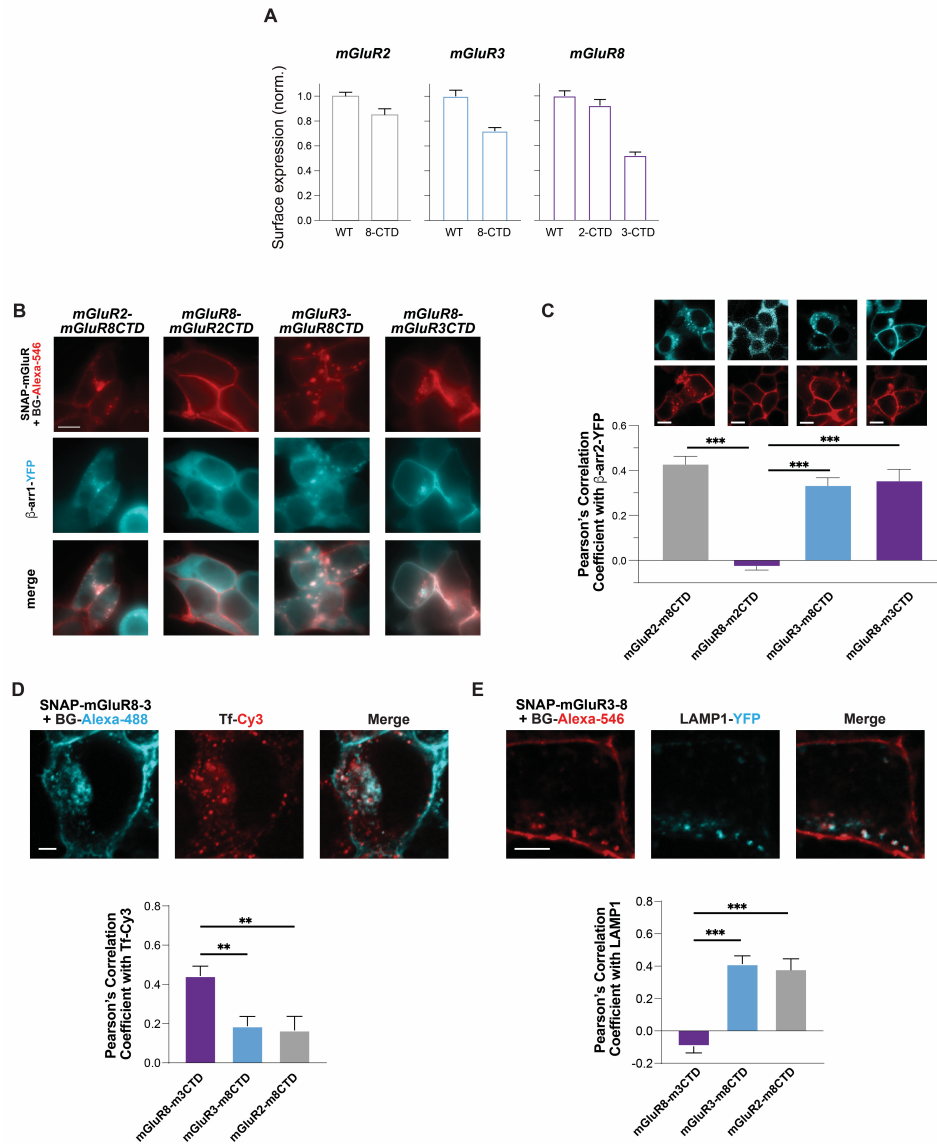


Fig. S2. Further analysis of C-terminal domain chimeras. (A) Quantification of surface expression for mGluR2-, mGluR3-, and mGluR8- C-terminal domain chimeras. Surface fluorescence is normalized to the respective wild type mGluR. (B) Fluorescence images of live cells expressing SNAP-mGluR2-mGluR8CTD, SNAP-mGluR8-mGluR2CTD, mGluR3-mGluR8CTD or mGluR8-mGluR3CTD (red) co-expressed with β -arr1-YFP (green) following treatment with glutamate. (C) Pearson's correlation coefficient analysis from confocal images comparing the top 10% of pixels between CTD chimeric receptors and β -arr2-YFP. Representative scanning confocal images are shown. Scale bar is 10 μ m. (D) Scanning confocal images of SNAP-mGluR8-mGluR3CTD (green) and Tf-Cy3 (red). Pearson's correlation coefficient (PCC) analysis comparing the top 10% of pixels between SNAP-mGluR8-mGluR3CTD with Tf-Cy3. (E) Scanning confocal images of SNAP-mGluR3-mGluR8CTD (red) and Lamp1-YFP (green). Pearson's correlation coefficient (PCC) analysis comparing the top 10% of pixels between SNAP-mGluR3-mGluR8CTD with Lamp1-YFP. Scale bars are 10 μ m. Data are represented as mean \pm SEM. One-way ANOVA, ** $p < 0.01$, *** $p < 0.001$.

