Comparison of Strategies for Non-perturbing Labeling of α-Synuclein to Study Amyloidogenesis

Conor M. Haney,[a] Rebecca F. Wissner,[a] John B. Warner,[a] Yanxin J. Wang,[a] John J. Ferrie,[a] Dustin J. Covell,[b] Richard J. Karpowicz, Jr.,[b] Virginia M.-Y. Lee,[b] and E. James Petersson[a]

[a]*Department of Chemistry; University of Pennsylvania; 231 South 34th Street, Philadelphia, Pennsylvania 19104-6323 USA.*

[b]*Center for Neurodegenerative Disease Research, University of Pennsylvania, 3600 Spruce Street, Philadelphia, PA 19104*

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General Information. *p*-Azidophenylalanine (Azf,Z) was purchased from Bachem (Torrance, CA, USA). *O*-Propargyl tyrosine (Ppy, π) was synthesized as previously described.[1] 5(6)'-carboxytetramethylrhodamine was purchased from Novabiochem, EMD Millipore (Darmstadt, Germany). Fluorescein-5-maleimide (Fam) was purchased from TCI America (Portland, Oregon). 5(6)'-carboxytetramethylrhodamine-azide, 5-isomer (Raz) was purchased from Lumiprobe (Hallandale Beach, FL, USA). Dibenzocyclooctyne-amine and carboxyrhodamine110-azide (Faz) were purchased from ClickChemistryTools (Scottsdale, AZ, USA). Tetramethylrhodamine dibenzocyclooctyne (Rco) was synthesized as described.[2] Fluorescein dibenzocyclooctyne (Fco) was synthesized using a similar procedure, described below. Ni-NTA resin was purchased from Qiagen (Valencia, CA) or from Fisher Scientific (Pittsburgh, PA, USA). *E. coli* BL21(DE3) cells were purchased from Stratagene (La Jolla, CA, USA). Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA). Bovine Factor Xa protease was purchased from Haematologic Technologies (Essex Junction, VT, USA). QuikChange® site-directed mutagenesis kits were purchased from Stratagene. DNA oligomers were purchased from Integrated DNA Technologies, Inc (Coralville, IA, USA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA). MilliQ filtered (18 MΩ) water was used for all solutions (Millipore, Billerica, MA, USA). The pEG6 plasmid, containing His_{10}-tagged *E. coli* AaT, was a gift from Alexander Varshavsky (California Institute of Technology). The pDULE2-pXF plasmid (previously referred to as pDULE2-pCnf) was a gift from Ryan Mehl (Oregon State University).[3] Preparation of the pTXB1-αS_{TAG136}-intein-H_6 plasmid containing αS with a C-terminal fusion to the *Mycobacterian xenopi* GyrA intein and C-terminal His_6 tag was described previously.[4] The construction of all other plasmids used in this work is described below. Low resolution electrospray ionization mass spectra (LRMS) were

collected with a Waters LCT Premier XE liquid chromatograph/mass spectrometer (Milford, MA, USA). Matrix-assisted laser desorption/ionization (MALDI) mass spectra were collected with a Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer (Billerica, MA, USA). UV/vis absorbance spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Gel images were obtained with a Typhoon FLA 7000 (GE Lifesciences, Princeton, NJ, USA). Fluorescence spectra were collected with a Tecan M1000 plate reader or on a PTI QuantaMaster fluorometer. Fluorescence polarization data were collected with a Tecan F200 plate reader (Mannedorf, Switzerland). Transmission electron microscopy (TEM) images were collected on a FEI TecnaiT12 electron microscope (Hillsboro, OR, USA). Mice for this study were procured from Charles River Laboratories (Raleigh, NC, USA). All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Fibrils were sonicated using a Diagenode Biorupter[TM] (Diagenode Inc. USA North America, Denville, NJ, USA). Rabbit polyclonal antibody anti-LAMP1 (ab24170) was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal antibodies Syn204[5] and 81A[6] as well as the rabbit polyclonal antibody SynA were generated and characterized in the laboratory of Virginia M. Y.-Lee at the University of Pennsylvania. Secondary antibodies were acquired from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA). Coverslips were affixed to slides using Fluoromount G[TM] (eBioscience, Inc., San Diego, CA, USA). Neuronal immunocytochemical images were acquired on a Leica DMI6000 microscope using a 100X oil immersion objective (Leica Microsystems Inc., IL, USA), an Olympus IX 81 microscope equipped with a Yokogawa CSU X1 spinning disc confocal scan head using a 100x oil immersion objective (Olympus America, Inc., Center

Valley, PA, USA), or a Perkin Elmer Lamina™ slide scanner (PerkinElmer, Inc., Santa Clara, CA, USA) using a 20X objective. Scanned slides were analyzed using Indica Labs HALO™ software (Indica Labs, Inc., Corrales, NM, USA) and graphs were generated using GraphPad PRISM 4™ (GraphPad Software, Inc., La Jolla, CA, USA).

Fluorescein Dibenzocyclooctyne (Fco). 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (72 μmol), H-hydroxybenzotriazole (72 μmol), and Et_3N (144 μmol) were added to a solution of 5(6)'-carboxyfluorescein (36.2 μmol) in 2 mL dimethylformamide. The reaction was stirred on ice for 10 minutes and then dibenzocyclooctyne-amine (36.2 μmol) was added. The reaction was allowed to warm to room temperature overnight with stirring. Solvent was removed under vacuum with a liquid nitrogen trap. The reaction was purified using 100 mL silica gel in 1:9 methanol/dichloromethane with 1 % acetic acid. Product fractions were concentrated by rotary evaporation and dried under vacuum overnight, affording Fco as an orange solid in 66 % yield. LRMS calcd for $C_{39}H_{27}N_2O_7$ $(M+H)^+$ 635.18, found 635.45.
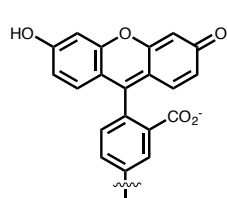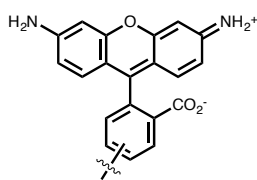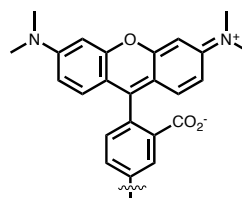
	Fluorescein	Carboxyrhodamine110	Tetramethylrhodamine
Used in:	Fam, Fak, Fco	Faz	Raz, Rco

Construction of pRK172 αS Cys Mutant Expression Plasmids. A plasmid containing the human wild-type αS gene cloned between NdeI and HindIII in the expression vector pRK172 was provided by Dr. Virginia Lee (Perelman School of Medicine, University of Pennsylvania). QuikChange® mutagenesis was used to mutate Ser_9 to Cys_9 and Glu_{114} to Cys_{114} to generate

pRK172_αS-C$_9$ and pRK172_αS-C$_9$, respectively. The sequence of each mutant plasmid was confirmed by DNA sequencing analysis. DNA primers for generating each mutant are shown in Figure S1.

Construction of pT-T7 αS TAG Expression Plasmids. A plasmid containing the human wild-type αS gene cloned between NdeI and HindIII in pT-T7 vector was provided by Dr. Elizabeth Rhoades (Department of Chemistry, University of Pennsylvania). Higher yields were observed for Uaa-containing proteins when expressed from the pT-T7_αS vector in comparison to Uaa expression from pRK172_αS vectors. Thus, all TAG-containing mutant constructs were generated in pT-T7_αS. QuikChange® mutagenesis was used to mutate Tyr$_{39}$ and Phe$_{94}$ to TAG in the pT-T7 construct to yield pT-T7_αS-TAG$_{39}$ and pT-T7_αS-TAG$_{94}$, respectively. A second round of QuikChange® mutagenesis reactions was performed to mutate pT-T7-TAG$_{94}$ to pT-T7_αS-C$_9$TAG$_{94}$ and pT-T7_αS-TAG$_{39}$ to pT-T7_αS-TAG$_{39}$C$_{114}$. The sequence of each mutant plasmid was confirmed by DNA sequencing analysis. DNA primers for each mutagenesis step are shown in Figure S1.

Construction of pET-16b_αS$_{2-140}$K$_2$ Plasmids. The construction of the pET-16b_H$_{10}$-αS$_{2-140}$K$_2$ plasmid has been described previously.[2] QuikChange® mutagenesis using the primers in Figure S1 was then used to mutate Tyr$_{39}$ and Phe$_{94}$ to TAG in the pET-16b_H$_{10}$-αS$_{2-140}$K$_2$ construct to yield pET-16b_H$_{10}$-αS$_{2-140}$K$_2$TAG$_{39}$ and pET-16b_H$_{10}$-αS$_{2-140}$K$_2$TAG$_{94}$, respectively. The sequence of each mutant plasmid was confirmed by DNA sequencing analysis.

Construction of pTXB1_αS_intein-H$_6$ Plasmid. A previously described pTXB1-aS$_{TAG136}$_intein-H$_6$ plasmid containing the human wild-type αS gene with a C-terminal *Mycobacterium xenopi* GyrA intein and His$_6$ fusion was reverted to the wild-type αS sequence

(TAG$_{136}$ to Tyr) using QuikChange$^{®}$ mutagenesis. The resulting sequence was confirmed by DNA sequencing analysis. DNA primers for generating this mutant are shown in Figure S1.

Construction of pTXB1_αS_intein-H$_6$ Cys Mutant Plasmid. QuikChange$^{®}$ mutagenesis was used to mutate Ser$_9$ to Cys$_9$ to generate the pTXB1-αS-C$_9$_intein-H$_6$ plasmid. The resulting sequence was confirmed by DNA sequencing analysis. DNA primers for generating this mutant are shown in Figure S1 and are the same as described above.

Construction of pTXB1_αS_intein-H$_6$ TAG Mutant Plasmid. QuikChange$^{®}$ mutagenesis was used to mutate Tyr$_{39}$ to TAG in the pTXB1_αS-TAG39_intein-H$_6$ in the pTXB1 construct described above containing the wild-type αS sequence. The pTXB1_αS-C$_9$-TAG$_{39}$_intein-H$_6$ plasmid was generated identically starting from the pTXB1-αS-C$_9$_intein-H$_6$ plasmid. The sequence of each mutant was confirmed by DNA sequencing analysis. DNA primers used for mutagenesis are shown in Figure S1 and are the same as described above.

DNA Oligomers Used for αS QuikChange® Mutagenesis

i.   Mutation S_9C

Forward: 5' – GTATTCATGAAAGGACTTTGCAAGGCCAAGGAGGGAGTTG – 3'

Reverse: 5' – CAACTCCCTCCTTGGCCTTGCAAAGTCCTTTCATGAATAC – 3'

ii.   Mutation $E_{114}C$

Forward: 5' – CCCCACAGGAAGGAATTCTGTGCGATATGCCTGTGGATCCTGA – 3'

Reverse: 5' – TCAGGATCCACAGGCATATCGCACAGAATTCCTTCCTGTGGGG – 3'

iii.   Mutation $Y_{39}TAG$

Forward: 5' – AAAAGAGGGTGTTCTCTAGGTAGGCTCCAAAACCAA – 3'

Reverse: 5' – TTGGTTTTGGAGCCTACCTAGAGAACACCCTCTTTT – 3'

iv.   Mutation $F_{94}TAG$

Forward: 5' – GCATTGCAGCAGCCACTGGCTAGGTCAAAAAGGACCAGTTGGG – 3'

Reverse: 5' – CCCAACTGGTCCTTTTTGACCTAGCCAGTGGCTGCTGCAATGC – 3'

v.   Mutation $TAG_{136}Y$

Forward: 5' – ATGCAGGCTTCAGGTTCATAGTCTTGATACCCTTC – 3'

Reverse: 5' – GAAGGGTATCAAGACTATGAACCTGAAGCCTGCAT – 3'

Fig. S1. DNA Oligomers Used for Quikchange® Mutagenesis.

Overexpression and Purification of αS and αS Cys Mutants. pRK172_αS, pRK172_αS-C_9, and pRK172_αS-C_{114} were transformed into competent *E. coli* BL21(DE3) cells. Single colonies were used to inoculate 5 mL of LB media supplemented with ampicillin (Amp, 100 µg/mL). The primary culture was incubated at 37 °C with shaking at 250 rpm for 4 h. The primary culture was used to inoculate 1 L of LB media containing Amp (100 µg/mL) which was then grown overnight at 37 °C with shaking at 250 rpm. The cells were harvested by centrifugation at 5000 x g for 15 min, and the resulting pellet was resuspended in lysis buffer (40 mM tris(hydroxymethyl)aminomethane (Tris), 5 mM ethylenediaminetetraacetic acid (EDTA),

pH 8.0) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 units/mL DNase I – Grade II. Following sonication, the cell lysate was boiled for 20 minutes prior to centrifugation for 20 minutes at 30,000 x g, 4 °C. The cleared supernatant was dialyzed against FPLC purification buffer (20 mM Tris, 100 mM NaCl pH 8.0) overnight at 4 °C. The resulting solution was purified by gel filtration over a Superdex 75 16/600 column followed by ion-exchange chromatography using a HiTrap Q HP column (5 mL) on an ÄKTA FPLC over a 100 min NaCl gradient (0 to 500 mM NaCl in 20 mM Tris, pH 8.0). The fractions containing the product were identified by MALDI MS, pooled, and dialyzed into αS buffer (20 mM Tris, 100 mM NaCl, pH 7.5) overnight. Proteins containing Uaas and with the C-terminal intein fusion present were quantified by the BCA assay (PierceTM BCA Protein Assay Kit, ThermoFischer Scientific) using bovine serum albumin (BSA) standards. Wild type αS (WT αS) and αS Cys mutants were quantified by UV-Vis absorbance at 280 nm ($\varepsilon_{280} = 5120$ M^{-1} cm^{-1}).[7]

Synthesis of αS-C$^{Fam}_9$ (1b) and αS-C$^{Fam}_{114}$ (2b). Prior to performing the labeling reactions, the concentration of each protein (αS-C$_9$ (1a) and αS-C$_{114}$ (2a)) was adjusted to approximately 1.0 mg/mL in αS buffer containing tris(2-carboxyethyl)phosphine TCEP (1 mM). Fluorescein-5-maleimide (Fam, 5 equiv) was then added to the protein from a fresh 10 mM stock solution prepared in DMSO. The labeling reaction was carried out at 37 °C for 4 h without shaking. After the first 4 h, an additional 5 equiv were added and the reaction was allowed to incubate for an additional 2-4 hours at 37 °C until quantitative conversion was observed by MALDI MS. The resulting Fam-labeled protein was dialyzed overnight against purification buffer and purified over a HiTrap Q HP column on an ÄKTA FPLC over a 100 min NaCl gradient (0 to 500 mM NaCl in 20 mM Tris, pH 8.0). The fractions containing the labeled product were identified by MALDI MS and dialyzed against water overnight at 4 °C. The dialyzed, labeled proteins were

further purified by reverse-phase HPLC using a Vydac 218TP C4 semi-prep column over the following gradient: isocratic at 95% aqueous phase for 5 min, and then ranging from 95% to 75% aqueous phase over 5 min, then to 40% aqueous phase over 20 min, then to 0% aqueous phase over 5 min. The purified proteins were then concentrated using an Amicon (Millipore) Ultra 0.5 mL 3 kDa spin column, exchanged into αS buffer, and stored at – 80 °C.

Mass Spectrometry Analysis of Fam Labeling Reactions. Fam labeling was periodically monitored using MALDI MS. Complete labeling was consistently observed within 8 h.
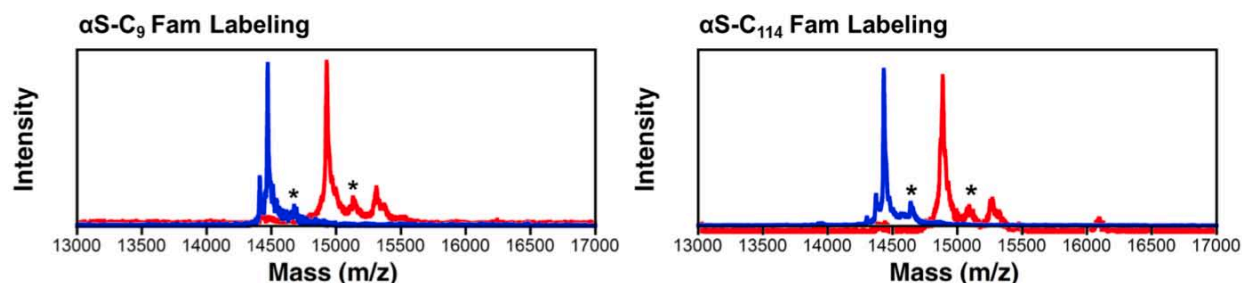

Fig. S2. MALDI MS of Crude Fam Labeling Reactions. Blue trace: Cys mutant prior to the labeling reaction. Red trace: Crude reaction mixture post labeling with a total of 10 equiv of Fam maleimide. The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

MALDI Analysis of HPLC Purified αS-C$^{Fam}_9$ (1b), αS-C$^{Fam}_{114}$ (1b). MALDI MS of the purified proteins consistently yielded masses that were 15-20 Da higher than the calculated mass. MALDI MS analyses of trypsin digested fragments suggest that this increase in mass corresponds to hydrolytic ring-opening of the maleimide group (see below).
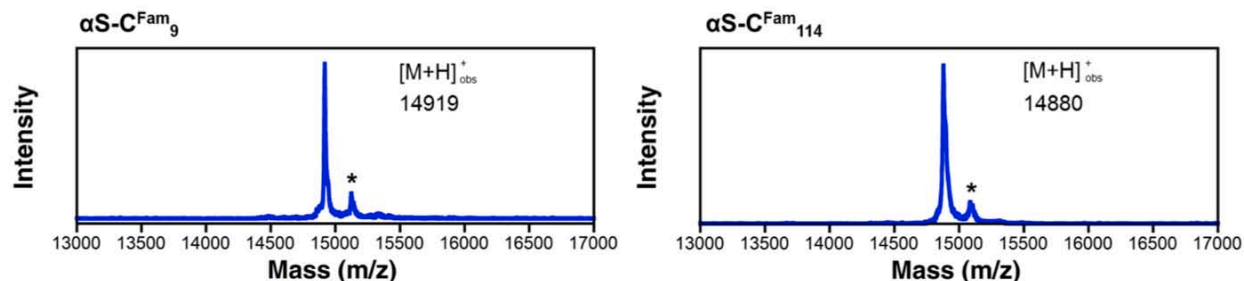

Fig. S3. MALDI MS of HPLC Purified αS-C$^{Fam}_9$ (1b) and αS-C$^{Fam}_{114}$ (1b). Expected [M+H]$^+$ of αS-C$^{Fam}_9$ and αS-C$^{Fam}_{114}$ are 14906 and 14864, respectively. Expected [M+H]$^+$ of ring-opened αS-C$^{Fam}_9$ and αS-C$^{Fam}_{114}$ are 14921 and 14880, respectively. The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

Trypsin Digestion of αS-C$^{Fam}_9$ (1b) and αS-C$^{Fam}_{114}$ (2b). αS mutants (10−50 μg) were incubated with 5 μL aliquots of sequencing-grade modified trypsin (0.1 mg/mL) in αS buffer at 37 °C for 4 h. After the incubation period, an aliquot (1.0 μL) of the digestion reaction was removed and analyzed by MALDI MS. MALDI MS analysis of the trypsin digestion fragments show that Fam labeling is both site-specific and quantitative.
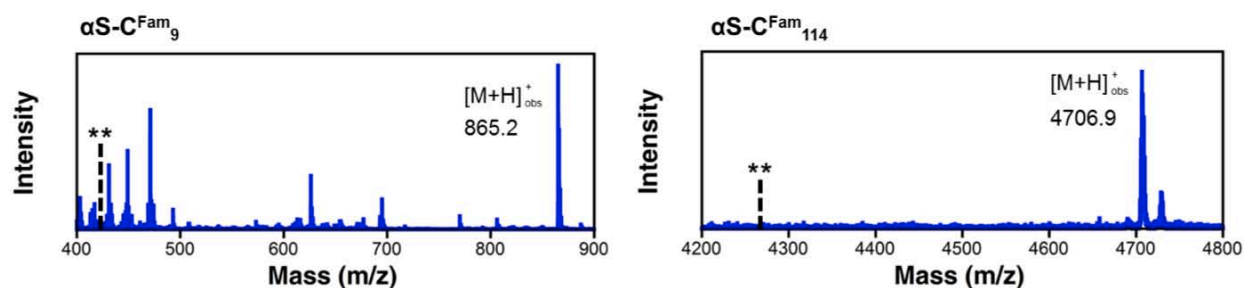

Fig. S4. MALDI MS of Trypsin Fragments of αS-C$^{Fam}_9$ (1b) and αS-C$^{Fam}_{114}$ (2b). Expected [M+H]$^+$ of αS-C$^{Fam}_9$ Frag. 7-10 and αS-C$^{Fam}_{114}$ Frag. 103-140 are 847.3, and 4687.6, respectively. Expected [M+H]$^+$ of maleimide ring-opened αS-C$^{Fam}_9$ Frag. 7-10 and αS-C$^{Fam}_{114}$ Frag. 103-140 865.3 and 4705.6, respectively. The double asterisk (**) corresponds to the expected mass of the unmodified fragment.

Overexpression and Purification of αS-Z$_{94}$ (3a), αS-π$_{39}$ (4a), αS-π$_{94}$ (5a), αS-C$_9$π$_{94}$ (7a) and αS-π$_{39}$C$_{114}$ (8a). pT-T7_αS-TAG$_{39}$ and pT-T7_αS-TAG$_{94}$ were transformed into competent *E. coli* BL21(DE3) cells harboring the orthogonal tRNA$_{CUA}$ and pXF-tRNA synthetase pair encoded by the pDULE2-pXF plasmid. Transformed cells were selected on the basis of Amp and streptomycin (Strep) resistance. Single colonies were used to inoculate 5 mL of LB media supplemented with Amp (100 μg/mL) and Strep (100 μg/mL). The primary 5 mL culture was incubated at 37 ºC with shaking at 250 rpm for 5-6 hours. The primary culture was then used to inoculate 1 L of a variant of M9 minimal media described previously. When the OD$_{600}$ of the secondary culture reached 0.8, the corresponding Uaa was added (220 mg for Ppy or 206 mg for AzF, final concentration of 1 mM). After 10 minutes, 1 mL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration of 1 mM) was added and the culture was

incubated overnight at 37 ºC with shaking at 250 rpm. The cells were harvested at 5000 x g for 15 min and the resulting pellet was resuspended in lysis buffer. Following sonication, the cell lysate was boiled for 20 minutes prior to centrifugation for 20 minutes at 30,000 x g, 4 ºC. The cleared supernatant was dialyzed against FPLC purification buffer overnight at 4 °C and purified by size-exclusion and ion-exchange chromatography as described above. Following FPLC purification of αS-Z_{94} (**3a**), αS-π_{39} (**4a**), and αS-π_{94} (**5a**), the protein-containing fractions were identified by MALDI-MS and pooled prior to click labeling. Following FPLC purification of αS-$C_9\pi_{94}$ (**7a**) and αS-$\pi_{39}C_{114}$ (**8a**), the proteins were dialyzed into αS buffer overnight prior to Fam labeling.

Synthesis of αS-π^{Raz}_{39} (4b) and αS-π^{Raz}_{94} (5b). Prior to performing the labeling reactions, the concentration of FPLC-purified αS-π_{39} and αS-π_{94} was adjusted to 1.0 mg/mL. The catalyst mixture was prepared by combining (for each mL of reaction) 1.25 µL of 80 mM Cu_2SO_4, 30 µL of 50 mM Tris-(hydroxypropyltriazolylmethyl)amine (THPTA) and 30 µL of freshly prepared 100 mM sodium ascorbate. This mixture was allowed to incubate at room temperature for 5 min. After adding 5 equiv of carboxytetramethylrhodamine azide (Raz azide) to the protein solution, the Cu(I)-containing catalyst mixture was added, and the reaction was allowed to incubate for 4 hours at 37 ºC. Following the labeling reaction, excess dye was removed by performing buffer exchange using an Amicon (Millipore) Ultra 0.5 mL 3 kDa spin column. Next, the proteins were purified by reverse-phase HPLC as previously described. The purified proteins were then concentrated, exchanged into αS buffer, and stored at – 80 ºC until further use.

MALDI MS of Raz Labeling Reactions. Click labeling of αS-π^{Raz}_{39} (**4b**) and αS-π^{Raz}_{94} (**5b**) was periodically monitored using MALDI MS. Following treatment with a total of 5 equivalents of Raz azide in the presence of Cu(I), near quantitative labeling was observed within 4 h.
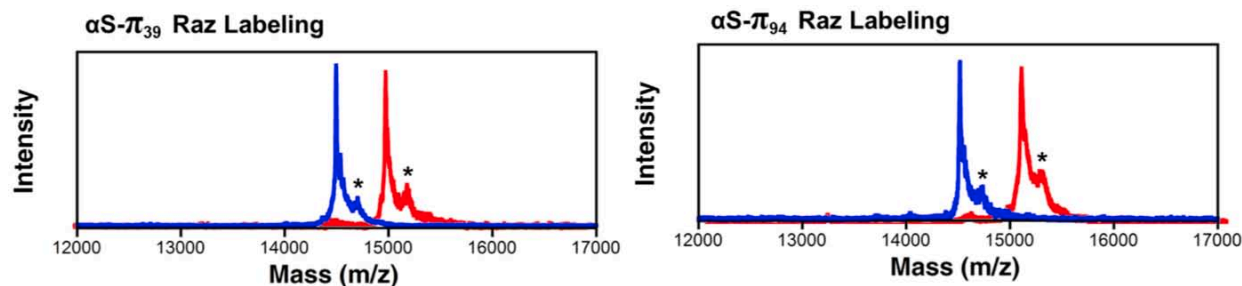
Fig. S5. MALDI MS of Crude Raz Labeling Reactions. Blue trace: Cys mutant prior to the labeling reaction. Red trace: Crude reaction mixture post labeling with 5 equiv of Raz in the presence of Cu(I). The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

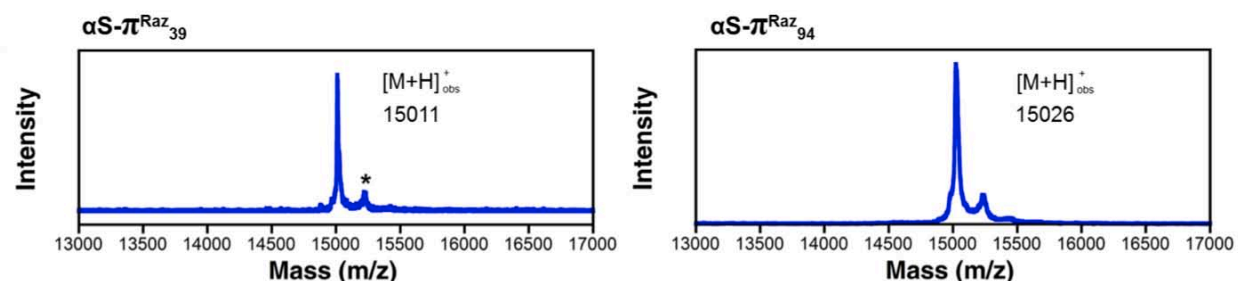**MALDI MS of HPLC Purified αS-π^{Raz}_{39} (4b) and αS-π^{Raz}_{94} (5b).**

Fig. S6. MALDI Traces of HPLC Purified αS-π^{Raz}_{39} (4b) and αS-π^{Raz}_{94} (5b). Expected [M+H]$^+$ of αS-π^{Raz}_{39} and αS-π^{Raz}_{94} are 15012 and 15027, respectively. The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

Trypsin Digestion of αSπ^{Raz}_{39} (4b) and αSπ^{Raz}_{94} (5b). Trypsin digestion was performed as described above. MALDI MS analysis of the trypsin digestion fragments show that Raz labeling is both site-specific and quantitative.
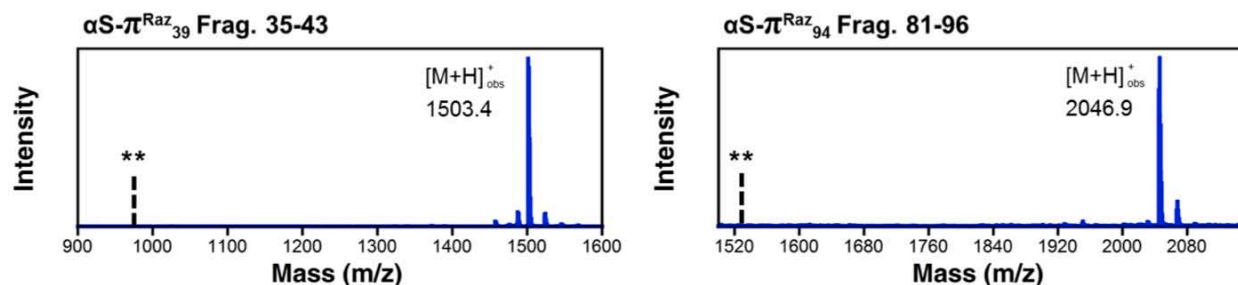

Fig. S7. MALDI MS of Trypsin Fragments of αS-π^{Raz}_{39} (4b) and αS-π^{Raz}_{94} (5b). Expected [M+H]$^+$ of αS-π^{Raz}_{39} Frag. 35-43 and αS-π^{Raz}_{94} Frag 81-96 are 1503.8 and 2047.0, respectively. The double asterisk (**) corresponds to the expected mass of the unmodified fragment.

Synthesis of αS-C$^{Fam}_9$π$^{Raz}_{94}$ (7c) and αS-π$^{Raz}_{39}$C$_{114}$ (8c). First, αS-C$_9$π$_{94}$ (**7a**) and αS-π$_{39}$C$_{114}$ (**8a**) were labeled with Fam and purified over a HiTrap Q HP column as described above to yield αS-C$^{Fam}_9$π$_{94}$ (**7b**) and αS-π$_{39}$C$^{Fam}_{114}$ (**8b**). The fractions containing the labeled product were identified by MALDI MS and pooled. Next, Cu-catalyzed click labeling with Raz azide was performed as described for the singly-labeled variants. Following the click reaction, excess dye was removed by performing buffer exchange, and the doubly-labeled proteins were purified by reverse-phase HPLC as described above. The purified proteins were then concentrated, exchanged into αS buffer, and stored at − 80 °C until further use.
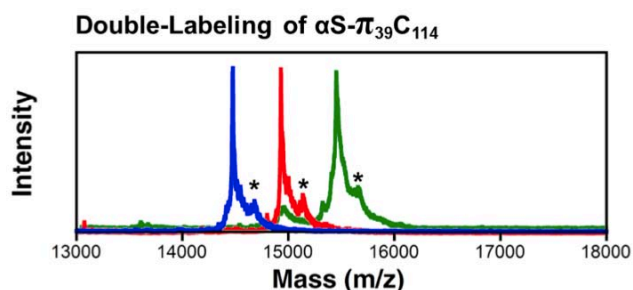

Fig. S8. MALDI MS of Crude Double Labeling Reactions. Blue trace: αS-π$_{39}$C$_{114}$ prior to the labeling reaction. Red trace: Crude reaction mixture post labeling with 10 equiv of Fam maleimide. Green trace: Crude reaction mixture post labeling with 5 equiv of Raz azide in the presence of Cu(I). The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

MALDI MS of HPLC Purified αS-C$^{Fam}_9$π$^{Raz}_{94}$ (7c) and αS-π$^{Raz}_{39}$C$_{114}$ (8c).
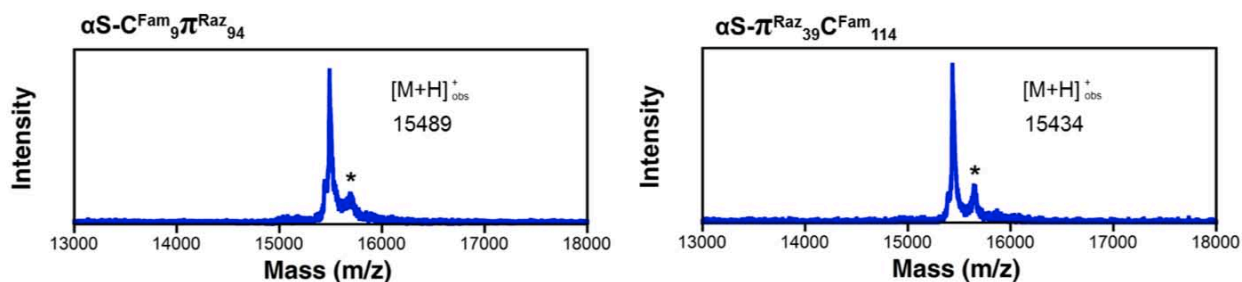
Fig. S9. MALDI MS Traces of HPLC Purified αS-C$^{Fam}_9$π$^{Raz}_{94}$ (7c) and αS-π$^{Raz}_{39}$C$^{Fam}_{114}$ (8c). Expected [M+H]$^+$ of αS-C$^{Fam}_9$π$^{Raz}_{94}$ and αS-π$^{Raz}_{39}$C$^{Fam}_{114}$ are 15470 and 15413, respectively. Expected [M+H]$^+$ of maleimide ring-opened αS-C$^{Fam}_9$π$^{Raz}_{94}$ and αS-π$^{Raz}_{39}$C$^{Fam}_{114}$. are 15488 and 15431, respectively. The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

Trypsin Digestion of αS-C$^{Fam}_9$π$^{Raz}_{94}$ (7c) and αS-π$^{Raz}_{39}$C$^{Fam}_{114}$ (8c). Trypsin digestion was performed as described above. MALDI MS analysis of the trypsin digestion fragments show that Fam and Raz incorporation are both site-specific and quantitative. In each Fam labeled fragment, a mass adduct of +18 Da was observed. This increase in mass is consistent with hydrolytic ring-opening of the maleimide group.
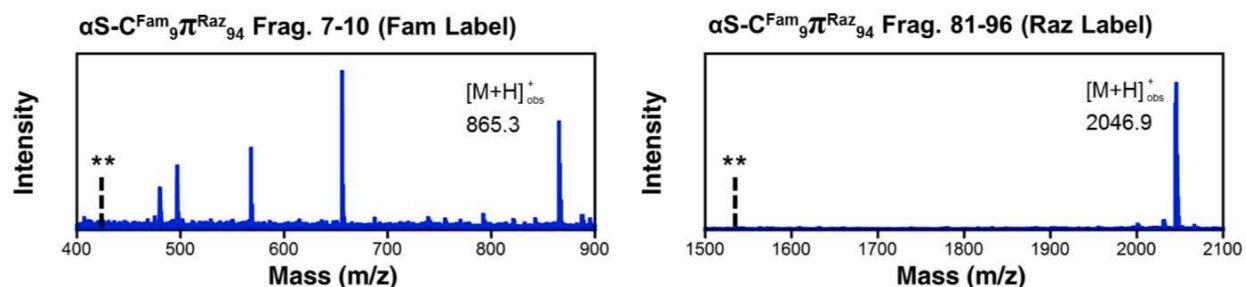

Fig. S10. MALDI MS of Trypsin Fragments of αS-C$^{Fam}_9$π$^{Raz}_{94}$ (7c). Expected [M+H]$^+$ of Frag. 7-10 and Frag 81-96 are 847.3 and 2047.0, respectively. Expected [M+H]$^+$ of maleimide ring-opened Frag. 7-10 is 865.3. The double asterisk (**) corresponds to the expected mass of the unmodified fragment.
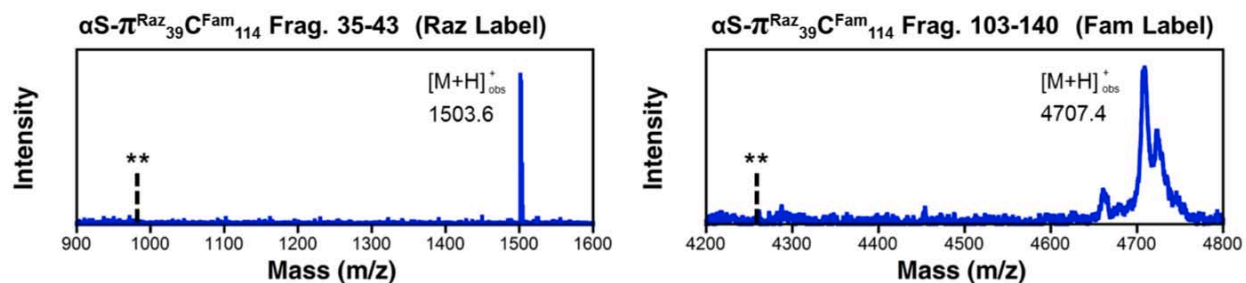
Fig. S11. MALDI MS of Trypsin Fragments of αS-π$^{Raz}_{39}$ C$^{Fam}_{114}$ (8c). Expected [M+H]$^+$ of Frag. 35-43 and Frag 103-140 are 1503.8 and 4687.6, respectively. Expected [M+H]$^+$ of maleimide ring-opened Frag. 103-140 is 4705.6. The double asterisk (**) corresponds to the expected mass of the unmodified fragment.

Synthesis of αS-D$^S_2$C$^{Fam}_9$ (6e). Protein fragment αS$_{9-140}$-C$_9$ (6b) and the synthetic thioester peptide Ac-αS$_{1-8}$-D$^S_2$-SR (6c) were prepared in accordance with previously described protocols.[3, 8] αS$_{9-140}$-C$_9$ (6b) (1 equiv, 0.10 µmol) was dissolved in 200 µL of degassed ligation buffer (6 M guanidinium hydrochloride, 200 mM sodium phosphate, 20 mM TCEP, 1% v/v thiophenol, pH 7.5) and incubated for 5 min. The reduced protein fragment was transferred to a microcentrifuge

tube containing 2 equiv of the dried N-terminal peptide thioester Ac-αS$_{1-8}$-D$^S_2$-SR (**6c**), purged with argon, and allowed to incubate for 24 h at 37 ℃ with shaking at 600 rpm. MALDI MS analysis of the crude ligation reaction showed quantitative conversion to the full length product Ac-αS-D$^S_2$C$_9$ (**6d**) (see below). Following ligation, the reaction solution was dialyzed into αS buffer. Next, the ligated protein was labeled with Fam as previously described. MALDI MS analysis of the crude reaction showed nearly quantitative conversion to the Fam-containing product αS-D$^S_2$C$^{Fam}_9$ (**6e**) (see below). Following the labeling reaction, excess dye was removed by performing buffer exchange using a spin column. The doubly-labeled protein was purified by reverse-phase HPLC as described above. The purified protein was concentrated, exchanged into αS buffer, and stored at − 80 ℃.