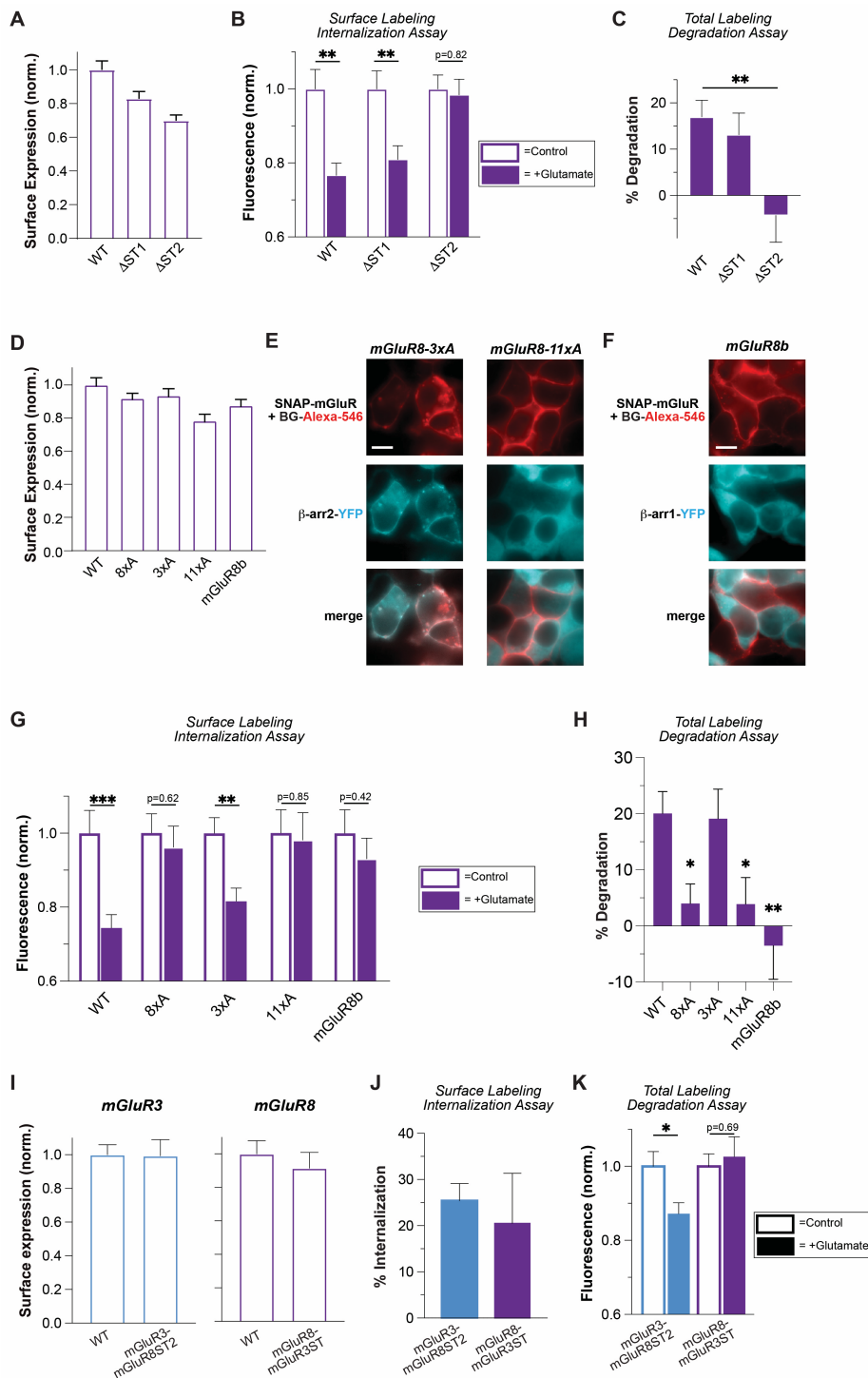


Fig. S3. Further analysis of the C-terminal domain of mGluR8. (A) Quantification of surface expression in SNAP-tagged mGluR8 containing deletion of either ST1 or ST2 regions. Surface fluorescence is normalized to wild type mGluR8. (B) Surface fluorescence levels from live cells expressing SNAP-mGluR8 containing deletion of either ST1 or ST2 regions treated with 60 min agonist (1 mM Glu) or control (antagonist). Values are normalized to the fluorescence of a given receptor under control/antagonist conditions. (C) Quantification of total fluorescence intensity from ST-rich region deleted SNAP tagged mGluR8. (D) Quantification of surface expression in SNAP tagged mGluR8 containing different alanine mutations in the C-terminal domain as well as SNAP tagged mGluR8b. Surface fluorescence is normalized to the wild-type mGluR8. (E) Fluorescence images of live cell expressing SNAP tagged mGluR8-3xA (red, left) and mGluR8-11xA (red, right) co-expressed with β -arr2-YFP (green). Merged image is shown below. (F) Fluorescence images of live cell expressing SNAP tagged mGluR8b (red) co-expressed with β -arr1-YFP (green). Merged image is shown below. (G) Surface fluorescence levels from live cells expressing SNAP-mGluR8

containing different mutations in the C-terminal domain treated with 60 min agonist (1 mM Glu) or control (antagonist). Values are normalized to the fluorescence of a given receptor under control/antagonist conditions. **(H)** Quantification of total fluorescence intensity from mGluR8 alanine mutants and mGluR8b. **(I)** Quantification of surface expression in SNAP tagged mGluR3 with mGluR8ST2 region (left) or mGluR8 with mGluR3ST region (right). Surface fluorescence is normalized to the respective wild type mGluRs. **(J)** Quantification of surface fluorescence intensity from SNAP tagged mGluR3-mGluR8ST2 and mGluR8-mGluR3ST. **(K)** Total fluorescence levels from live cells expressing SNAP-mGluR3-mGluR8ST2 or SNAP-mGluR8-mGluR3S treated with 60 min agonist (1 mM Glu) or control (antagonist). Values are normalized to the fluorescence of a given receptor under control/antagonist conditions. Scale bars are 10 μ m. Data are represented as mean \pm SEM. Unpaired t-tests (B, G, K) or One-way ANOVA (C, H), * $p < 0.05$, ** $p < 0.01$.