Mass Spectrometry Analysis of αS-D$^S_2$C$^{Fam}_9$ Synthesis.
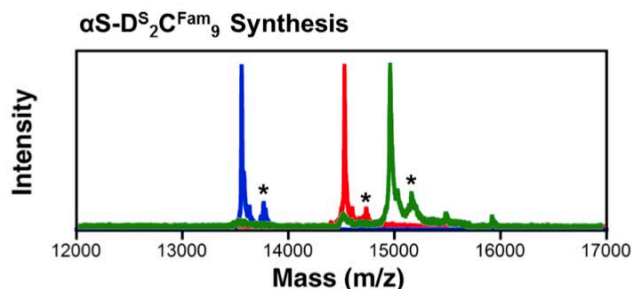
Fig. S12. MALDI MS of Crude Ligation and Fam Labeling Reactions Towards αS-D$^S_2$C$^{Fam}_9$ (6e). Blue trace: αS$_{9-140}$-C$_9$ (6b). Red trace: Formation of αS-D$^S_2$C$_9$ (6d) following ligation of αS$_{9-140}$-C$_9$ (6b) with Ac-αS$_{1-8}$-D$^S_2$-SR (6c). Green trace: Post Fam labeling of αS-D$^S_2$C$_9$ (6d) to generate αS-D$^S_2$C$^{Fam}_9$ (6e). The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

Synthesis of αS$_{2-140}$-K$_2$ζ^{Fco}$_N$ (S1d) and αS$_{2-140}$-K$_2$ζ^{Rco}$_N$ (S1e).   Note: The protein labeling procedures reported here have not been optimized since the AaT labeling method was deemed less useful for constructing libraries of doubly labeled proteins than combinations of Cys/Uaa labeling.   pET-16b_αS$_{2-140}$K$_2$ was transformed into *E. coli* BL21 (DE3) cells. Transformed cells were selected on the basis of Amp resistance. Single colonies were used to inoculate 4 mL of LB media supplemented with Amp (100 µg/mL). The primary culture was used to inoculate 1 L of LB media containing Amp (100 µg/mL) which was then incubated at 37 °C with shaking at 250 rpm. Upon reaching an OD$_{600}$ value of 0.8, protein expression was induced by the addition of IPTG (1 mM) followed by incubation at 25 °C with shaking at 250 rpm for 16 hours. The cells were then harvested by centrifugation at 4,200 x g for 15 min and the resulting pellet was resuspended in Factor Xa cleavage buffer (100 mM NaCl, 50 mM Tris, 5 mM CaCl$_2$, pH 8.0) containing protease inhibitor cocktail, 1 mM PMSF, and 10 units/mL DNAse1–Grade II. Following sonication, the cell lysate was boiled for 20 minutes prior to centrifugation for 20 minutes at 30,000 x g, 4 °C. The cleared supernatant was then incubated with Ni-NTA resin for 1 h on ice with gentle shaking. The Ni-NTA resin was first rinsed with Factor Xa cleavage buffer followed and then washed with Factor Xa buffer containing 50 mM imidazole. The protein was eluted with Factor Xa buffer containing 250 mM imidazole. SDS-PAGE analysis was performed to analyze the purity of the eluted protein-containing fractions.   Selected fractions were then dialyzed against Factor Xa cleavage buffer containing 100 mM urea at 4 °C. Next, the His$_{10}$ purification tag was removed from His$_{10}$-αS$_{2-140}$-K$_2$ (**S1a**) by incubation of the purified protein (0.5 mg/mL) with Bovine Factor Xa (80 Units) overnight at 37 °C. Cleavage of the His$_{10}$ tag was monitored by MALDI MS. Cleaved protein (αS$_{2-140}$-K$_2$, **S1b**) was dialyzed and loaded onto a HiTrap Q HP column equilibrated with FPLC purification buffer. αS$_{2-140}$-K$_2$ (**S1b**) was eluted

over a linear gradient of FPLC purification buffer containing sodium chloride from 0 M to 1 M over 120 min. Protein fractions were analyzed by MALDI MS. N-terminal modification with azidohomoalanine (Aha) was achieved using the following conditions: αS_{2-140}-K_2 (**S1b**) (0.5 mg/mL), ATP (2.5 mM), Aha (2.0 mM), *E. coli* total tRNA (2 mg/mL), *E. coli* Met*RS (0.1 mg/mL, expressed and purified as described in as described Wagner *et al.*[9]), and AaT (0.1 mg/mL, expressed and purified as described Wagner *et al.*[9]) in 50 mM HEPES pH 8.0, 150 mM KCl, 10 mM MgCl$_2$. The reaction was incubated at 37 °C for 1.5 h. Upon completion, the reaction was diluted 1:1 in FPLC purification buffer and purified using a HiTrap Q HP column to yield αS_{2-140}-$K_2\zeta_N$ (**S1c**). Protein fractions were analyzed by MALDI MS, pooled, and dialyzed into FPLC purification buffer.
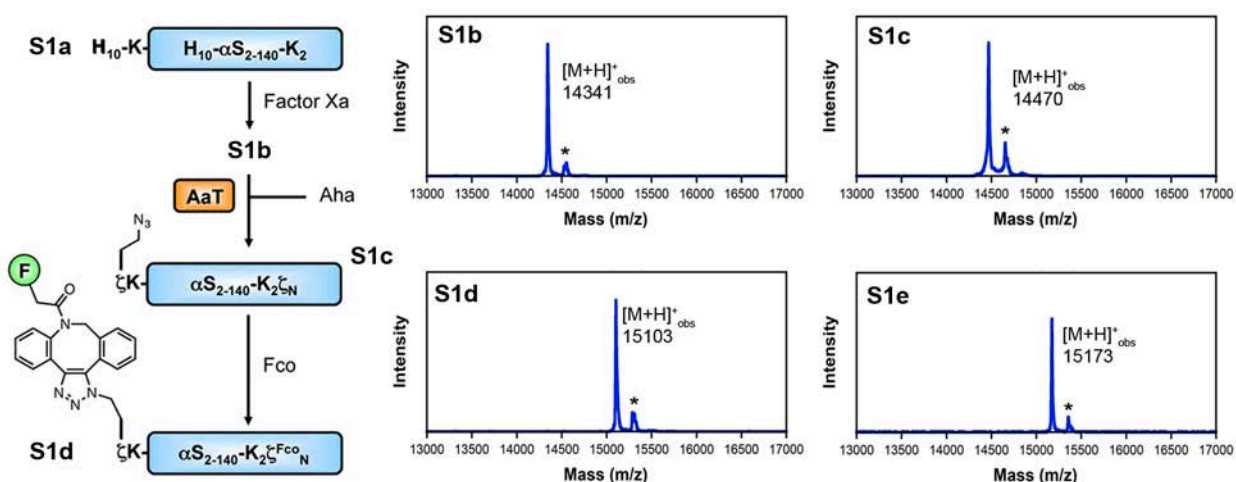

Fig. S13. MALDI MS Analysis of N-Terminal Labeling Using AaT. Expected [M+H]$^+$ of αS_{2-140}-K_2 (**S1b**), αS_{2-140}-$K_2\zeta_N$ (**S1c**), αS_{2-140}-$K_2\zeta^{Fco}{}_N$ (**S1d**), and αS_{2-140}-$K_2\zeta^{Rco}{}_N$ (**S1e**) are 14341, 14469, 15103, and 15159, respectively. Observed mass of **S1e** indicates oxidation, possibly at methionine. The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

αS_{2-140}-$K_2\zeta_N$ (**S1c**) was adjusted to 0.5 mg/mL from above and urea was added to a final concentration of 1.0 M. Protein was labeled with 3 equiv of either Fco (to generate αS_{2-140}-$K_2\zeta^{FK1}{}_N$, **S1d**) or Rco (to generate αS_{2-140}-$K_2\zeta^{Rco}{}_N$, **S1e**) at 37 °C for 3 h. Excess dye was removed by dialysis against 20 mM Tris pH 8.0 (2 x 5 L) with the first dialysis at room

temperature and the second at 4 °C. The labeled protein was purified using a HiTrap Q HP column and fractions were analyzed by MALDI-MS. Combined fractions were concentrated and exchanged into phosphate buffered saline (PBS, 12 mM NaH_2PO_4, 50 mM NaCl, 4.7 mM KCl, pH 7.3) using Amicon Ultra 0.5 Centrifugal 3 kDa spin columns and stored at -80 °C.

Synthesis of $\alpha S_{2\text{-}140}\text{-}K_2\zeta^{Fco}{}_N Z^{Rco}{}_{39}$ (S2e) and $\alpha S_{2\text{-}140}\text{-}K_2\zeta^{Fco}{}_N Z^{Rco}{}_{94}$ (S3e). Plasmid encoding either $H_{10}\text{-}\alpha S_{2\text{-}140}\text{-}K_2TAG_{39}$ or $H_{10}\text{-}\alpha S_{2\text{-}140}\text{-}K_2TAG_{94}$ were co-transformed with the pDULE2pXf plasmid into *E. coli* BL21 (DE3) cells. Single colonies were selected and grown in 4 mL of LB containing Amp (100 μg/mL) and Strep (100 μg/mL) for 4 hours at 37 °C with shaking at 250 rpm. The 4 mL primary culture was used to inoculate 1 L of M9 minimal media and grown at 37 °C with shaking at 250 rpm until the OD_{600} value reached 1.0. Protein expression was induced with 0.75 mM Azf and 1.0 mM IPTG and cells were incubated at 25 °C with 250 rpm shaking for 16 hours. Cultures and purified protein containing Azf were shielded from light using foil coverings. Cell lysis and Ni-NTA purification were performed as described for $H_{10}\text{-}\alpha S_{2\text{-}140}\text{-}K_2$ (**S1a**) to yield $H_{10}\text{-}\alpha S_{2\text{-}140}\text{-}K_2Z_{39}$ (**S2a**) or $H_{10}\text{-}\alpha S_{2\text{-}140}\text{-}K_2TAG_{94}$ (**S3a**) . Protein concentration was adjusted to 0.5 mg/mL with Factor Xa cleavage buffer. The His_{10} purification tag was removed by incubation of purified αS (0.5 mg/mL) with Factor Xa in the presence of 100 mM urea overnight at 37°C. Upon completion of Factor Xa cleavage, the reaction was quenched by adjusting the urea concentration to 1.0 M.

$\alpha S_{2\text{-}140}\text{-}K_2Z_{39}$ (**S2b**) and $\alpha S_{2\text{-}140}\text{-}K_2Z_{94}$ (**S3b**) were labeled *in situ* with 3 equiv of Rco at 37 °C for 3 hours. Excess dye was removed by dialysis against 20 mM Tris pH 8.0 (3 x 5 L) with the first dialysis at room temperature and the subsequent dialysis at 4 °C. Protein was purified using a HiTrap Q HP column and fractions were analyzed by MALDI-MS. $\alpha S_{2\text{-}140}\text{-}K_2Z^{Rco}{}_{39}$ (**S2c**) and $\alpha S_{2\text{-}140}\text{-}K_2Z^{Rco}{}_{94}$ (**S3c**) were then N-terminally modified with Aha as described above for $\alpha S_{2\text{-}}$

$_{140}K_2$ (**S1b**) to yield $\alpha S_{2\text{-}140}K_2\zeta_N Z^{Rco}_{39}$ (**S2d**) and $\alpha S_{2\text{-}140}K_2\zeta_N Z^{Rco}_{94}$ (**S3d**). Reactions were diluted

1:1 with FPLC purification buffer and purified using a HiTrap Q HP column as described above.

Protein fractions were analyzed by MALDI-MS and adjusted to 0.5 mg/mL in FPLC purification

buffer. Protein was N-terminally labeled with 3 equivalents of Fco in the presence of 1.0 M urea

at 37 °C for 3 h to yield $\alpha S_{2\text{-}140}K_2\zeta^{Fco}_N Z^{Rco}_{39}$ (**S2e**) and $\alpha S_{2\text{-}140}K_2\zeta^{Fco}_N Z^{Rco}_{94}$ (**S3e**). Excess dye was

removed by dialysis against MilliQ water (3 x 5 L) with the first dialysis at room temperature

and the subsequent dialysis at 4 °C. Doubly labeled protein was concentrated using Amicon

Ultra 0.5 Centrifugal 3 kDa spin columns and purified by HPLC using a C4 column. Fractions

were analyzed by MALDI MS and combined. Protein was exchanged into PBS pH 7.3 using

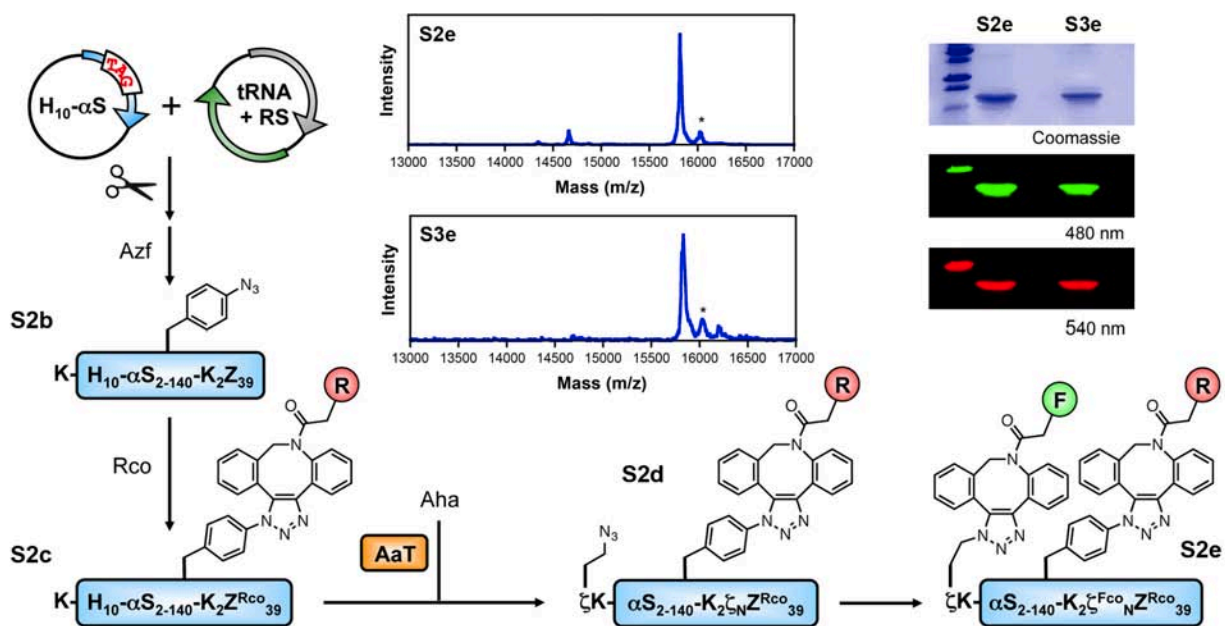Amicon Ultra 0.5 Centrifugal 3 kDa spin columns. Concentrated protein was stored at -80 °C.

Fig. S14. Double Labeling Using Amber Suppression and N-Terminal AaT Transfer. Azf was incorporated into αS and modified with tetramethylrhodamine-dibenzocyclooctyne (Rco). Then Aha was attached by AaT-mediated transfer and modified with fluorescein-dibenzocyclooctyne (Fco). Molecular weights of gel markers are 17, 25, 32, 47 kDa. 25 kDa marker in gel is fluorescently stained for alignment. MALDI MS: Calcd m/z for $\alpha S_{2\text{-}140}$-$K_2\zeta^{Fco}_N Z^{Rco}_{39}$ (**S2e**) whole protein [M+H]$^+$: 15818, Obs 15817. Calcd m/z for $\alpha S_{2\text{-}140}$-$K_2\zeta^{Fco}_N Z^{Rco}_{94}$ (**S3e**) whole protein [M+H]$^+$: 15834, Obs 15833. The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

Overexpression and Purification of αS-C₉ Intein Constructs. pTXB1_αS-C₉_Mxe-H₆ plasmid was transformed into competent E. coli BL21(DE3) cells. A single colony was used to inoculate 5 mL of LB media supplemented with ampicillin (Amp, 100 μg/mL). The primary culture was incubated at 37 °C with shaking at 250 rpm for 4-5 h. The primary culture was used to inoculate 1 L of LB media containing Amp (100 μg/mL) which was grown at 37 °C with shaking at 250 rpm and induced by the addition of IPTG (1 mM final concentration) when it eached an OD_{600} of 0.8-1; the culture was subsequently incubated overnight (18 h) at 18 °C. The cells were harvested by centrifugation at 4000 x g for 20 min at 4 °C, and the resulting pellet was resuspended in 15 mL lysis buffer (40 mM tris(hydroxymethyl)aminomethane (Tris), 5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 supplemented with one Roche protease inhibitor cocktail pill (cOmplete mini tablets, EDTA-free, Easy Pack, Roche Cat. #04693159001). Cells were lysed by sonication and the resulting lysate was centrifuged for 20 min at 14,000 x g, 4 °C to pellet insoluble debris. The cleared supernatant was incubated for 1 h on ice with 3 mL Ni-NTA resin. The slurry was loaded into a fritted column and the liquid allowed to flow through; the resin was then washed with 15 mL 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, then with 20 mL 50 mM HEPES 5 mM imidazole, pH 7.5. The desired protein (αS-C₉-Mxe-H₆, **S4a**)was then eluted with 50 mM HEPES, 300 mM imidazole pH 7.5. At this point, the intein can be cleaved by addition of β-mercaptoethanol (βME, 200 mM final concentration) overnight (18 h) at room temperature for post-hydrolysis Fam labeling, or the resulting eluent may be dialyzed overnight in 3 L 20 mM Tris pH 8.0 at 4 °C for Fam labeling with the intein intact. Following βME cleavage of the intein, the protein-containing solution was dialyzed overnight (18 h) at 4 °C in 20 mM Tris, pH 8.0. The resulting dialysate was incubated with Ni-NTA resin (3 mL) for 1 h on ice, and the slurry was loaded into a fritted column. The

flow-through was collected, and the resulting solution containing αS-C$_9$ can be labeled with Fam and purified as described above to produce αS-C$^{Fam}_9$ (**1b**) from pRK172_αS-C$_9$ (data not shown).

Synthesis of αS-C$^{Fam}_9$ with Intein Fusion. The concentration αS-C$_9$-Mxe-H$_6$ (**S4b**) in 20 mM Tris pH 8.0 was determined by BCA assay (Pierce^{TM} BCA Protein Assay Kit, used according to manufacturer's specifications and with bovine serum albumin (BSA) standard solutions). αS-C$_9$-intein-H$_6$ (**S4a**, 148 µM, 5 mL) was treated with tris(2-carboxxyethyl)phosphine (TCEP, 1 mM final concentration) for 10 min at room temperature; following this time, 5 equiv. for fluorescein-5-maleimide (Fam, 183 µL from a freshly prepared 20 mM stock in DMSO, 740 µM final concentration) was added and the solution incubated at 37 °C. Labeling was assessed by MALDI-TOF MS and the reaction incubated until quantitative conversion to αS-C$^{Fam}_9$-Mxe-H$_6$ (**S4b**) was observed (8 h). The solution was then dialyzed overnight (16 h) at 4 °C in 20 mM Tris pH 8, then incubated with 2 mL Ni-NTA resin on ice for 1 h. The resulting slurry was loaded into a fritted column, and the liquid containing excess Fam was allowed to flow through. The desired protein was then eluted with 6 mL 50 mM HEPES, 300 mM imidazole pH 7.5. The eluent was treated with βME (200 mM final concentration) for 18 h at room temperature, then dialyzed for 8 h at 4 °C against 20 mM Tris pH 8. The dialysate was then incubated with 2 mL Ni-NTA resin on ice for 1 h, the slurry loaded into a fritted column, and the resulting αS-C$^{Fam}_9$ (**1b**) containing flow through collected. The flow through was dialyzed overnight (16 h) at 4 °C against 20 mM Tris pH 8, then purified over a HiTrap Q HP column on an ÄKTA FPLC over a 100 min NaCl gradient (0 to 500 mM NaCl in 20 mM Tris, pH 8.0). Fractions containing the desired product were identified by MALDI-TOF MS and pooled. The resulting solution was further purified by reverse-phase HPLC using a Vydac 218TP

C4 semi-prep column over the following gradient between 0.1 % TFA in water (aqueous phase) and 0.1 % TFA in acetonitrile: 95% aqueous phase for 5 min, then gradient between 95% and 70% aqueous phase over 5 min, then a gradient to 55% aqueous phase over 40 min, then 0% aqueous phase over 5 min. Fractions containing purified protein were identified by MALDI-TOF MS and the resulting fraction pool was diluted 5-fold with 20 mM Tris, 100 mM NaCl pH 7.5 and concentrated using an Amicon Ultra 0.5 mL 3 kDa spin column.