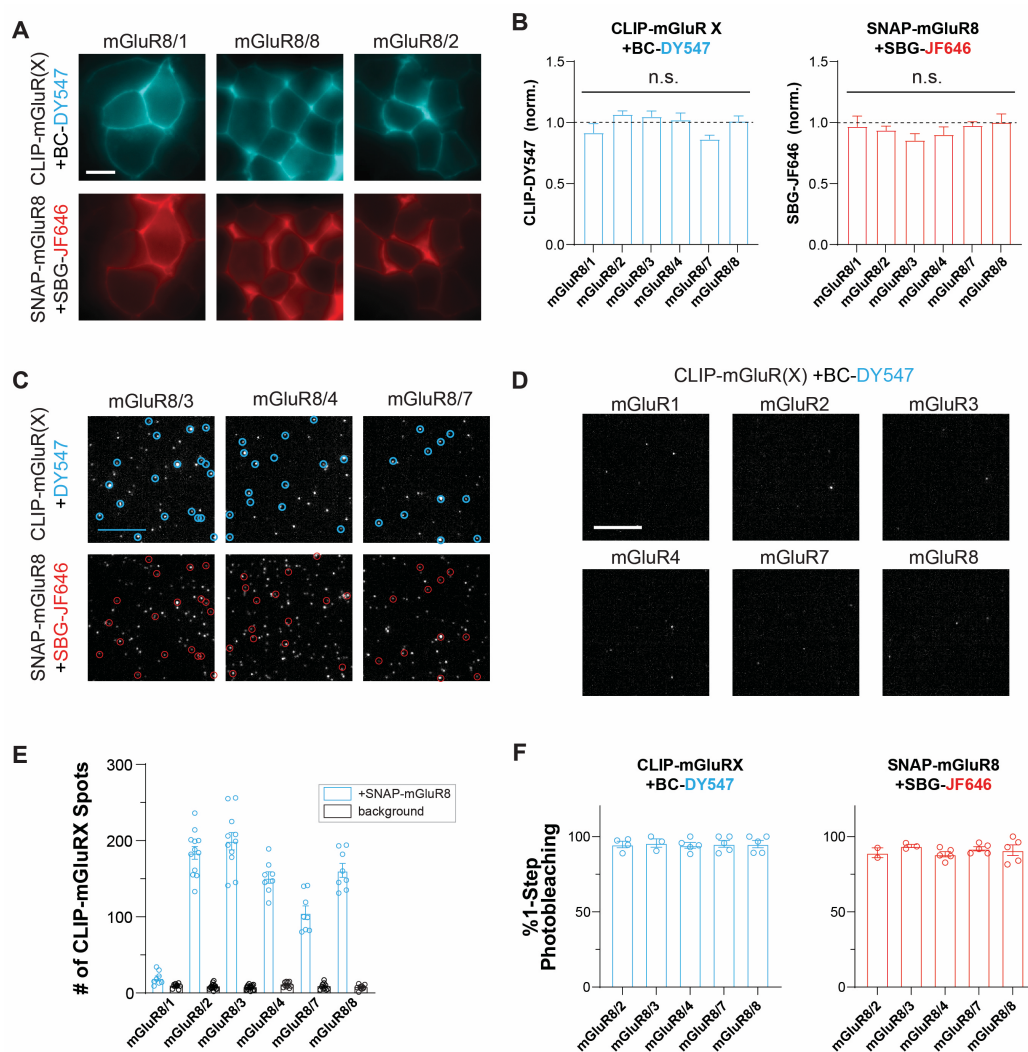


Fig. S4. Further analysis of mGluR co-expression and mGluR8 heterodimerization. (A) Fluorescence live cell images of SNAP-tagged mGluR8 (red) co-expressed with CLIP-tagged mGluR1, mGluR8 or mGluR2 (green). (B) Quantification of fluorescence intensity of cells with HA-SNAP-mGluR8 co-expressed with CLIP-tagged mGluRs. Fluorescence intensity is normalized to the homodimer condition expressing HA-SNAP-mGluR8 and CLIP-mGluR8. (C) Representative single molecule fluorescence images of HA-SNAP-mGluR8 with CLIP-mGluR3, -mGluR4, or -mGluR7. Co-localized spots are circled in green for CLIP-mGluRs and red for SNAP-mGluR8. (D) Representative single molecule images showing minimal non-specific binding of CLIP-mGluR1, -mGluR2, -mGluR3, -mGluR4, -mGluR7 or -mGluR8 when expressed alone and applied to a passivated coverslip coated in anti-HA antibodies. (E) Quantification of total number of CLIP-mGluRX spots (X = 1, 2, 3, 4, 7, 8) when expressed alone (black) or with SNAP-mGluR8 (green) (F) Quantification of %1-Step photobleaching step for colocalized HA-SNAP-mGluR8 (red) and CLIP-mGluRX spots (X = 2, 3, 4, 7, 8, green), indicating strict 1:1 heterodimerization. “n.s.” is from One-way ANOVA test. Data are represented as mean \pm SEM.