Analysis of Fam Labeling Reaction of αS-C$_9$-Mxe-H$_6$ (S4a). Fam labeling was periodically monitored using MALDI-MS to assess reaction progess; quantitative labeling was observed within 8 h. Confirmation of labeling and the extent of background labeling of the intein Cys was assessed by SDS-PAGE.
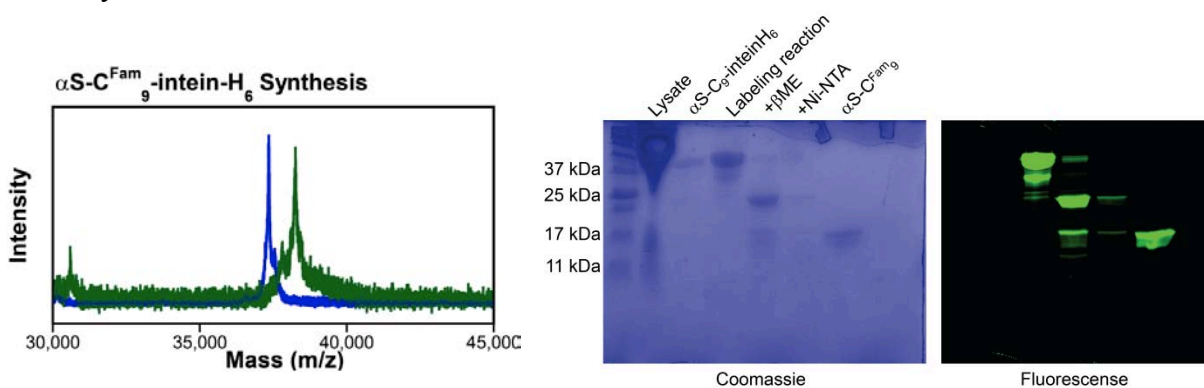

Fig. S15. MALDI MS and SDS-PAGE Analysis of αS-C$_9$-Mxe-H$_6$ (S4a) Labeling. Left: Crude MALDI-MS analysis of Cys labeling reaction. Blue trace: αS-C$_9$-intein-H$_6$ prior to labeling. Green trace: Crude labeling reaction with 5 equiv of Fam maleimide after 8 hours showing near-complete consumption of starting material. Right: SDS-PAGE gel of αS-C$_9$-intein-H$_6$ prior to and during labeling and purification.

MALDI Analysis of HPLC Purified αS-C$^{Fam}_9$ (1b) from Intein Construct. MALDI MS of the purified protein yielded a mass 17 Da higher than the calculated mass. MALDI MS analysis of trypsin digested fragments suggests that this increase in mass corresponds to hydrolytic ring opening of the maleimide group.
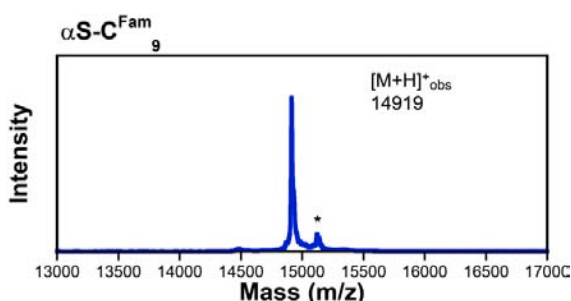

Fig. S16. MALDI MS of HPLC Purified αS-C$^{Fam}_9$ (1b) Produced from Intein Fusion. Expected [M+H]$^+$ of αS-C$^{Fam}_9$ is 14903 Da. Expected [M+H]$^+$ of ring-opened αS-C$^{Fam}_9$ is 14921 Da. The asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da).

Trypsin Digestion of αS-C$^{Fam}_9$ (1b) from Intein Fusion. αS mutant (20 µg) was incubated with 5 µL of sequencing-grade modified trypsin (0.1 mg/mL) in αS buffer at 37 °C for 2 hours. After the incubation period, an aliquot (1.0 µL) of the digestion reaction was removed and analyzed by MALDI MS. MALDI MS of the trypsin digestion fragment (αS$_{7-10}$-C$^{Fam}_9$) show that Fam labeling is site-specific and quantitative.
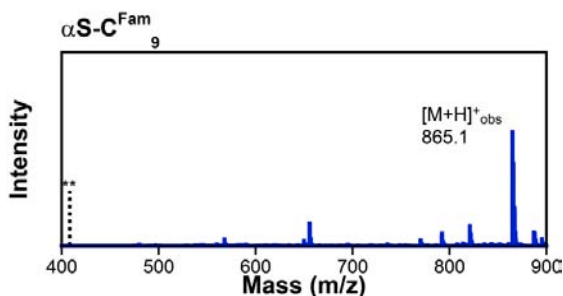

Fig. S17. MALDI MS of Trypsin Fragments of αS-C$^{Fam}_9$ (1b). Expected [M+H]$^+$ of αS-C$^{Fam}_9$ fragment 7-10 is 847.3. Expected [M+H]$^+$ of maleimide ring-opened αS-C$^{Fam}_9$ fragment 7-10 is 865.3. The double asterisk (**) corresponds to the expected mass of the unmodified fragment.

Overexpression and Purification of αS-C$_9$-π_{39}-Mxe-H$_6$ (9a). pTXB1_αS-C$_9$-TAG$_{39}$_intein-H$_6$ was transformed into competent *E. coli* BL21(DE3) cells harboring the orthogonal tRNA$_{CUA}$ and pXF-tRNA synthetase pair encoded by the pDULE2-pXF plasmid. Transformed cells were selected on the basis of ampicillin and streptomycin resistance. A single colony was used to inoculate 5 mL LB media supplemented with ampicillin (100 µg/mL) and streptomycin (100 µg/mL) and the primary culture was incubated at 37 °C for with shaking at 250 rpm for 5-6 hours. The primary culture was then used to inoculate 500 mL of a variant of M9 minimal media described previously containing ampicillin and streptomycin (100 µg/mL each). When the OD$_{600}$ of the secondary culture reached 0.9, propargyltyrosine (Ppy, π) was added (110 mg solubilized in H$_2$O with 3-5 drops of 1 M NaOH; final concentration of 1 mM). After 10 minutes, 500 µL of 1 M isopropyl β-D-1-thiogalactopyranose (IPTG) was added and the culture incubated overnight (16 h) at 18 °C. The cells were harvested by centrifugation at 4000 x g for 20 min at 4 °C. The resulting pellet was resuspended in 15 mL lysis buffer (40 mM tris(hydroxymethyl) aminomethane (Tris), 5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 supplemented with one Roche protease inhibitor cocktail pill (cOmplete mini tablets, EDTA-free, Easy Pack, Roche Cat. #04693159001). Cells were lysed by sonication and the resulting lysate was centrifuged for 20 min at 14,000 x g, 4 °C to pellet insoluble debris. The cleared supernatant was incubated for 1 h on ice with 3 mL Ni-NTA resin. The slurry was loaded into a fritted column and the liquid allowed to flow through; the resin was then washed with 15 mL 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, then with 20 mL 50 mM HEPES 5 mM imidazole, pH 7.5. The desired protein was then eluted with 50 mM HEPES, 300 mM imidazole pH 7.5. Following elution of αS-C$_9\pi_{39}$-MxeH$_6$ (**9a**), βME was added to a final concentration of 200 mM and the solution incubated overnight (18 h) at room temperature. The βME-cleaved

protein was then dialyzed for 8 hours at 4 °C against 20 mM Tris, pH 8.0. Following this time, the dialysate was incubated with 3 mL Ni-NTA resin on ice for 1 h. The Ni-NTA slurry was then loaded into a fritted column and the flow-through containing $\alpha S\text{-}C_9\pi_{39}$ (**9b**) collected. The flow-through was dialyzed overnight (14 h) at 4 °C against 20 mM Tris pH 8.0 and used directly in labeling reactions as described below.

Synthesis of $\alpha S\text{-}C^{Fam}_9\text{-}\pi^{Raz}_{39}$ (9d) from Intein Construct. The concentration of semi-crude $\alpha S\text{-}C_9\text{-}\pi_{39}$ (**9b**) in 20 mM Tris pH 8.0 obtained above was determined by BCA assay (Pierce^{TM} BCA Protein Assay Kit, used according to manufacturer's specifications and with bovine serum albumin (BSA) standard solutions; concentration was determined to be 2 mg/mL (140 µM)). The protein (5 mL volume) was labeled with fluorescein-5-maleimide as follows: TCEP was added to a final concentration of 1 mM, then 5 equiv Fam was added (313 µL from a freshly prepared 25 mM stock in DMSO; final concentration 700 µM). The reaction was incubated a 37 °C and periodically monitored by MALDI MS; complete labeling was observed within 10 h to yield $\alpha S\text{-}C^{Fam}_9\text{-}\pi_{39}$ (**9c**). The labeled protein was then dialyzed against 3 L 20 mM Tris pH 8.0 overnight at 4 °C to remove excess dye. The following day, the dialysate was used in Cu(I)-catalyzed click labeling as follows, assuming negligible change in protein concentration: catalyst mixture (1.25 µL/mL 80 mM CuSO$_4$, 30 µL/mL 50 mM Tris-(hydroxypropyltriazolylmethyl)amine (THPTA) and 30 µL/mL 100 mM sodium ascorbate) was prepared and allowed to rest 5 min at room temperature. Following this time, 5 equiv of carboxytetramethylrhodamine azide (Raz; 70 µL from a 50 mM stock in DMSO, final concentration 700 µM) was added, then the catalyst mixture described above. The reaction was allowed to incubate at 37 °C and monitored periodically by MALDI MS; complete labeling was observed in 6 h to yield $\alpha S\text{-}C^{Fam}_9\text{-}\pi^{Raz}_{39}$ (**9d**). The reaction was then dialyzed overnight at 4 °C in 3 L 20 mM Tris pH 8.0. The dialysate was then purified

on an ÄKTA FPLC system over a 100 min NaCl gradient (0 to 500 mM NaCl in 20 mM Tris pH 8.0) using a HiTrap Q HP column (5 mL). Fractions containing the desired product were identified by MALDI MS and pooled. The desired fractions were further pruified by reverse phase HPLC HPLC using a Vydac 218TP C_4 semi-prep column over the following gradient between 0.1 % TFA in water (aqueous phase) and 0.1 % TFA in acetonitrile: 95% aqueous phase for 5 min, then gradient between 95% and 70% aqueous phase over 5 min, then a gradient to 55% aqueous phase over 40 min, then 0% aqueous phase over 5 min. Fractions containing purified protein were identified by MALDI-TOF MS and the resulting fraction pool was diluted 5-fold with 20 mM Tris, 100 mM NaCl pH 7.5 and concentrated using an Amicon (Millipore) Ultra 0.5 mL 3 kDa spin column.

Analysis of Labeling Reactions for αS-C$_9$-π_{39} (9b) from Intein Construct. Labeling reactions were periodically monitored by MALDI MS. Complete labeling was observed within 10 h and 6 h for Fam and Cu-catalyzed Raz labeling, respectively. Analysis of the labeling reactions by SDS-PAGE shows flourescence corresponding to both Fam and Raz following labeling.
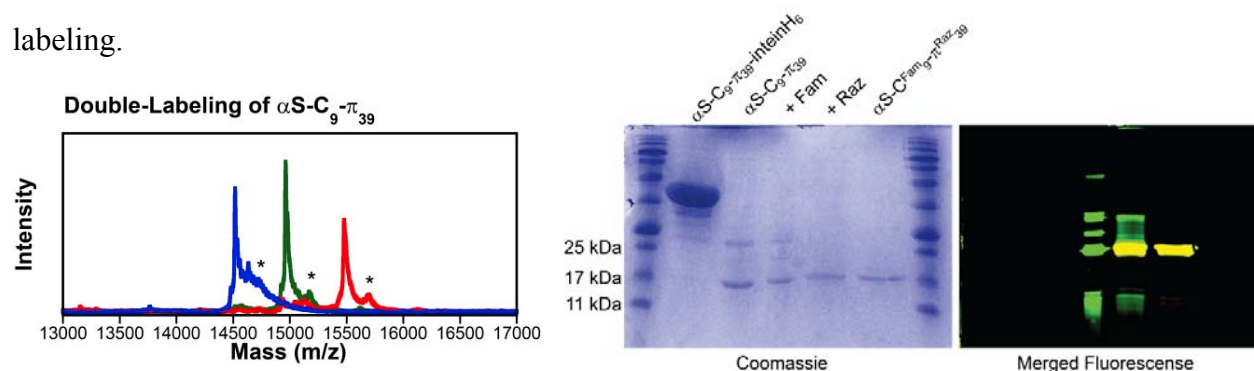

Fig. S18. MALDI MS and SDS-PAGE Analysis of αS-C$_9\pi_{39}$ (9b) Labeling. Left: Crude MALDI-MS analysis of Fam and Raz labeling reactions. Blue trace: αS-C$_9$-π_{39} (**9b**) prior to labeling. Green trace: Crude labeling reaction with 5 equiv of Fam maleimide after 10 hours showing complete consumption of starting material. Red trace: Crude Cu(I)-catalyzed labeling reaction with Raz after 6 hours showing near-complete labeling. Asterisk (*) indicates a matrix adduct observed in all MALDI spectra of αS (+207 Da). Right: SDS-PAGE gel of αS-C$_9$-π_{39}-Mxe-H$_6$ (**9a**) prior to and following βME intein cleavage and labeling.

Aggregation Analysis of Singly Labeled αS Constructs. Wild-type αS, αS-C$^{Fam}_9$ (**1b**), αS-C$^{Fam}_{114}$ (**2b**) and αS-π$^{Raz}_{94}$ (**5b**) produced as described above in 20 mM Tris, 100 mM NaCl pH 7.5 were used to determine if singly-labeled αS perturbs aggregation kinetics as follows. The concentration of each protein was determined by UV-Vis absorbance. αS wild type was quantified based on tyrosine absorbance at 280 nm (ε_{280} = 5120 M^{-1} cm^{-1})[7]; αS-C$^{Fam}_9$ and αS-C$^{Fam}_{114}$ were quantified by fluorescein absorbance at 494 nm (ε_{494} = 68000 M^{-1} cm^{-1})[10]; αS-π$^{Raz}_{94}$ was quantified by tetramethylrhodamine absorbance at 555 nm (ε_{555} = 87000 M^{-1} cm^{-1}; extinction coefficient from the manufacturer). Samples were prepared in triplicate in 20 mM Tris, 100 mM NaCl pH 7.5 as: 100 μM wild-type αS; 95 μM wild-type αS with 5 μM αS-C$^{Fam}_9$; 95 μM wild-type αS with 5 μM αS-C$^{Fam}_{114}$; or 95 μM wild-type αS with 5 μM αS-π$^{Raz}_{94}$. Samples were prepared in Eppendorf tubes and placed into an Ika MS3 (Ika, Wilmington, NC, USA) orbital shaker at 37 °C and shaken at 1500 RPM; each sample was parafilmed at the top of the Eppendorf tube throughout the assay to ensure minimal solvent evaporation. At each time point (0, 2, 4, 8, 12, 24, 32 and 48 hours) the following procedure was followed: each sample was vortexed, then centrifuged briefly, then 10 μL of each sample was removed and added to 140 μL 20 μM Congo Red solution in water. The samples were allowed to rest at room temperature for 20 minutes, then transferred to a 96-well black Corning CoStar clear-bottomed plate. Absorbance measurements (230-700 nm) were collected on a Tecan M1000 plate reader. The presence of αS fibrils was determined by the ratio of Congo Red absorbance at 540 nm over absorbance at 480 nm. The triplicate samples of each protein mixture were averaged and plotted as mean ± standard deviation. Primary data can be found in Figure S18. Following completion of the assay, fibrils were pelleted by centrifugation (90 min, 13200 x g, 4 °C) and the supernatant removed. The resulting fibril pellet was stored at − 20 °C for later TEM analysis. Congo Red

analysis of 25% αS-C$^{Fam}_{114}$ was carried out as described above for 5% labeled protein aggregation; aggregation progress was assayed at 0, 4, 8, 12, 24, 32, and 48 hours.
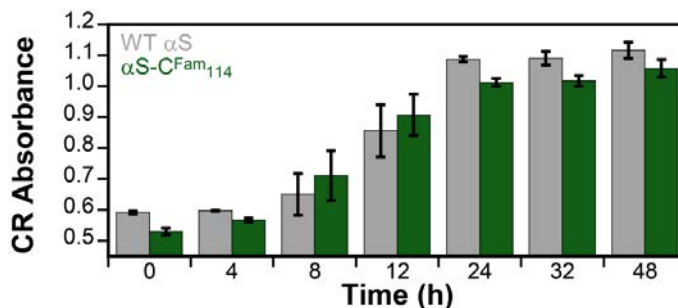

Fig. S19. Aggregation Analysis of αS-C$^{Fam}_{114}$ at 25% Labeled Protein Content. Fibrils formed from 100% WT αS or as a mixture of 75% WT αS with 25% singly-labeled αS-C$^{Fam}_{114}$. Aggregation was monitored by the ratio of Congo Red (CR) absorbance at 540 nm/480 nm and plotted as mean and standard deviation (error bars) from three replicate samples.