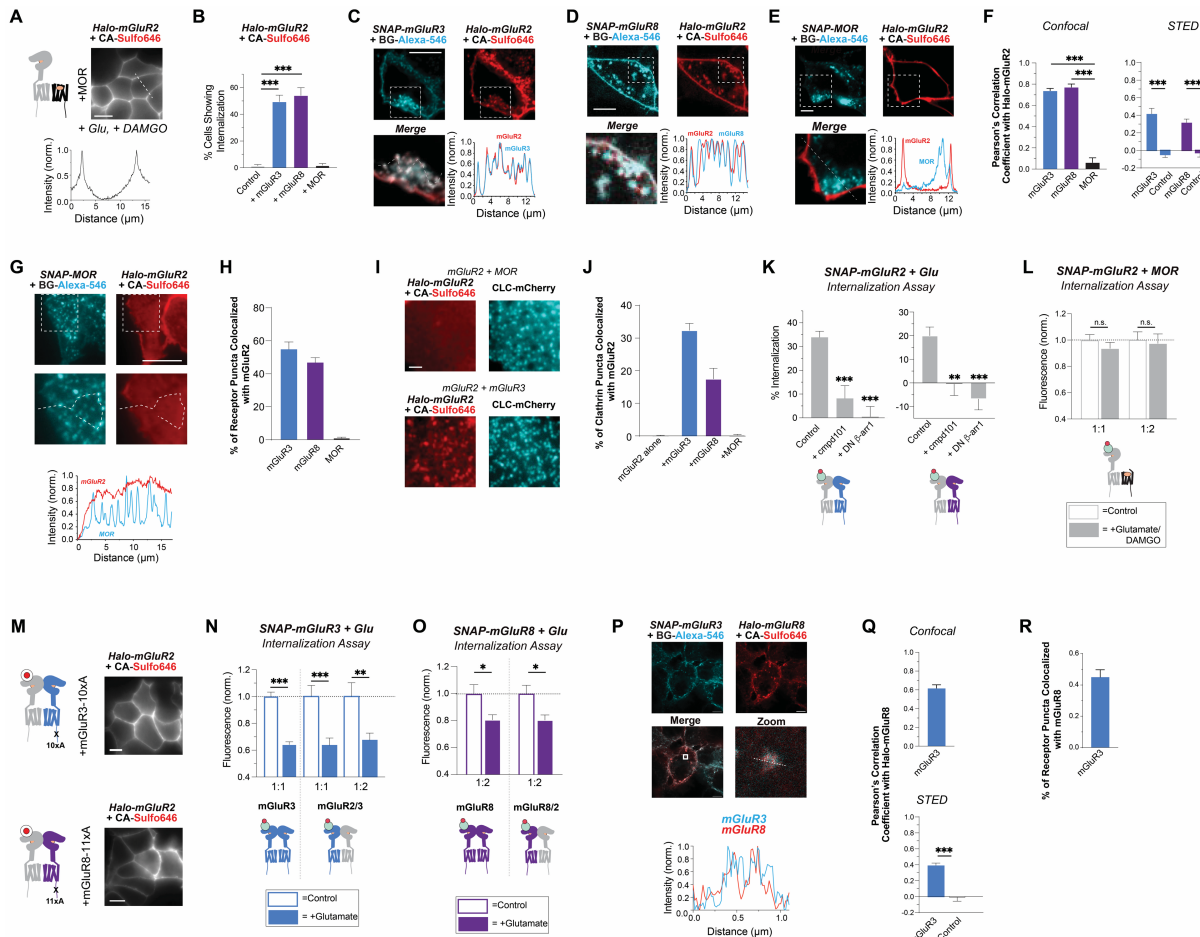


Fig. S5. Further analysis of the effects of mGluR co-expression on internalization. (A) Fluorescence live cell images of Halo-mGluR2 labeled with CA-Sulfo646 co-expressed with either untagged MOR 30 min after 1 mM Glu and 10 μM DAMGO co-application. Line scan profiles (dotted lines) are shown below. (B) Quantification of numbers of cells in Fig. 5A and fig. S5A showing internalization as determined by blind analysis. At least 7 individual fluorescence images showing 10-30 cells were quantified. (C to E) Scanning confocal images of live cells expressing SNAP-mGluR3 and Halo-mGluR2 (C), SNAP-mGluR8 and Halo-mGluR2 (D), or SNAP-MOR and Halo-mGluR2 (E) labeled with BG-Alexa546 for SNAP and CA-Sulfo646 for Halo. Line scans from the dotted line from the zoomed in, merged image. (F) Pearson's correlation coefficient analysis from confocal (left) and STED (right) images comparing the top 10% of pixels between Halo-mGluR2 and other receptors. For the control groups in the STED graph, one of the channels was rotated by 90 degrees. (G) TIRF images of cells expressing Halo-mGluR2 with SNAP-MOR following 10-15 min of Glu and DAMGO treatment. Zoomed images are from the dotted box area. Line scan profiles are from the dotted line. (H) Quantification of the percentage of receptor puncta colocalized with mGluR2 from TIRF images. (I) TIRF images of cells expressing Halo-mGluR2 alone or with mGluR3 together with CLC-mCherry as a clathrin marker. (J) Quantification of the percentage of clathrin puncta colocalized with Halo-mGluR2 when expressed alone or with mGluR3, mGluR8 or MOR. (K) Quantification of SNAP-mGluR2 internalization when co-expressed with either mGluR3 or mGluR8 and treated with cmpd101 or additional co-expression with DN-β-Arr1 using surface labeling assay upon co-expression. (L) Quantification of SNAP-mGluR2 internalization when co-expressed with μOR and treated with Glu and DAMGO at 1:1 or 1:3 DNA ratio using surface labeling assay upon co-expression (M) Fluorescence images of cells expressing Halo-mGluR2 labeled with CA-Sulfo646 when co-expressed with either SNAP-mGluR3-10x (top) or SNAP-mGluR8-11x (bottom) followed by 1 mM Glu incubation. (N and O) Quantification of SNAP-mGluR3 (J) or SNAP-mGluR8 (K) internalization when co-expressed with Halo-mGluR2, -mGluR3, or -mGluR8 at 1:1 or 1:2 DNA ratio using surface labeling assay upon co-expression. (P) STED images of cells expressing Halo-mGluR8 with SNAP-mGluR3 following 30 min of Glu treatment. Zoomed images are from the dotted box area. Line scan profiles show the co-localization of receptor puncta. (Q) Pearson's correlation (PCC) analysis from scanning confocal (top) or STED (bottom) images of cells expressing Halo-mGluR8 with mGluR3 or MOR. For the control group in the STED graph, one of the channels was rotated by 90 degrees. (R) Quantification of TIRF images from cells expressing Halo-mGluR8 with mGluR3 showing the percentage of mGluR3 puncta colocalized with mGluR8.

Unpaired t-tests (N, O, Q) or One-way ANOVA (F, K), * p < 0.05, ** p < 0.01.

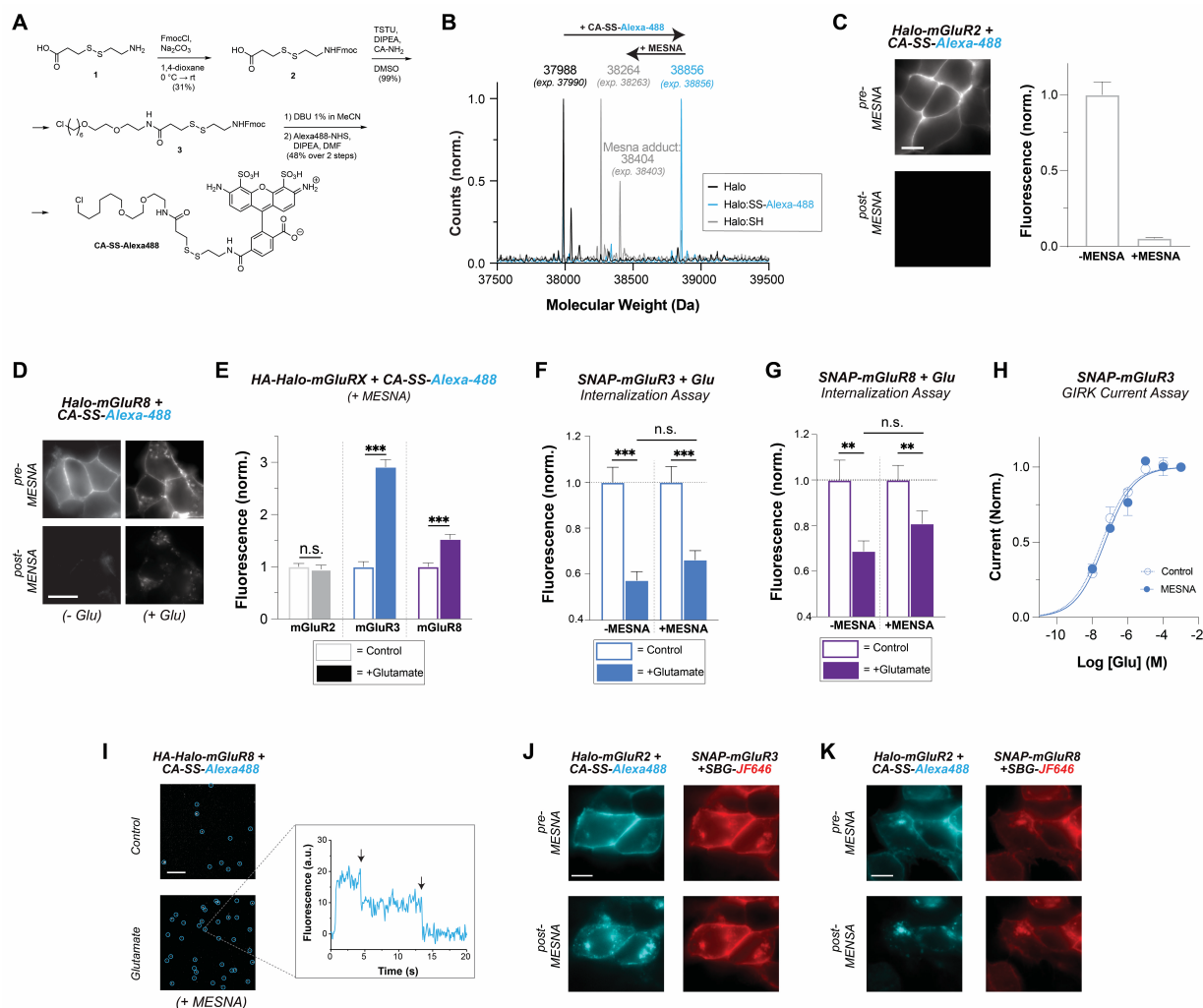


Fig. S6. Further analysis of cleavable Halo-dye and single molecule detection of internalized mGluRs. (A) Synthetic scheme for CA-SS-Alexa-488. **(B)** Mass spectra of Halo tag itself (black), labeled with CA-SS-Alexa-488 (green), and after MESNA treatment (grey). **(C)** Fluorescence images of live cells expressing Halo-mGluR2 labeled with CA-SS-Alexa-488 before and after 100 mM MESNA treatment. Quantification is shown on the right and normalized to pre-MESNA condition. **(D)** Fluorescence images of live cells expressing Halo-mGluR8 labeled with CA-SS-Alexa-488 incubated or not with 1 mM Glu for 30 min and before and after MESNA treatment. **(E)** Quantification of fluorescence intensity from cells expressing Halo-mGluR2, -mGluR3, or -mGluR8 labeled with CA-SS-Alexa-488 treated with 1 mM Glu or antagonist for 30 min followed by MESNA treatment. **(F, G)** Quantification of surface fluorescence levels from cells expressing SNAP-mGluR3 (F) or SNAP-mGluR8 (G) after treatment of 1 mM Glu or antagonist with and without MESNA application. **(H)** Glutamate dose dependent SNAP-mGluR3 GIRK current with and without MESNA application. **(I)** Representative SiMPull images of HA-Halo-mGluR8 labeled with CA-SS-Alexa-488 followed by 1 mM Glu incubation (vs. control) and MESNA treatment. A representative fluorescence intensity trace is plotted in a colocalized spot with arrows marking the photobleaching steps. **(J, K)** Fluorescence images of live cells showing Halo-mGluR2 labeled with CA-SS-Alexa-488 co-expressed with SNAP-mGluR3 (F) or SNAP-mGluR8 (G) labeled with SBG-JF646 and incubated with 1 mM Glu. Images are shown before and after MESNA treatment. Data are represented as mean \pm SEM.