Quantification of Labeled αS Incorporation. Triplicate samples of WT αS, or 5% αS-C$^{Fam}_9$ (**1b**), αS-C$^{Fam}_{114}$ (**2b**) and αS-π^{Raz}_{94} (**5b**) in 95% WT αS were prepared as described above. A 30 μL aliquot was removed and stored at -80 °C to use as a quantification standard, and the remainder used in fibril formation by shaking at 1500 rpm, 37 °C for 48 h. Following this time, insoluble material was pelleted by centrifugation (13,200 rpm, 90 min, 4 °C). The supernatant was removed and the resulting pellet resuspended in an equal volume of buffer. The resuspended pellet (10 μL aliquot) was combined with sodium dodecyl sulfate (2 μL from a 150 mM stock in water; 25 mM final concentration) and boiled for 20 min. The samples were then cooled on ice for 20 min, then 3 μL loading dye added. Monomeric samples for calibration were prepared by serial 2-fold dilution of 10 μL with water; 2 μL water was then added to each sample, followed by 3 μL loading dye. The samples were then analyzed by SDS-PAGE (18% acrylamide gel run at 150 V for 1.5 h). Fluorescence images were acquired for samples containing **1b**, **2b**, or **5b** using a Typhoon FLA7000, and each gel stained with Coomassie Brilliant Blue for 2 h at room temperature, then destained for 2 h at room temperature and

imaged (Figure S21-S22). Gel quantification was performed using ImageJ software. The area of each fluorescent band or total protein by Coomassie staining was determined, and the monomeric standards used to generate a linear calibration; the protein present in the pellet samples was determined relative to the calibration curve, and the fraction relative to the first standard band calculated. The three samples were averaged and plotted as mean and standard deviation (Figure S20). Incorporation of labeled αS-C$^{Fam}_{114}$ at 25% labeled protein was performed by utilizing 25% αS-C$^{Fam}_{114}$ fibrils generated for cell transduction (*vide infra*). The aggregates were pelleted by centrifugation and resuspended in buffer to a total concentration of 100 µM (assuming complete sedimentation of protein); post-resuspension analysis was performed as described above. Separate standards were generated by diluting labeled αS-C$^{Fam}_{114}$ and WT to 25 µM and 75 µM, respectively, in an Eppendorf tube and used as described above for in-gel standards.
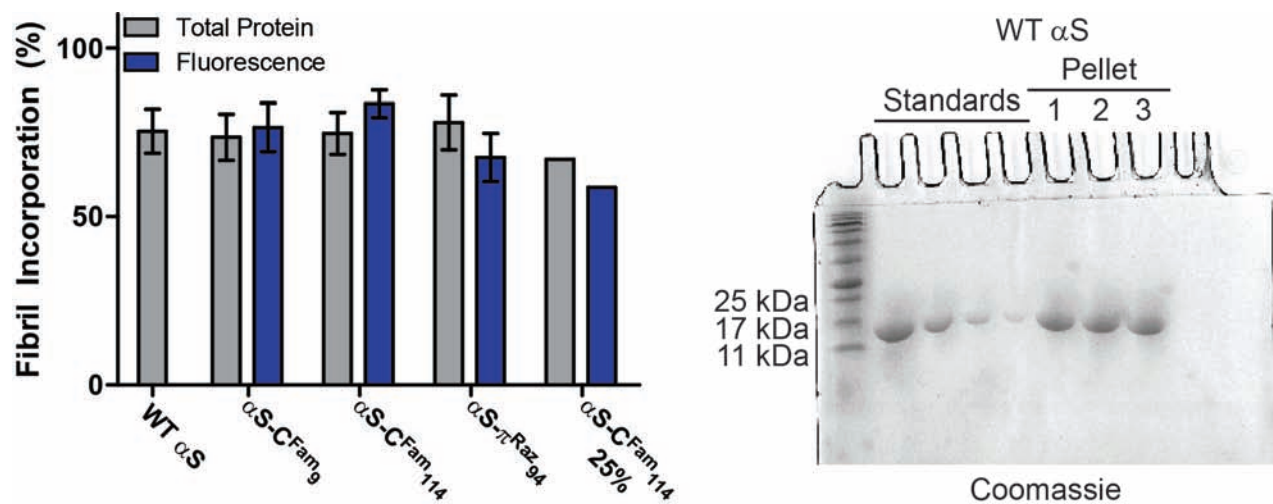

Fig. S20. Quantified Incorporation of αS into Fibrils. Left: Fibrils formed from triplicate samples of WT αS or 5% αS-C$^{Fam}_9$ (**1b**), αS-C$^{Fam}_{114}$ (**2b**), or αS-π^{Tmr}_{94} (**5b**) in 95% WT αS, or single sample of 25% αS-C$^{Fam}_{114}$ (**2b**) quantified by in-gel fluorescence or Coomassie staining relative to standard samples following SDS-PAGE; bars represent the average and standard deviation for triplicate samples. Right: Post aggregation SDS-PAGE gel of triplicate samples of WT αS.
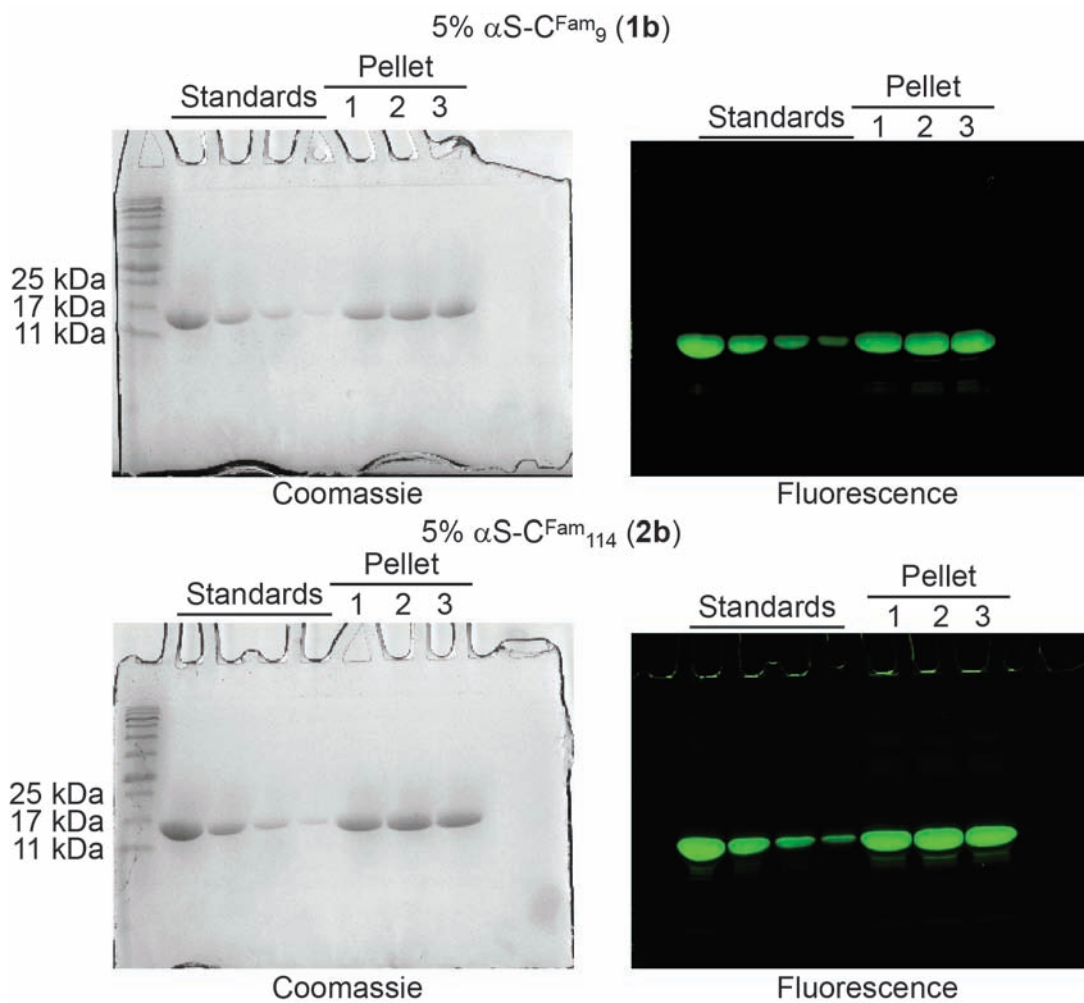
Fig. S21. Post-aggregation SDS-PAGE of triplicate samples of 5% αS-C$^{Fam}_9$ (**1b**), αS-C$^{Fam}_{114}$ (**2b**) in 95% WT αS.
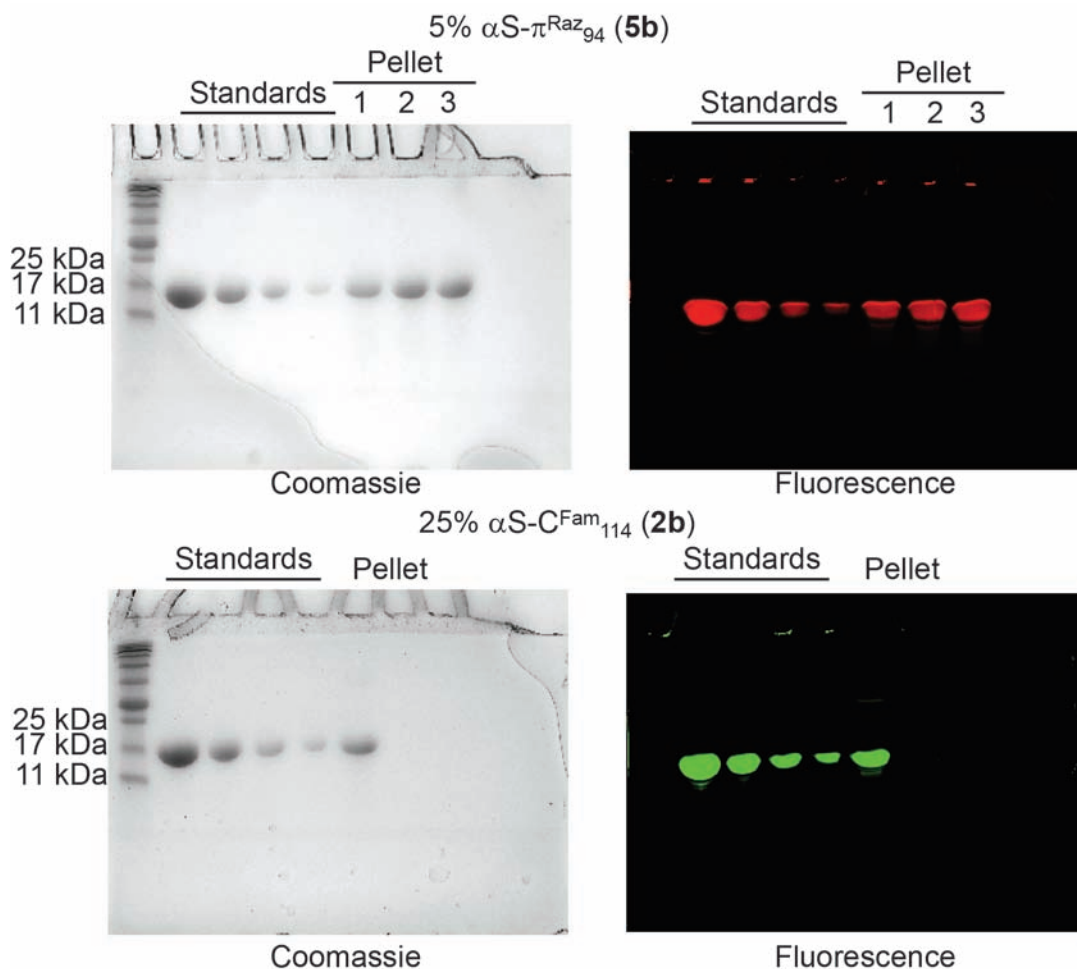
Fig. S22. Post-aggregation SDS-PAGE of triplicate samples of 5% αS-π^{Tmr}_{94} (**5b**) in 95% WT αS and a single sample of 25% αS-C$^{Fam}_{114}$ (**2b**) in 75% WT αS.

FRET Studies of αS Aggregation. Aggregation was performed using wild-type αS, αS-C$^{Fam}_9$, αS-π^{Raz}_{94}, and αS-C$^{Fam}_9$-π^{Raz}_{94} produced as described above. The concentration of each protein was determined by UV-Vis absorbance. αS wild type was quantified based on tyrosine absorbance at 280 nm (ε_{280} = 5120 M^{-1} cm^{-1})[7]; αS-C$^{Fam}_9$ was quantified by fluorescein absorbance at 494 nm (ε_{494} = 68000 M^{-1} cm^{-1}); αS-π^{Raz}_{94} and αS-C$^{Fam}_9$-π^{Raz}_{94} were quantified by tetramethylrhodamine absorbance at 555 nm (ε_{555} = 87000 M^{-1} cm^{-1}). Absorption spectra of the labeled αS samples used can be found in Figure S25. Samples were prepared by dilution of each

labeled protein and wild type with αS buffer (20 mM Tris, 100 mM NaCl pH 7.5) to a final concentration of 1 μM labeled protein and 99 μM wild type. Samples were prepared in Eppendorf tubes and placed into an Ika MS3 orbital shaker at 37 °C and shaken at 1500 RPM; each sample was covered with parafilm at the top of the Eppendorf tube throughout the assay to ensure minimal solvent evaporation. At each time point (0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 48 hours) the following procedure was followed: each sample was vortexed, then centrifuged briefly, then 10 μL of each sample was removed and added to 140 μL 20 μM Congo Red solution in water; an additional 15 μL of each sample was removed and added to 135 μL assay buffer for fluorescence measurements. Samples in Congo Red were allowed to rest at room temperature 20 minutes prior to measurement of absorption spectra (230-700 nm) in a 96-well black CoStar clear bottom plate on a Tecan M1000 plate reader. Samples in assay buffer were analyzed by pipetting into a 96-well black CoStar clear bottom plate; fluorescence polarization for αS-C^{Fam}_9 and αS-C^{Fam}_9-π^{Raz}_{94} was measured on a Tecan F200 plate reader with λ_{ex} = 485 nm and λ_{em} = 535 nm. Congo Red and fluorescence polarization data were fit to a sigmoidal expression in KaleidaGraph (Synergy Software, Reading, PA, USA; Fig. S23-S24); estimated $t_{1/2}$ values for each sample are provided in Table S1 Following fluorescence polarization measurements, samples of αS-C^{Fam}_9, αS-π^{Raz}_{94} and αS-C^{Fam}_9-π^{Raz}_{94} were analyzed by steady-state fluorescence and time correlated single photon counting (TCSPC) measurements using a PTI QuantaMaster fluorometer with multicell holder and Peltier temperature control at 20 °C in 1 mM quartz cuvettes. Conditions for steady-state flourescence are as follows: αS-C^{Fam}_9 and αS-C^{Fam}_9-π^{Raz}_{94}, fluorescein λ_{ex} = 485 nm; λ_{em} = 495-700 nm; αS-π^{Raz}_{94} and αS-C^{Fam}_9-π^{Raz}_{94} rhodamine λ_{ex} = 555 nm, λ_{em} = 565-700 nm; data was collected with 3 nm slit widths for excitation and emission, 1 nm step size, and 0.5 sec integration time. TCSPC measurements were

collected of αS-C$^{Fam}_9$ and αS-C$^{Fam}_9$-π^{Raz}_{94} using an LED with 486 nm excitation and 515 nm emission at 20 °C, 0-199 ns, peak channel count 10000. Following completion of the aggregation assay, the samples were split into two separate aliquots; fibrils in each aliquot were then pelleted by centrifugation (13200 x g for 90 min at 4 °C) and the supernatant removed. One aliquot was immediately resuspended in an equal volume (relative to supernatant) of αS buffer and the measurements described above repeated on the resuspended fibril sample. Measurements were performed as described above for the aggregation of αS-C$^{Fam}_9$ and αS-D$^S_2$-C$^{Fam}_9$ with data collected during aggregation at 0, 4, 8, 12, 24, 32, 48 hour time points and on resuspended fibrils (Figs. S29-S32, Table S3). The second aliquot was frozen at $-$ 20 °C until analysis by TEM.

Fitting of Aggregation Kinetics. Congo Red and fluorescence polarization data from the aggregation of wild-type αS, αS-C$^{Fam}_9$, αS-π^{Raz}_{94}, and αS-C$^{Fam}_9$-π^{Raz}_{94} were fit to a sigmoidal curve to estimate aggregation kinetics. Fitting was accomplished using Equation S1, where C_0 is the Congo Red absorbance ratio or mP value at t = 0; C_{48} is the Congo Red absorbance ratio or mP at t = 48; m is a fit parameter representative of the slope; $t_{1/2}$ is the half-maximum of the kinetic curve. In all fits, initial parameters were entered as follows for Congo Red and fluorescence polarization data, but values were not constrained. For Congo Red data, initial parameters were: C_0 = 0.5; C_{48} = 1; m = 1; $t_{1/2}$ = 8. For fluorescence polarization data, initial parameters were: C_0 = 80; C_{48} = 350; m = 1; $t_{1/2}$ = 8.

$$Y = C_0 + \frac{C_{48} - C_0}{1 + e^{\frac{t_{1/2} - t}{m}}} \qquad \text{(Eq. S1)}$$

Fluorescence Lifetime Measurement Fitting. Collected TCSPC measurements of αS-C$^{Fam}_9$ and αS-C$^{Fam}_9$-π^{Raz}_{94} were fit to either a monoexponential (0, 4 hour data) or biexponential (8-48

hour and resuspended fibril data) function; biexponential functions were used to maintain optimal time-decay window in the presence of significant scattering of incident light due to the presence of αS fibrils (see Fig. S27). For TCSPC measurements during aggregation of αS-C^{Fam}$_9$ and αS-D^S$_2$-C^{Fam}$_9$, collected data was fit to a monoexponential decay function. Data from steady-state measurements can be found in Figures S25 and S230. TCSPC data and lifetimes can be found in Figures S26-S27 or S31-32 and Tables S2 or S3, respectively. Data analysis was performed using FelixGX software 9PTI) and equations described previously[11] for an exponential decay model (Eq. S2):

$$I(t) = \sum_{i=1}^{n} A_i e^{-\frac{t}{\tau_i}} \qquad \text{(Eq. S2)}$$

In Eq. S2, A_i and τ_i are the amplitude and lifetime of the ith component, respectively. Reduced χ^2 values were calculated for each fit according to Equation S3:

$$\chi^2 = \frac{1}{N-p} \sum_{j=1}^{N} W(j)^2 [decay(j) - fit(j)]^2 \qquad \text{(Eq. S3)}$$

In Equation S3, N is the number of fit data points; j is the measured photon counts at a given delay time (i.e. a bin); p is the number of adjustable, fitted parameters; $W(j)$ is a Poisson weighting factor; $decay(j)$ is the experimentally determined decay curve; and $fit(j)$ is the fitted model decay curve. Weighted residuals were calculated according to Equation S4 using values as above:

$$R = W(j)[decay(j) - fit(j)] \qquad \text{(Eq. S4)}$$

Distance was calculated based on the quenching efficiency, taken from lifetime data according to Equation S5:

$$E_Q(\tau) = 1 - \left(\frac{\tau_{2L}}{\tau_{DL}}\right) \qquad \text{(Eq. S5)}$$

Above, τ_{2L} is the fluorescein lifetime obtained at a given time point for the doubly-labeled construct $\alpha S\text{-}C^{Fam}{}_9\pi^{Raz}{}_{94}$ and τ_{DL} is the fluorescein lifetime for the donor-labeled construct, $\alpha S\text{-}C^{Fam}{}_9$. Interfluorophore distance was then calculated from $E_Q(\tau)$ using Equation S6 using a calculated R_0 of 52 Å derived from experimental data:

$$R = R_0\left(\frac{1}{E_Q(\tau)} - 1\right)^{\frac{1}{6}} \qquad \text{(Eq. S6)}$$

The characteristic distance has been empirically derived through the relationship in Equation S7:

$$R_0{}^6 = \frac{9000(ln(10))\kappa^2\Phi_D \cdot J}{128\pi^5 n^4 N_A} \qquad \text{(Eq. S7)}$$

Here, κ^2 represents the geometrical factor that relates the orientation of the donor and acceptor transition dipole moments, approximated as 2/3, Φ_D is the quantum yield of the donor fluorophore, and J is the overlap integral of the donor fluorescence and acceptor absorbance spectra. Rearrangement and constant combination gives Equation S8:

$$R_0 = 0.211(\Phi_D\kappa^2 n^{-4} \cdot J)^{1/6} \qquad \text{(Eq. S8)}$$

J has been determined empirically from the absorbance and emission spectra of the TMR and FAM α-synuclein, respectively, through application of the integral in Equation S9:

$$J = \int_0^\infty f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \qquad \text{(Eq. S9)}$$

where $f_D(\lambda)$ is the normalized donor emission, $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor, at each wavelength (λ). The normalized donor emission is given by Equation S10:

$$f_D(\lambda) = \frac{F_D(\lambda)}{\int_0^\infty F_D(\lambda)d\lambda} \qquad \text{(Eq. S10)}$$

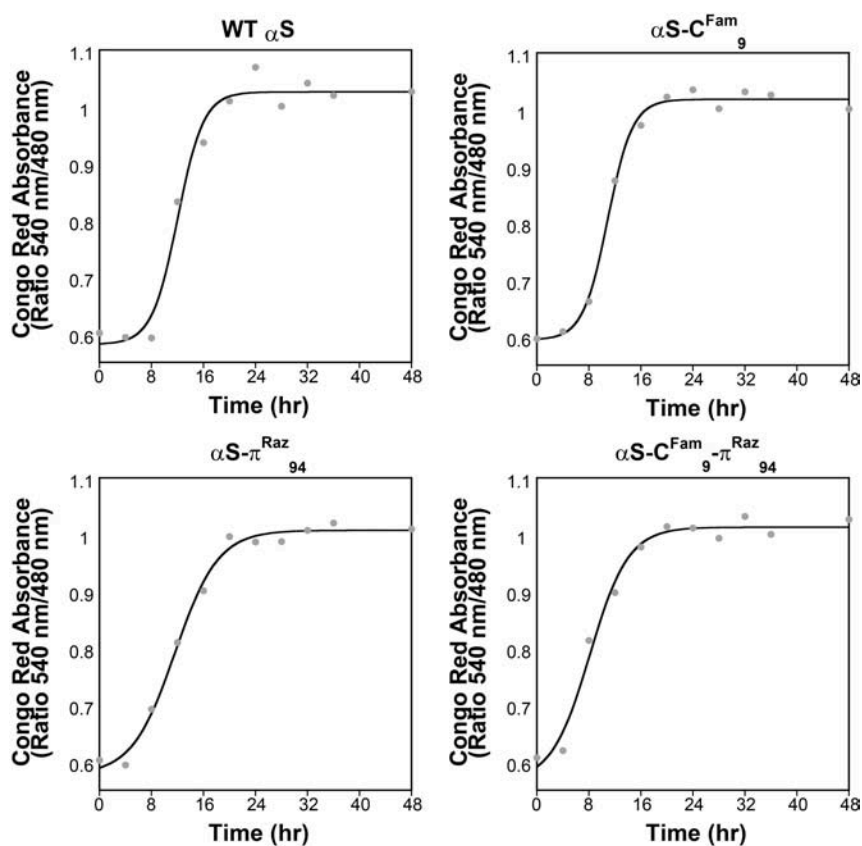where $F_D(\lambda)$ is the fluorescence emission spectrum of the donor dye.

Fig. S23. Congo Red Absorbance and Fitting from FRET Studies of Aggregation. Congo Red absorbance ratio (540 nm/480 nm) plotted for WT αS (top left), αS-C$^{Fam}_9$ (top right), αS-π^{Raz}_{94} (bottom left), and αS-C$^{Fam}_9$-π^{Raz}_{94} (bottom right). Solid lines represent sigmoidal fits according to Equation S1 and the parameters in Table S1.
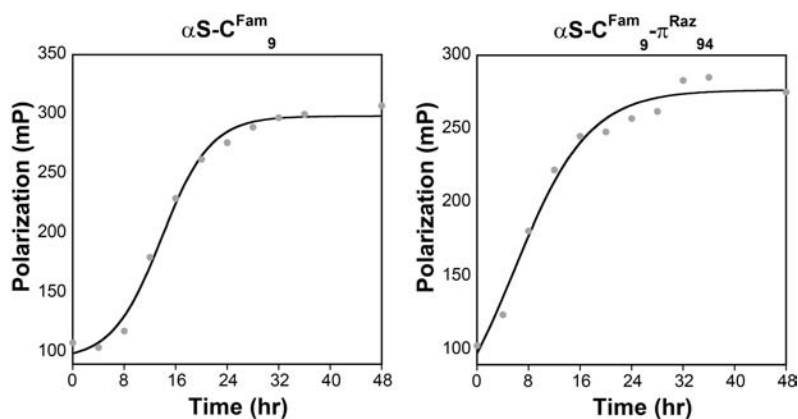
Fig. S24. Fluorescence Polarization and Fitting from FRET Studies of Aggregation. Fluorescence polarization (in mP value) plotted against time for αS-C$^{Fam}_9$ (left) and αS-C$^{Fam}_9$-π^{Raz}_{94} (right). Solid lines represent sigmoidal fits according to Equation S1 and the parameters in Table S1.

Table S1. Fit Parameters for αS Aggregation

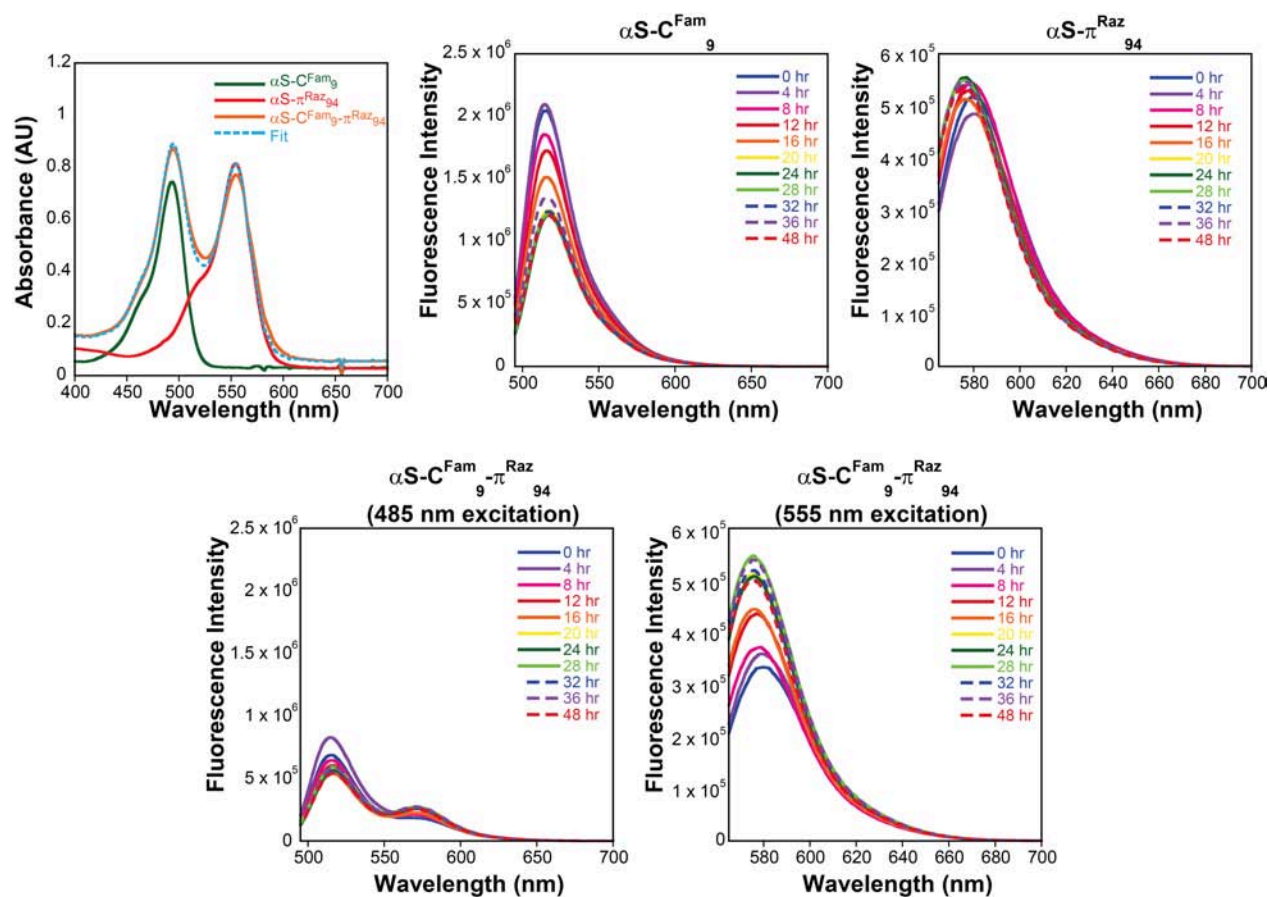| αS Sample	C_0	C_{48}	m	$t_{1/2}$ ($\pm$ σ)	R
Congo Red					
WT αS	0.58	1.03	1.9	12.0 $\pm$ 0.6	0.99
αS-C$^{Fam}_9$	0.59	1.02	1.9	10.9 $\pm$ 0.3	0.99
αS-π^{Raz}_{94}	0.58	1.01	3.3	11.6 $\pm$ 0.6	0.99
αS-C$^{Fam}_9$-π^{Raz}_{94}	0.56	1.01	2.9	8.2 $\pm$ 0.8	0.99
Fluorescence Polarization					
αS-C$^{Fam}_9$	94	298	3.8	13.8 $\pm$ 0.6	0.99
αS-C$^{Fam}_9$-π^{Raz}_{94}	31	276	5.8	6 $\pm$ 3	0.99

Fig. S25. Steady-State Fluorescence Data from FRET Studies of αS Aggregation. Top left: Absorbance measurements and minimized least-square fit (dashed blue line) of singly- and doubly-labeled αS constructs used in aggregation. Top middle: Steady state fluorescence data collected for αS-C^{Fam}_9 during the course of aggregation (0-48 hr) with excitation at 485 nm. Top right: Steady state fluorescence data collected for αS-π^{Raz}_{94} during the course of aggregation (0-48 hr) with excitation at 555 nm. Bottom left: Steady state fluorescence data collected for αS-C^{Fam}_9-π^{Raz}_{94} during the course of aggregation (0-48 hr) with 485 nm excitation. Bottom right: Steady state fluorescence data collected for αS-C^{Fam}_9-π^{Raz}_{94} during the course of aggregation (0-48 hr) with 555 nm excitation.
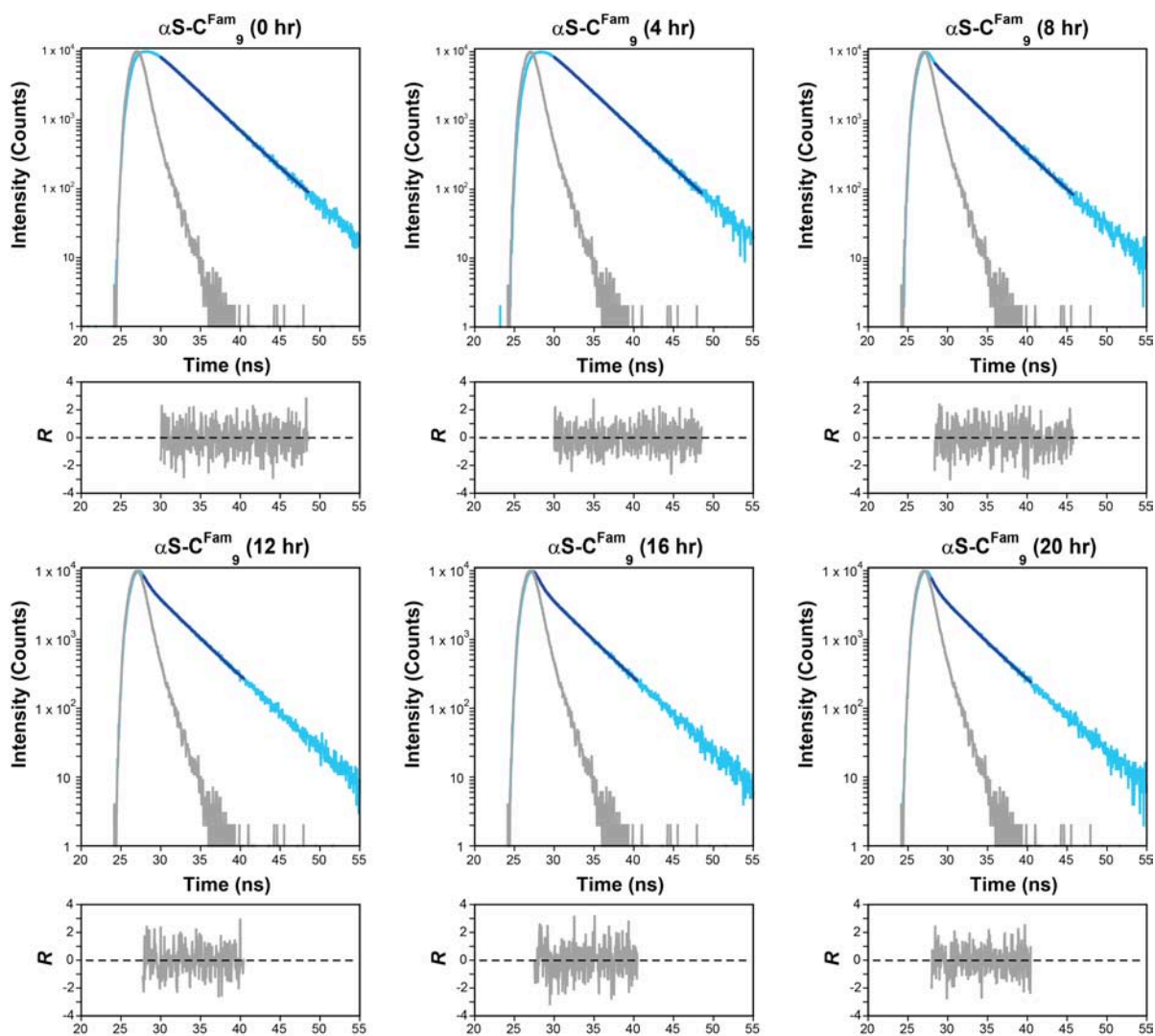
Fig. S26a. Fluorescence Lifetimes for αS-C^{Fam}₉ During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. Fits for 0 and 4 hour data are monoexponential; all other fits are bi-exponential. Weighted residuals are shown in grey below each fluorescence lifetime plot.
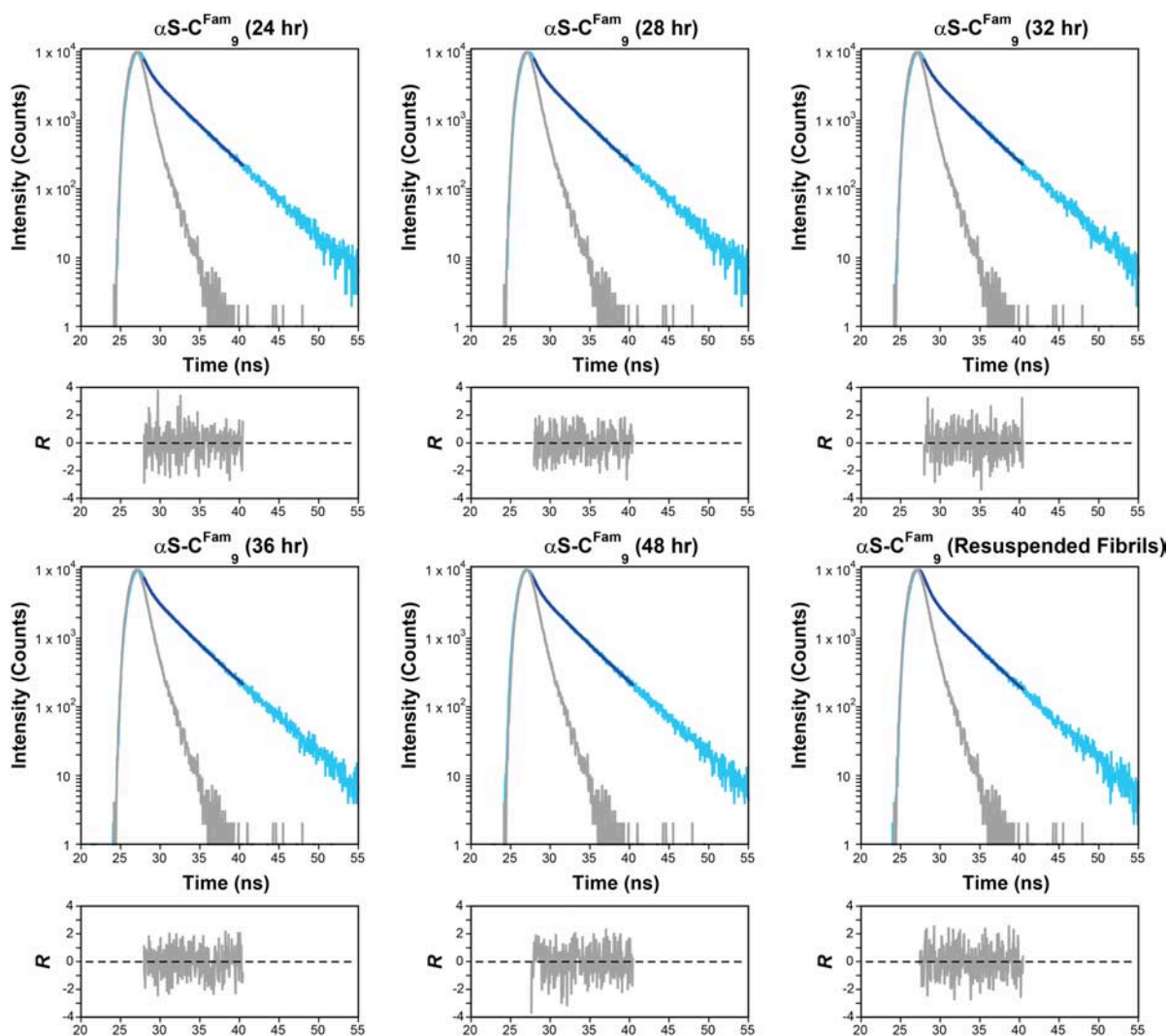
Fig. S26b. Fluorescence Lifetimes for αS-C$^{Fam}_9$ During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. Fits for 0 and 4 hour data are monoexponential; all other fits are bi-exponential. Weighted residuals are shown in grey below each fluorescence lifetime plot.
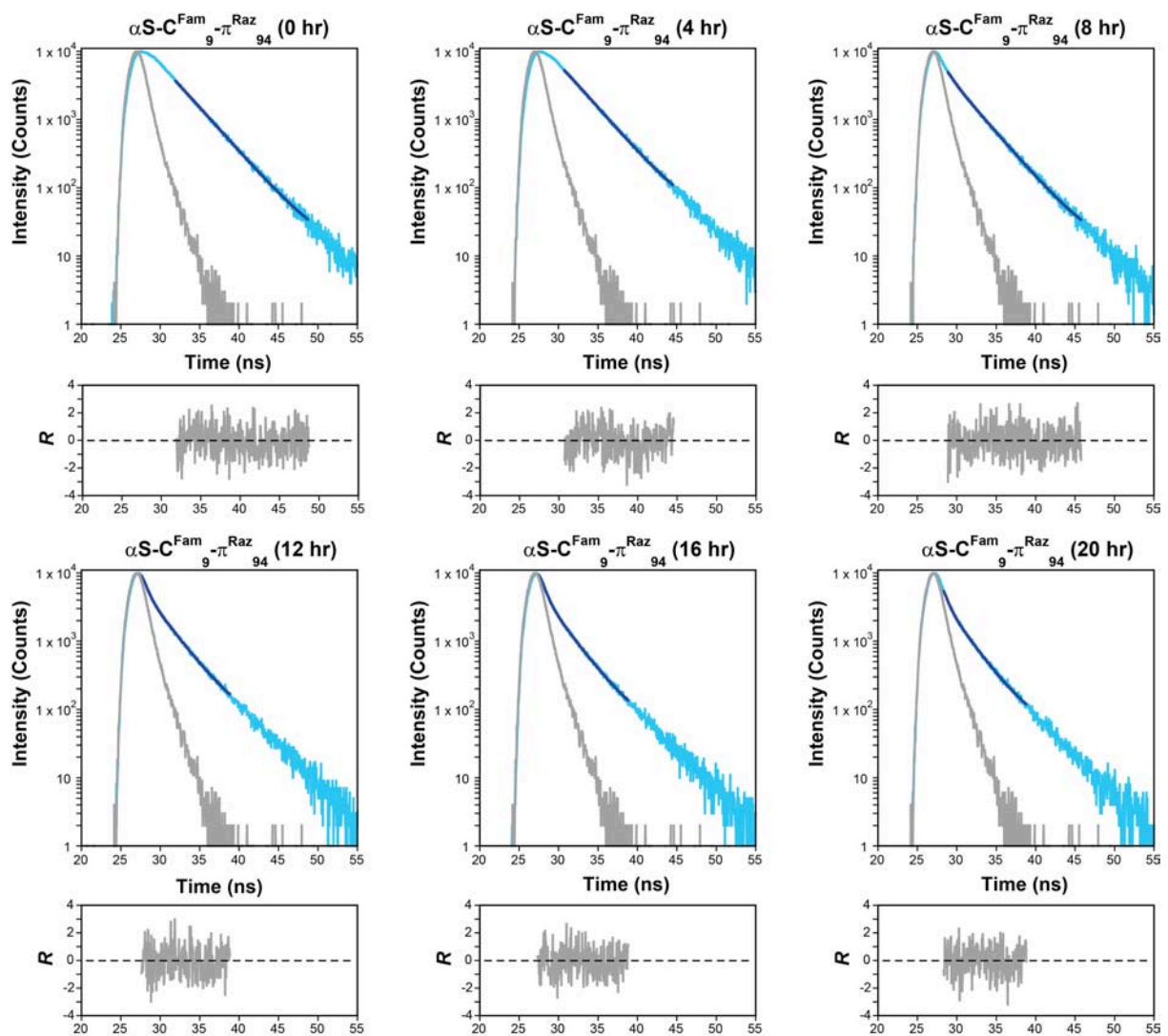
Fig. S27a. Fluorescence Lifetimes for αS-C$^{Fam}_9$-π$^{Raz}_{94}$ During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. Fits for 0 and 4 hour data are monoexponential, other fits are bi-exponential. Weighted residuals are shown in grey below each lifetime plot.
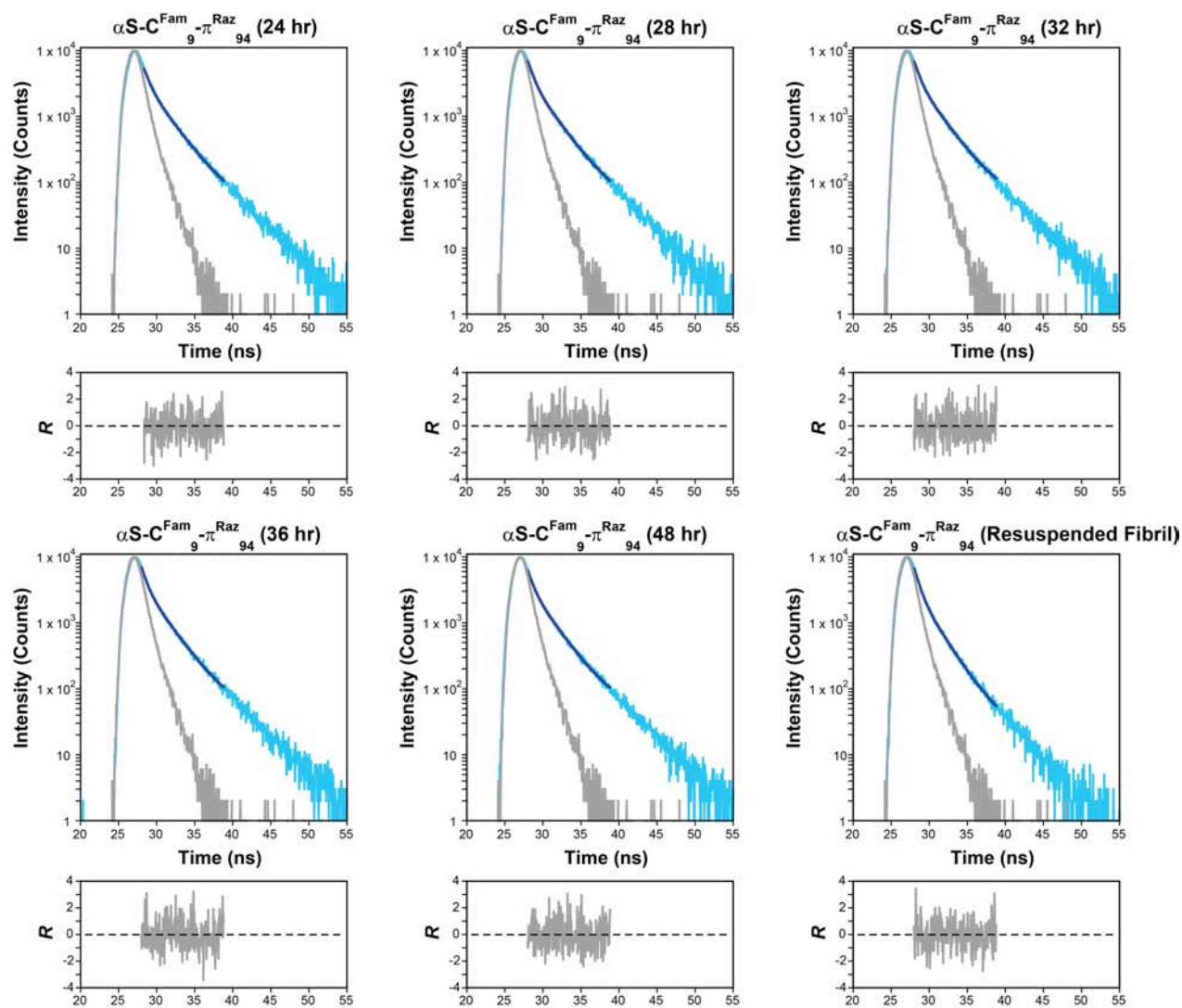
Fig. S27b. Fluorescence Lifetimes for αS-C$^{Fam}_9$-π$^{Raz}_{94}$ During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. Weighted residuals are shown in grey below each fluorescence lifetime plot.
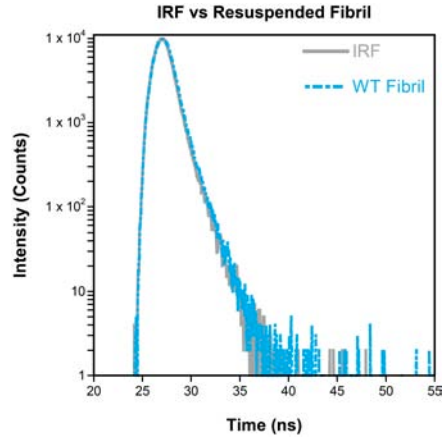
Fig. S28. Instrument Response Function (IRF) and Wild-type α**S Fibril TCSPC Data.** The IRF was recorded based on scattering of incident light collected at 515 nm as described above for measurements on fluorophore-labeled αS. WT αS fibril was collected based on a 10x dilution of resuspended WT αS fibril into aggregation buffer using the same collection parameters.