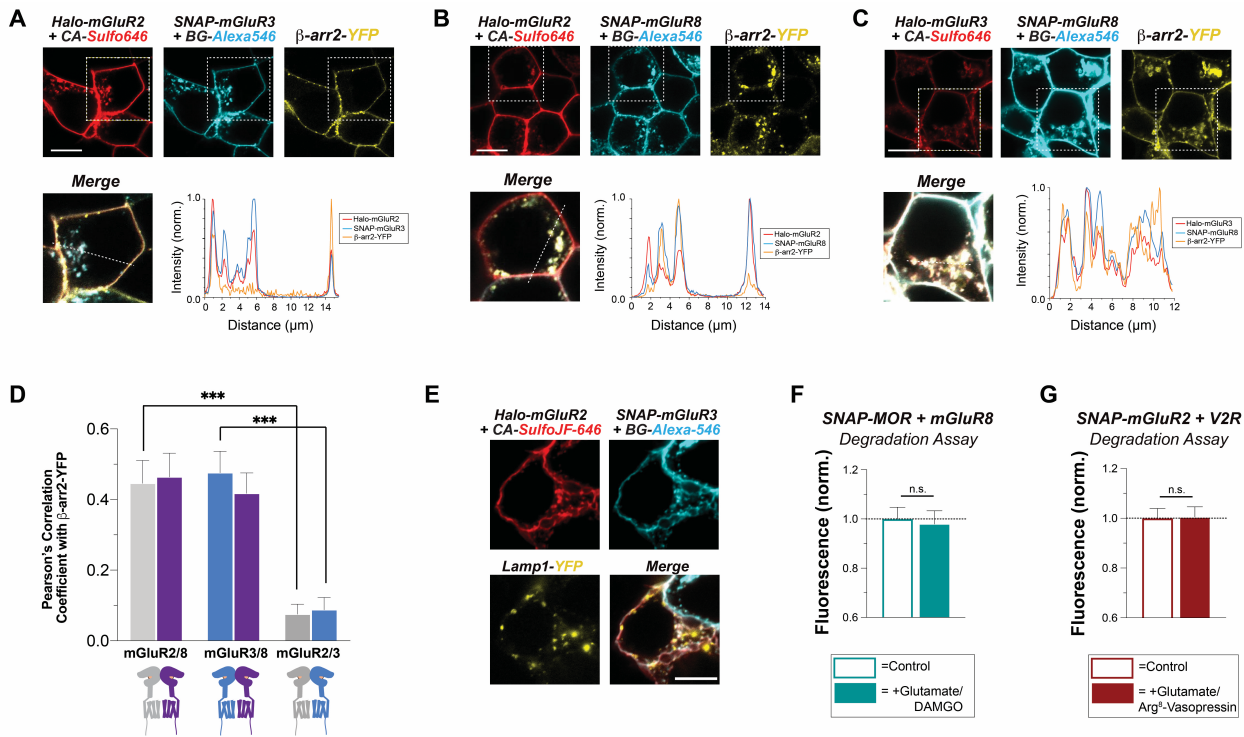
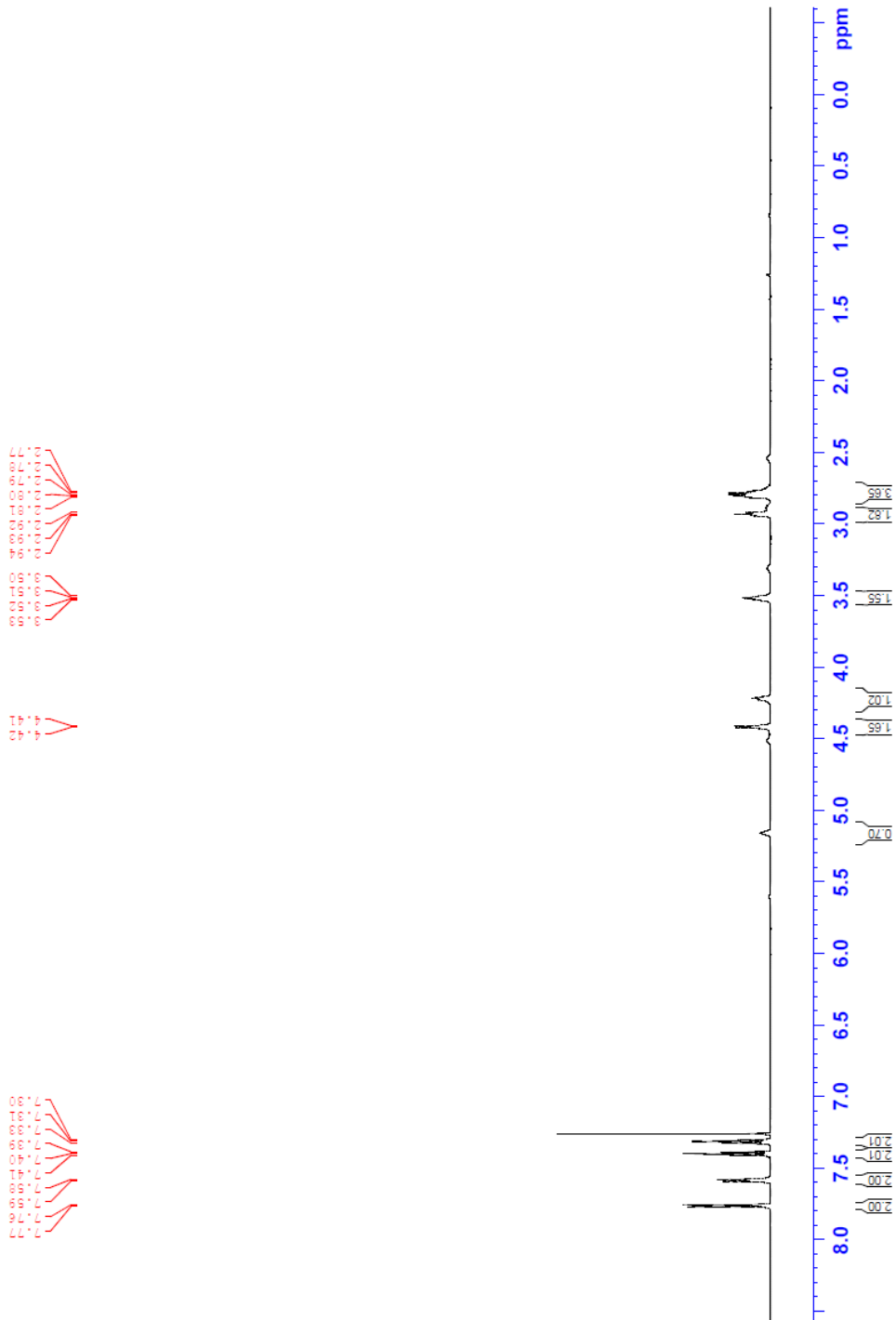


Fig. S7. Further analysis of the effects of mGluR heterodimerization on β -arrestin co-internalization and degradation. (A to C) Scanning confocal images of live cells expressing Halo-mGluR2 and SNAP-mGluR3 (A), Halo-mGluR2 and SNAP-mGluR8 (B), and Halo-mGluR3 and SNAP-mGluR8 (C) co-expressed with β -arr2-YFP. Zoomed in, merged image is from the dotted box area. Line scans are shown from the dotted line. (D) Pearson's correlation coefficient analysis from confocal images comparing the top 10% of pixels between Halo-mGluR2 (or Halo-mGluR3) and β -arr2-YFP for heterodimers of mGluR2/3, mGluR3/8, and mGluR2/3. (E) Scanning confocal images of fixed cells expressing Halo-mGluR2 labeled with CA-Sulfo646, SNAP-mGluR3 labeled with BG-Alexa546 (cyan) and Lamp1-YFP (green). Merged image is shown. (F) Quantification of total fluorescence from live cells expressing SNAP-MOR and untagged mGluR8 treated with 60 min agonist (10 μ M DAMGO and 1 mM Glu) or control (antagonist) before labeling with membrane permeable fluorophore, BG-JF549. Values are normalized to the fluorescence of a given receptor under control/antagonist condition. (G) Quantification of total fluorescence from live cells expressing SNAP-mGluR2 and untagged V2R treated with 60 min agonist (1 mM Glu and 100 nM Vasopressin) or control (antagonist) before labeling with membrane permeable fluorophore, BG-JF549. Values are normalized to the fluorescence of a given receptor under control/antagonist condition. Scale bars are 10 μ m. Data are represented as mean \pm SEM. Unpaired t-tests, n.s. for non-significance. Unpaired t-tests (F, G) or One-way ANOVA (D), ** p < 0.01, *** p < 0.001.