Table S2. TCSPC Fitting Data for α**S-C**$^{Fam}_9$ **and** α**S-C**$^{Fam}_9$**-**π^{Raz}_{94}**.**

αS-C$^{Fam}_9$		
Time (hours)	τ_1 (ns)	τ_2 (ns)	χ^2
0	3.9	-	1.02
4	4.0	-	0.82
8	3.9	0.08	1.06
12	3.8	0.02	0.98
16	3.9	0.02	1.16
20	3.8	0.03	0.95
24	3.9	0.06	1.06
28	3.8	0.03	0.91
32	3.8	0.04	1.15
36	3.8	0.05	1.03
48	3.8	0.01	1.19
Resuspended	3.7	0.07	1.14

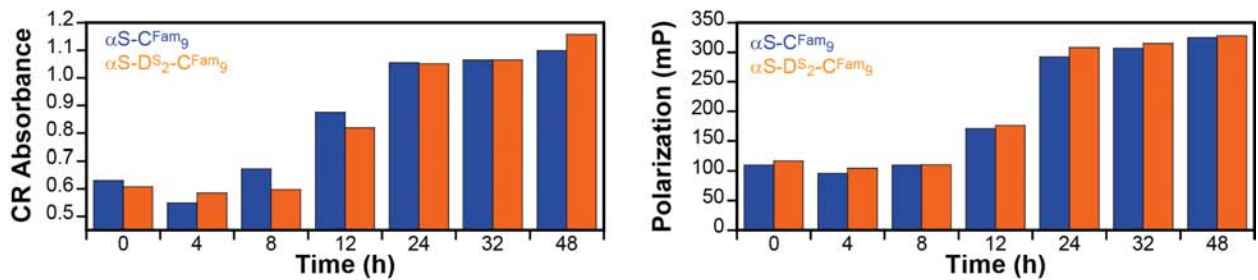| αS-C$^{Fam}_9$-π^{Raz}_{94}		
Time (hours)	τ_1 (ns)	τ_2 (ns)	χ^2
0	3.3	-	1.04
4	3.3	-	1.06
8	3.0	0.60	1.09
12	2.9	0.06	1.13
16	2.9	0.09	0.98
20	2.8	0.27	1.03
24	2.8	0.32	1.00
28	2.7	0.19	1.11
32	2.7	0.19	1.09
36	2.7	0.12	1.15
48	2.7	0.10	1.09
Resuspended	2.5	0.18	1.12

Figure S29. Aggregation analysis of αS-C$^{Fam}_9$ and αS-D$^S_2$-C$^{Fam}_9$. Fibrils formed from 1% aS-CFam9 or 1% aS-DS2-CFam9 in 99% wild type background. Aggregation was monitored by the ratio of Congo Red (CR) absorbance at 540nm/480 nm (left figure) and by fluorescence polarization (in mP value, right figure).
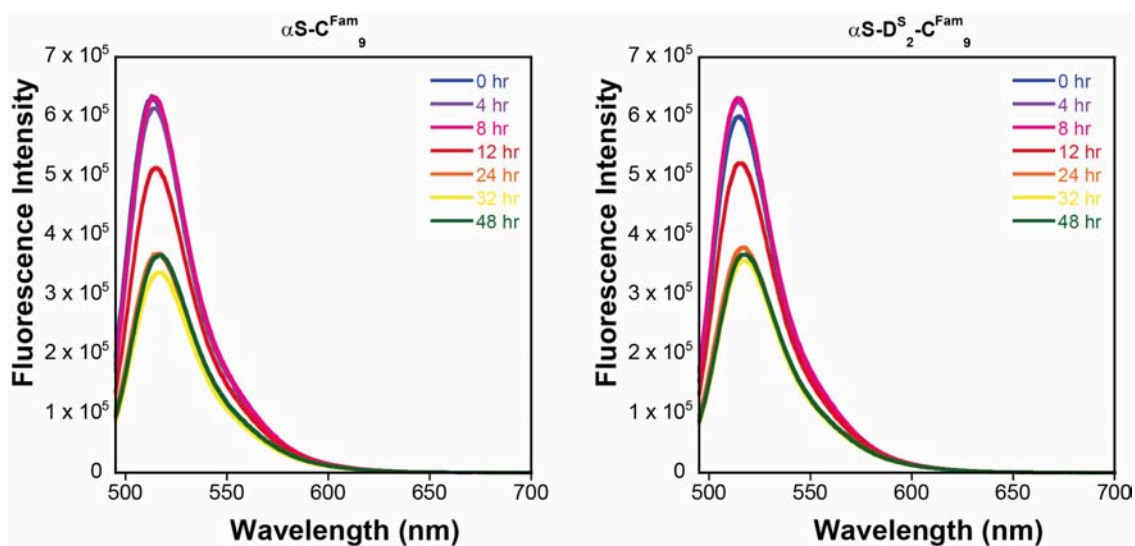

Figure S30. Steady-State Fluorescence Data from αS-C$^{Fam}_9$ and αS-D$^S_2$-C$^{Fam}_9$ Aggregation. Left: Steady state fluorescence data for αS-C$^{Fam}_9$ during the course of aggregation (0-48 hr) with excitation at 485 nm. Right: Steady state fluorescence data for αS-D$^S_2$-C$^{Fam}_9$ during the course of aggregation (0-48 hr) with excitation at 485 nm.
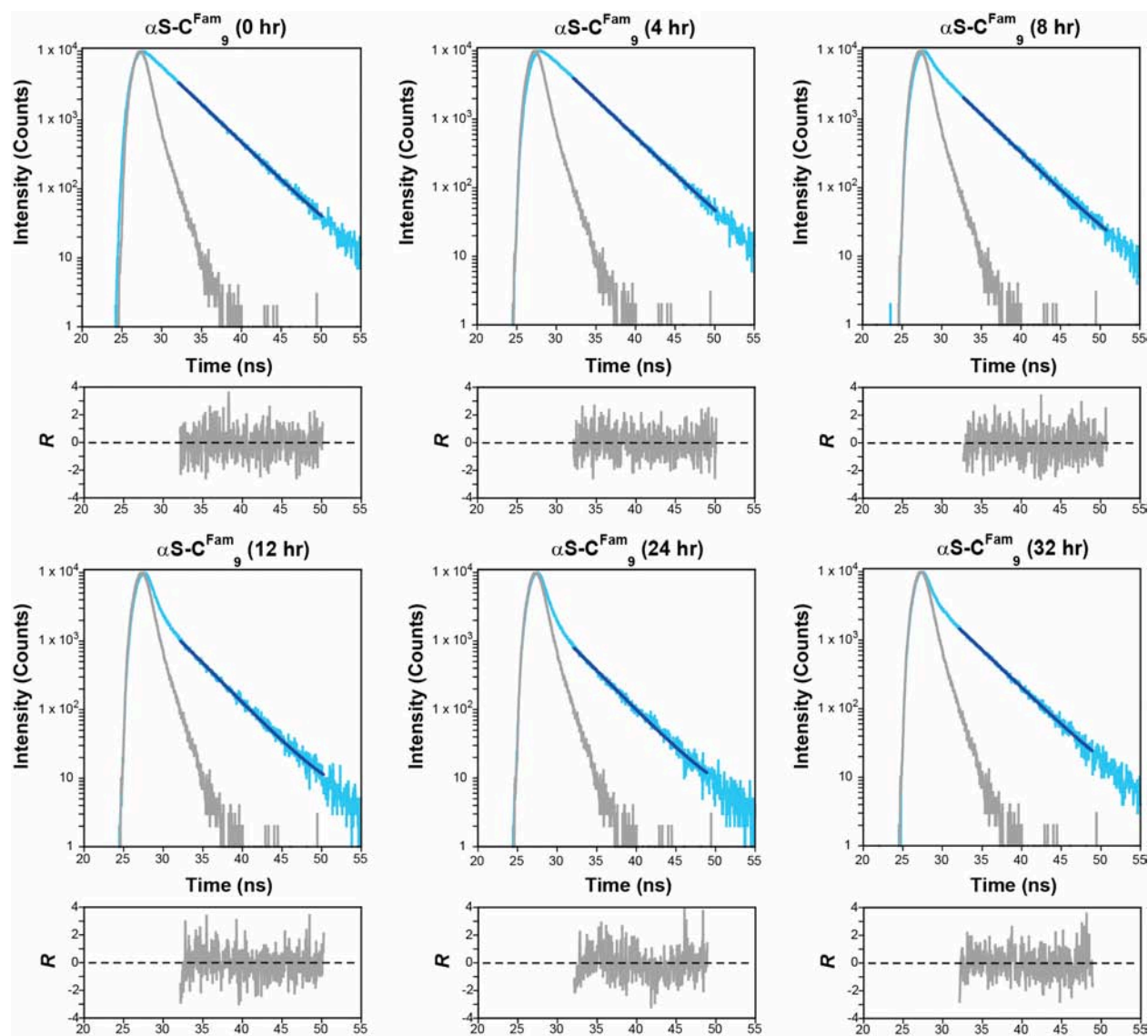
Figure S31a. Fluorescence Lifetimes for αS-C^{Fam}9 During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. All fits are monoexponential. Weighted residuals are shown in grey below each fluorescence lifetime plot.

Figure S31b. Fluorescence Lifetimes for αS-C^Fam_9 During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. All fits are monoexponential. Weighted residuals are shown in grey below each fluorescence lifetime plot.
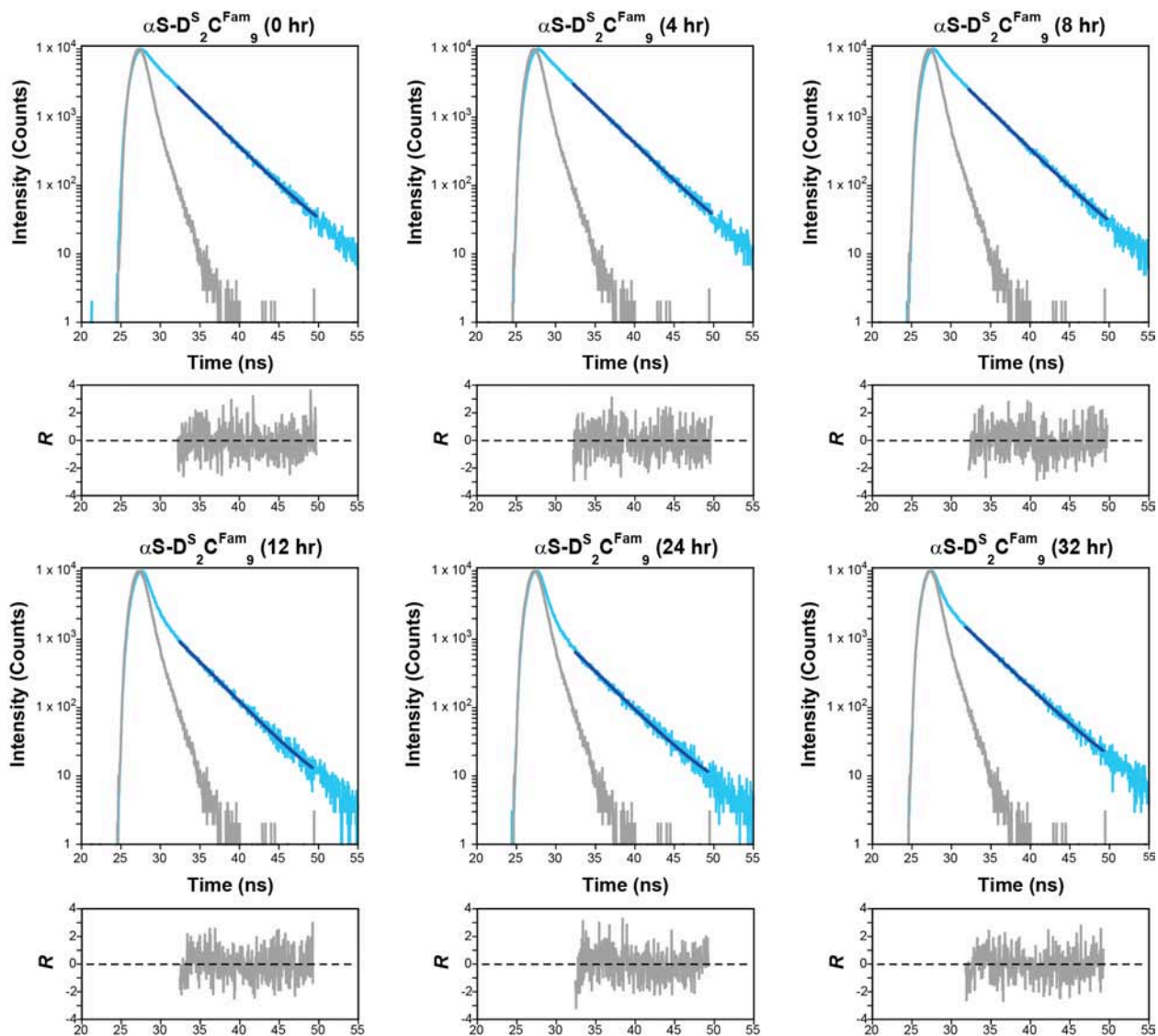
Figure S32a. Fluorescence Lifetimes for αS-D$^S_2$-C$^{Fam}_9$ During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. All fits are monoexponential. Weighted residuals are shown in grey below each fluorescence lifetime plot.
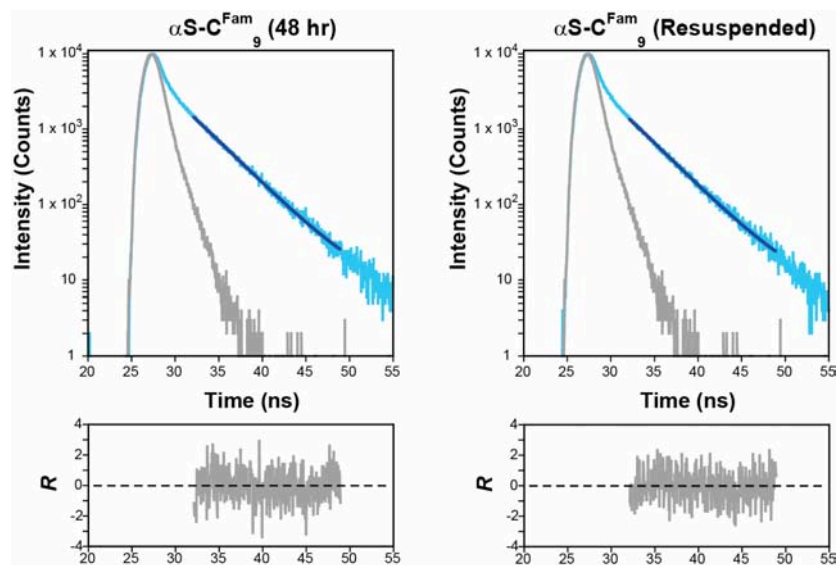
Figure S32b. Fluorescence Lifetimes for αS-C$^{Fam}_9$ During Aggregation. TCSPC data is shown in light blue; exponential fits to the data are shown in dark blue; the instrument response function (IRF) is shown in gray. All fits are monoexponential. Weighted residuals are shown in grey below each fluorescence lifetime plot.
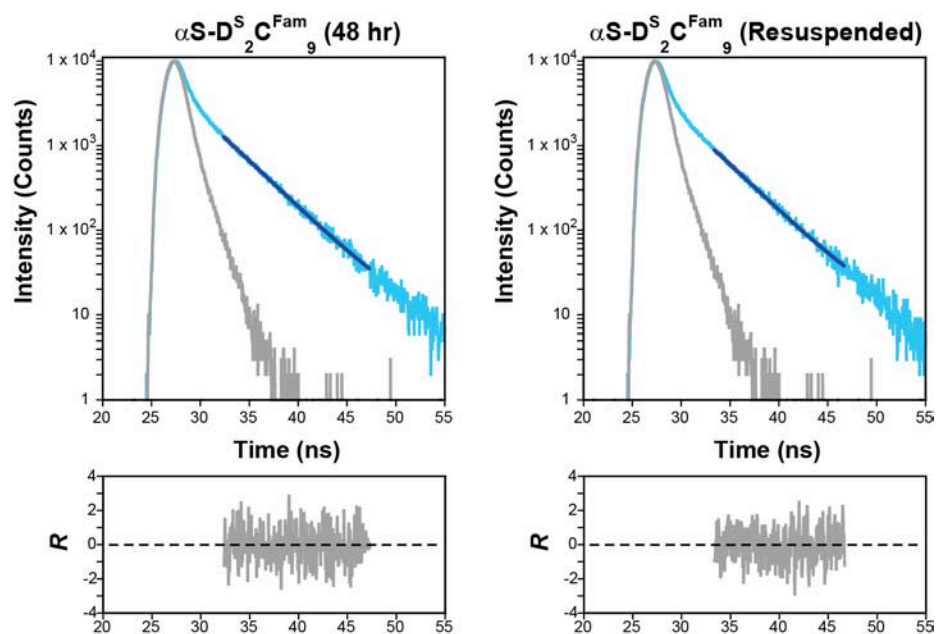
Table S3. TCSPC Fitting Data for αS-C$^{Fam}_9$ and αS-D$^S_2$-C$^{Fam}_9$.