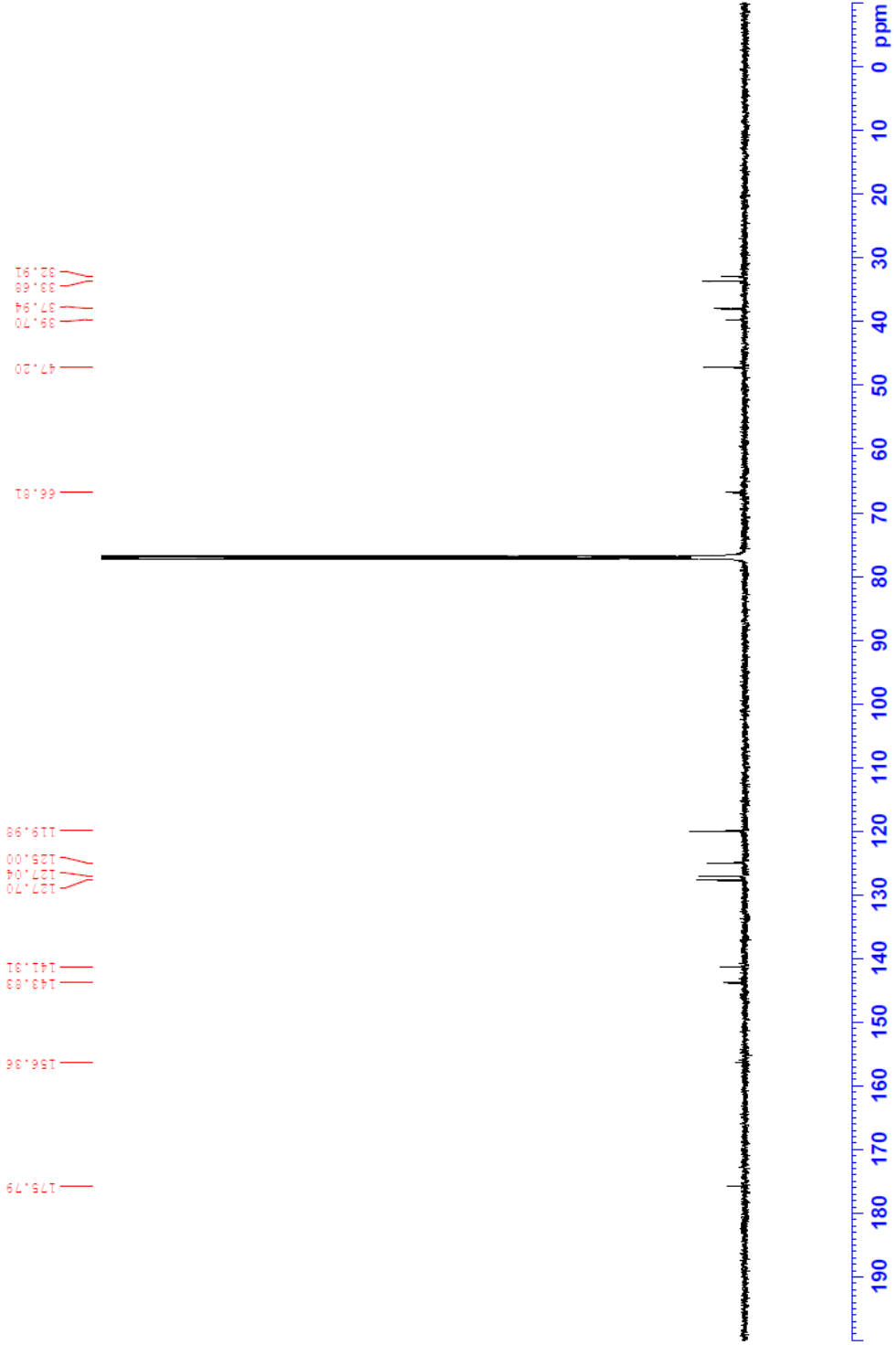
Supplementary Chemical Characterization

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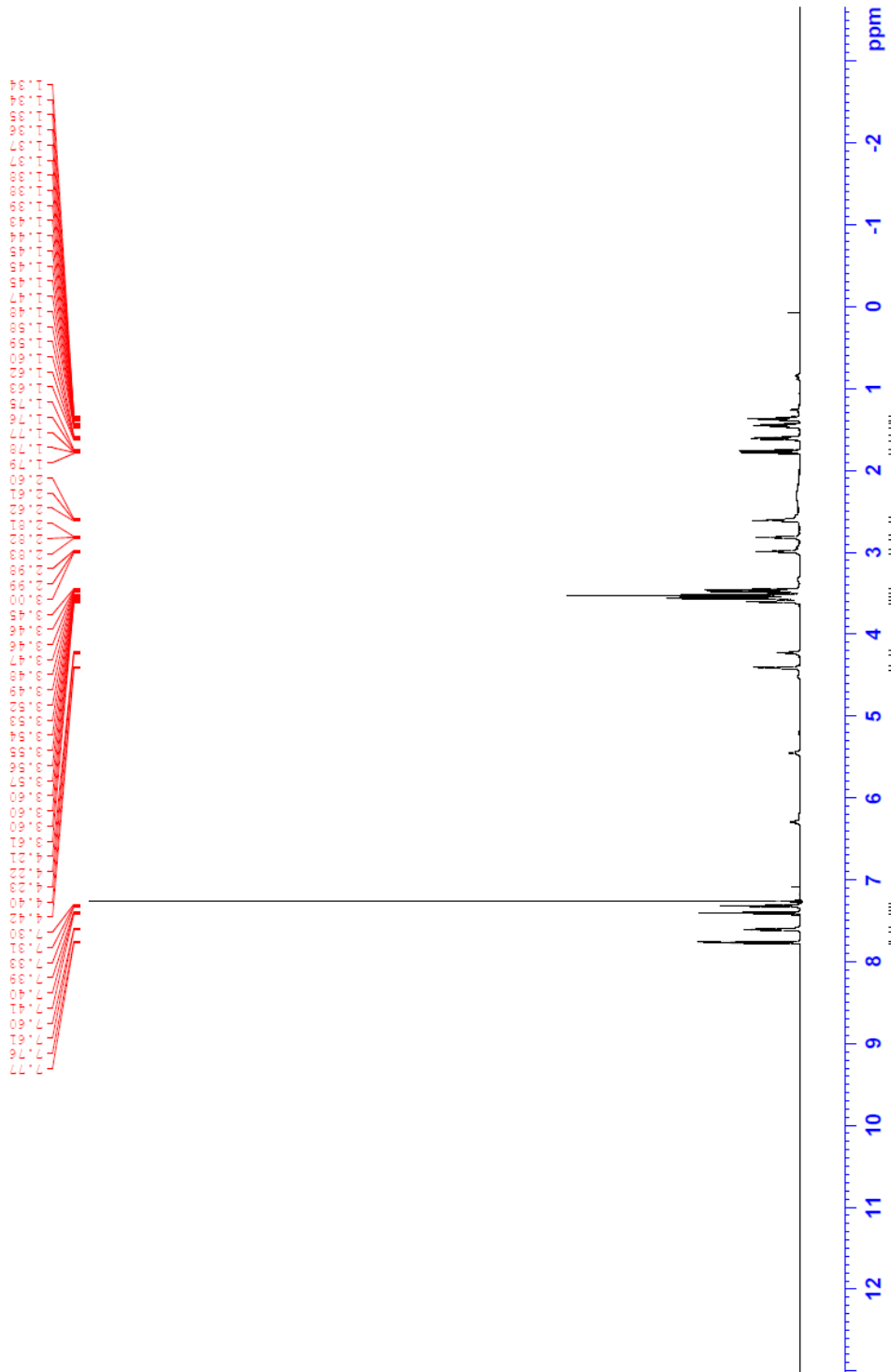
3-((2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)ethyl)disulfaneyl)propanoic acid (2):
¹H NMR:



1 ¹³C NMR:
2

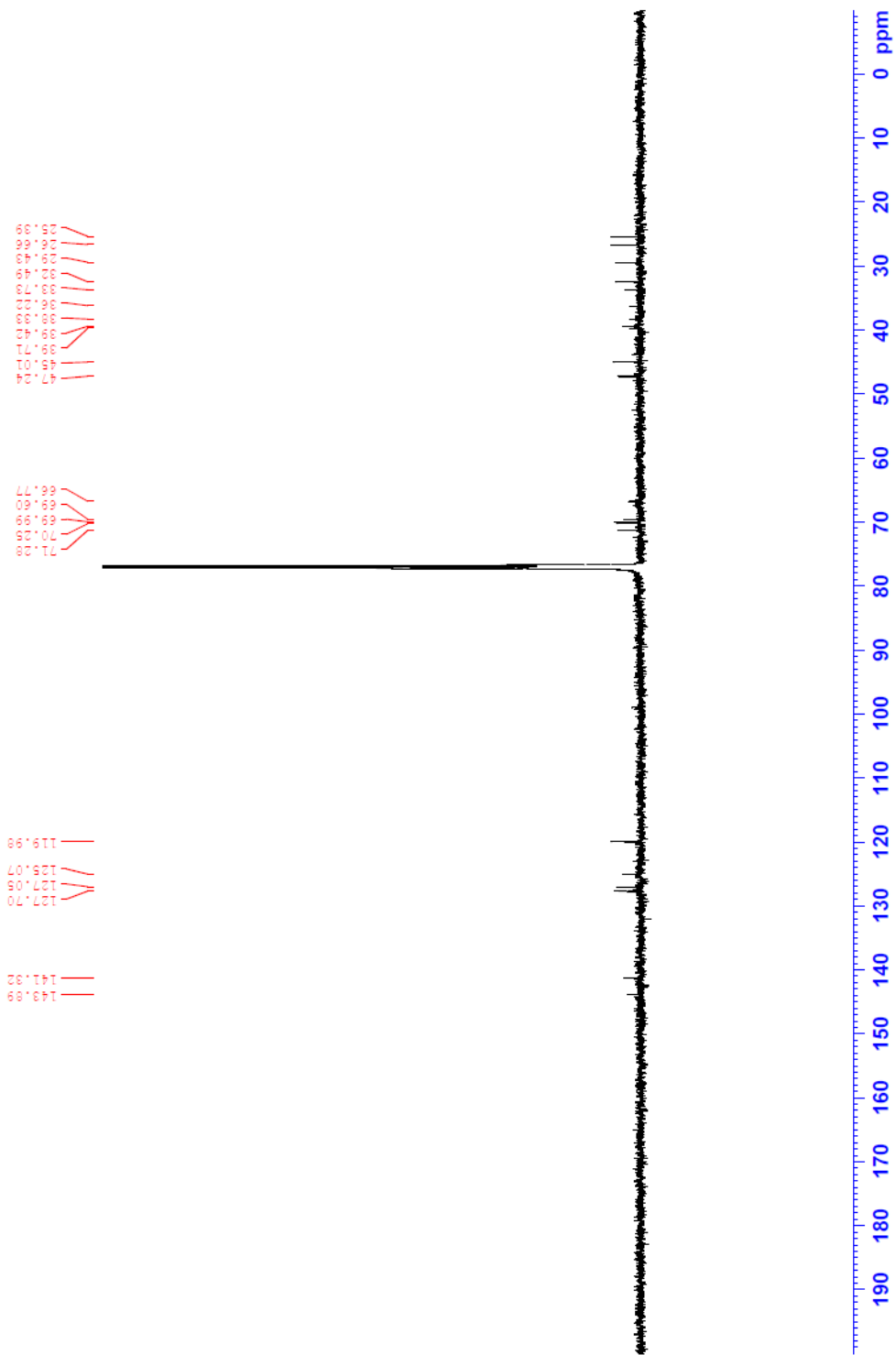


7 (9H-Fluoren-9-yl)methyl (20-chloro-7-oxo-11,14-dioxa-3,4-dithia-8-azaicosyl)carbamate (3):
8 ¹H NMR:



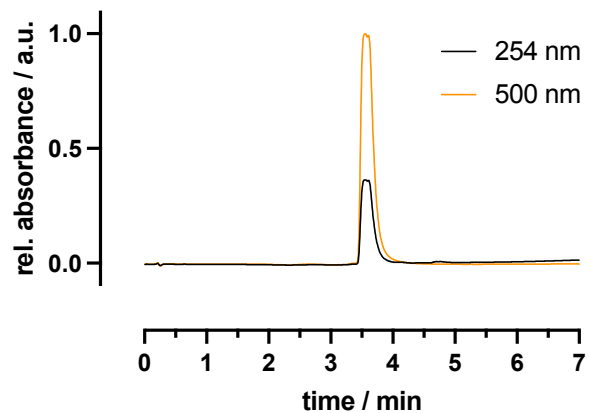
1
2
3

¹³C NMR:



8 2-(6-Amino-3-iminio-4,5-disulfo-3*H*-xanthen-9-yl)-4-((20-chloro-7-oxo-11,14-dioxa-3,4-dithia-8-
9 azaicosyl)carbamoyl)benzoate (CA-SS-Alexa-488):

0
1 LCMS:



Protocol for full protein mass spectrometry

An Eppendorf tube was charged with recombinantly expressed and purified Halo-tag dissolved in 100 μ L of a 50 mM HEPES / 50 mM NaCl solution (pH = 7.4) to a concentration of 1.0 μ M (1.0 equiv.) and subjected to QTOF mass spectrometry. Then 4.0 equiv. **CA-SS-Alexa-488** were added, and the mixture incubated for 15 minutes at rt, before again being subjected to QTOF. Then, an excess of MESNA (final concentration 100 mM) was added and subjected to QTOF.