αS-C$^{Fam}_9$		
Time (hours)	τ (ns)	χ^2
0	3.9	1.08
4	3.9	0.99
8	3.9	1.11
12	3.7	1.12
24	3.7	1.16
32	3.9	0.97
48	3.8	1.08
Resuspended	3.8	1.03
αS-D$^S_2$C$^{Fam}_9$		
Time (hours)	τ (ns)	χ^2
0	3.8	1.13
4	3.9	1.10
8	3.8	1.09
12	3.7	1.12
24	3.8	1.14
32	3.9	1.00
48	3.9	1.04
Resuspended	3.9	1.11

Transmission Electron Microscopy (TEM). TEM was carried out on an FEI Tecnai T12 instrument with an accelerating voltage of 80 kV. Fibril samples obtained from aggregation and centrifugation were resuspended in 20 mM Tris, 100 mM NaCl pH 7.5. Carbon Formvar coated 300-mesh Cu grids were inverted over a 10 µL drop of sample and allowed to rest for 2 minutes at room temperature. After this time, excess solution was wicked off and the grid was washed 2 x 10 sec with water. Excess solution was wicked off, and the grids were stained 3 x 15 sec with 2% w/v ammonium molybdate, pH 7.8 in water. The grids were allowed to dry for 2 min at room temperature, then imaged. Images were collected at magnification ranging from 6500 x to 42000 x. TEM image analysis was performed manually using Gatan Digital Micrograph software (Gatan, Inc.; Pleasanton, CA, USA). Fibril length analysis was performed on images collected at 21000 x magnification; width analysis was performed on images collected at 42000 x magnification. For length analysis, a minimum of 100 fibrils were analyzed for each sample, and the resulting data binned by length in 25 nm increments (Figures S33 and S34). For width analysis, a minimum of 50 fibrils were analyzed for each sample, and the resulting data averaged (Table S4); only isolated fibril areas were used in width analysis.
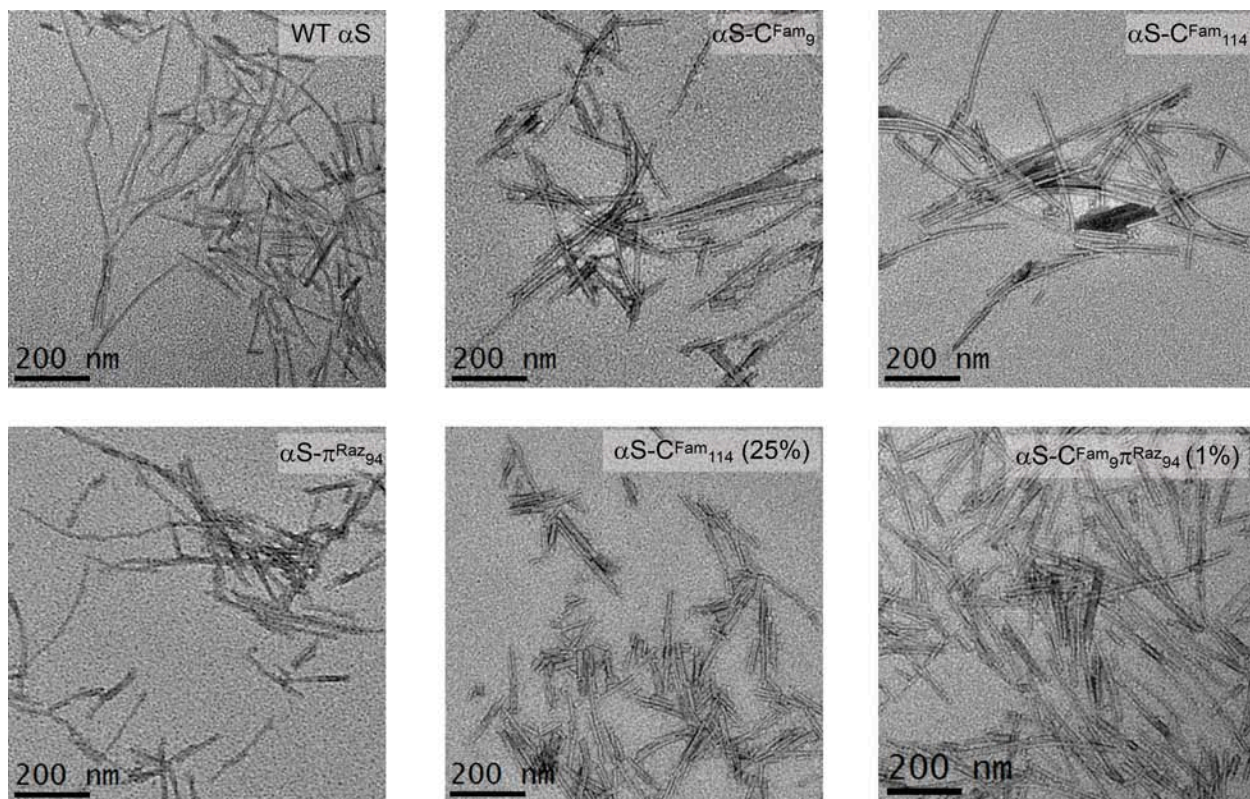
Figure S33. Representative TEM Images Used in Length Analysis. Images were collected at 21000 x magnification on a FEI Tecnai T12 electron microscope using resuspended fibril samples and grids prepared as described above.

Figure S34. Fibril Length Distribution of Labeled αS Variants. Lengths of aS fibrils were measured following 48 hrs of aggregation using either 100% WT protein (top left), 95% WT and 5% labeled protein, or percentages indicated above the graph.

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Table S4. Average Fibril Width from N = 50 Fibrils.
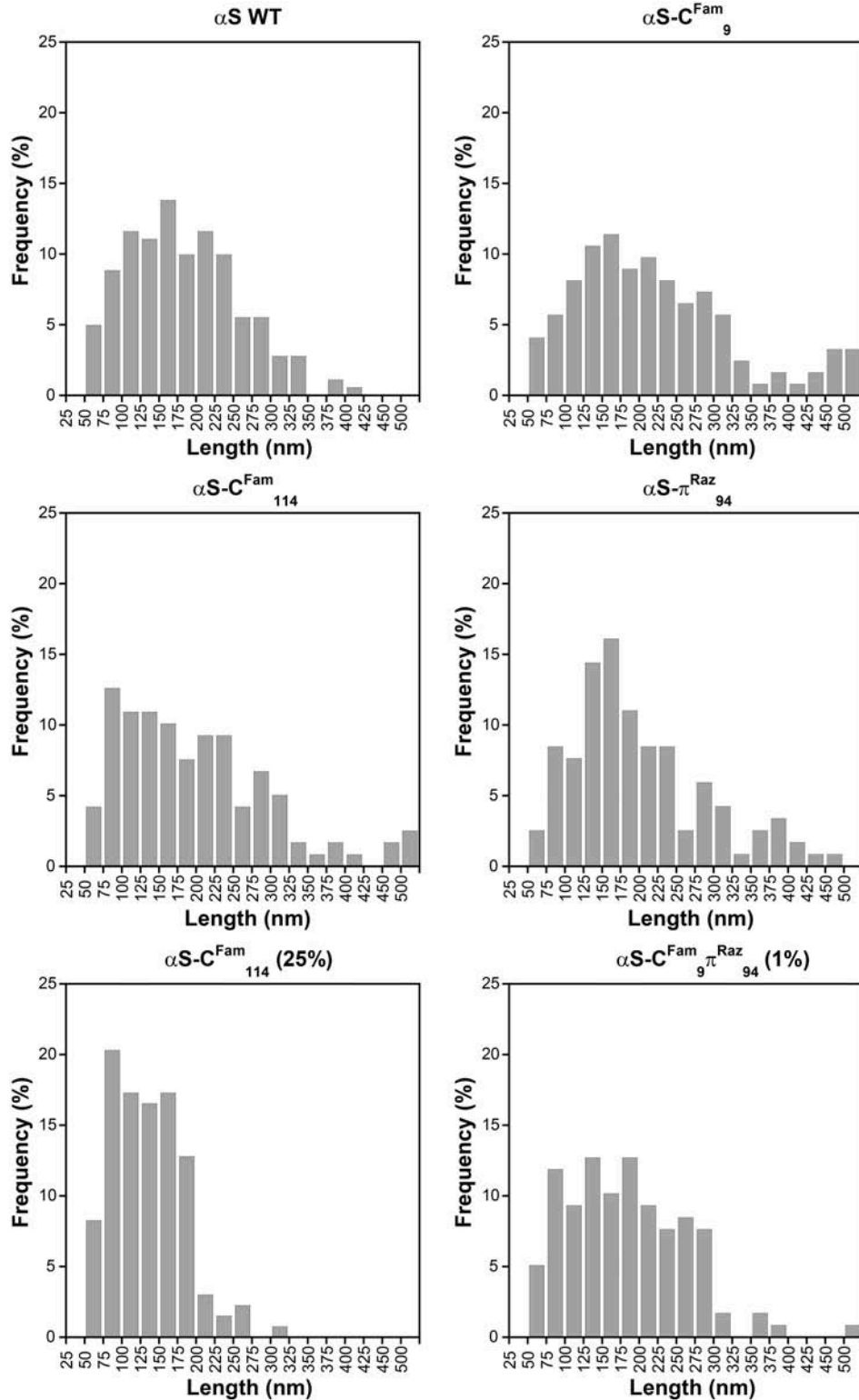
Fibril Composition	Width (nm) ± σ
WT αS	11.5 ± 1.7
5% αS-C$^{Fam}_9$	11.9 ± 1.6
5% αS-C$^{Fam}_{114}$	11.4 ± 1.6
5% αS-π$^{Raz}_{94}$	11.3 ± 1.7
25% αS-C$^{Fam}_{114}$	10.8 ± 1.2
1% αS- C$^{Fam}_9$π$^{Raz}_{94}$	10.9 ± 1.3

Primary Neuronal Cultures and Fibril Transduction for Fibril Internalization Assay.
Primary neuronal cultures were prepared from E15-E17 embryos of CD1 mice (Charles River Laboratories; Raleigh, NC, USA). All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Dissociated hippocampal neurons were plated onto poly-D-lysine coated 13mm coverslips in a 24 well plate at 100,000 cells/coverslip and allowed to mature for 7 d. Pre-formed fibrils (PFFs) were generated by diluting recombinant α-syn to 2.5 mg/ml in Dulbecco's PBS (Dulbecco's Phosphate Buffered Saline –Ca –Mg; Cellgro, Mediatech Inc. A Corning subsidiary, Manassas, VA) and incubating at 37°C with constant agitation at 1,000 rpm for 7 days in an Eppendorf Thermomixer C^{TM} with ThermoTop^{TM}. (Eppendorf North America, Hauppage, NY, USA). PFF transduction was performed at 7 d *in vitro*, whereby αS PFFs composed of 100% WT αS, 5% αS-C$^{Fam}_{114}$ + 75% WT αS, or 25% αS-C$^{Fam}_{114}$ + 75% WT αS were diluted to 2.3 μM (33.3 μg/ml) in PBS and sonicated for 10 min on the high setting with a Diagenode Biorupter™ (30s on, 30s off, 10 °C bath temp). Neurons were then treated with PBS or sonicated PFFs to give final αS concentrations of 69.2 nM (0.5 μg αS/coverslip) and returned to a 37 °C incubator for 6 h.

Immunocytochemistry of Internalized Fibrils. Internalization studies were performed as previously reported.[12] Briefly, neurons were fixed with pre-warmed 4% paraformaldehyde (PFA)

in PBS containing 4% sucrose for 15 min. The cells were washed five times with PBS and incubated with blocking buffer (3% BSA containing, 0.5% saponin in PBS) for at least 1 h at room temperature. Coverslips transduced with WT αS fibrils were incubated with antibodies recognizing LAMP1 (Abcam; Cambridge, MA, USA, ab24170, 1:5000) and human WT αS (Syn204, 1:500) diluted in blocking buffer overnight at 4 °C. Coverslips transduced with 5% αS-C^{Fam}_{114} + 75% WT αS or 25% αS-C^{Fam}_{114} + 75% WT αS fibrils were incubated with an antibody recognizing LAMP1 diluted in blocking buffer overnight at 4 °C. All coverslips were then washed 5 x 5 min with PBS, followed by staining with appropriate (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA, A21131 and A11037) Goat-anti-Rabbit Alexa Fluor© 594 secondary antibody for all coverslips and Goat-anti-Mouse IgG$_{2a}$ 488-conjugated secondary antibody for only the WT αS fibril transduced coverslips diluted in blocking buffer for 2 h at room temperature. Coverslips were then washed (5 x 5 min), briefly air dried, and mounted on to glass slides with Fluoromount G™ with DAPI and allowed to dry overnight, in the dark before imaging. For quantification, 20 – 50 cells per coverslip were randomly chosen by an investigator who was blinded to treatment conditions. Each experiment was done in triplicate, and repeated two times. 20-50 0.2 μm Z-stack 100X images per cell were obtained using a Leica DMI6000 microscope (Leica Microsystems Inc., IL, USA). Internalized or intracellular fibrils were defined as those which were positive for Syn204 or Fam and in the same plane as or in close proximity to LAMP1 inside the cell body. Co-localization of Syn204-positive or Fam-positive puncta with LAMP1 was noted on a per cell basis. Any Syn204-positive or Fam-positive PFFs near the membrane that spanned all planes in the Z-stack were excluded. The number of neurons positive for intracellular αS puncta and the number of cells with LAMP1 co-localizing intracellular αS puncta were counted and the percentage of cells with intracellular fibrils and the

percentage of cells with LAMP1 co-localizing intracellular fibrils was computed (Fig. S35). Representative images taken on an Olympus IX 81 microscope equipped with a Yokogawa CSU X1 spinning disc confocal scan head using a 100x oil immersion objective (Olympus America, Inc., Center Valley, PA, USA) for WT and 25% αS-C^{Fam}_{114} + 75% WT αS are shown in Figure 7 in the main text. Fam fluorescence from 5% αS-C^{Fam}_{114} + 95% WT αS samples was too dim for useful imaging.
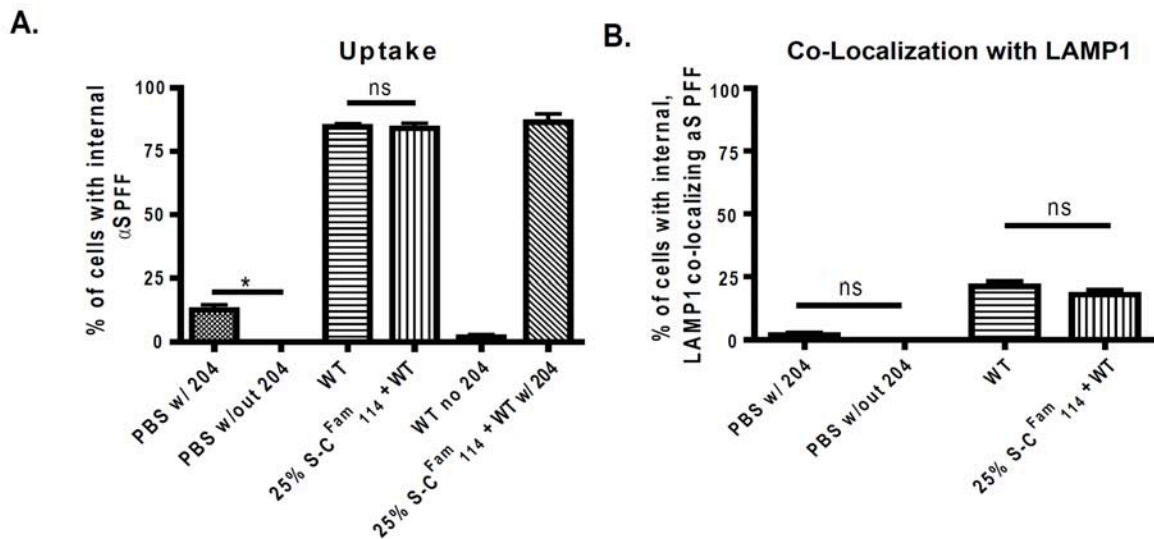

Figure S35. Quantification of αS PFF Internalization. **A.** Treatment of non-transgenic mouse primary hippocampal neurons with preformed WT or 25% αS-C^{Fam}_{114} + WT aS fibrils for 6 hr reveals that incorporation of the dye labeled monomer has no effect on the extent of fibril uptake and provides sufficient signal for study without the need for a secondary antibody labeled with fluorophore (WT vs 25% αS-C^{Fam}_{114} + WT vs 25% αS-C^{Fam}_{114} + WT w/ 204). Furthermore, leaving out the fluorophore labeled secondary antibody improves the signal-to-noise in this experiment (PBS w/ 204 vs PBS w/out 204) bar = SEM, * = p<0.05 **B.** Treatment of non-transgenic mouse primary hippocampal neurons with preformed WT or 25% αS-C^{Fam}_{114} + WT aS fibrils for 6 hr reveals that incorporation of the dye labeled monomer has no effect on the internal localization of fibrils as measured by co-localization with LAMP1 (WT vs 25% αS-C^{Fam}_{114} + WT). bar = SEM.

Primary Neuronal Cultures and Fibril Transduction for Effective Potency Determination. Primary neuronal cultures were prepared from E15-E17 embryos of CD1 mice (Charles River Laboratories; Raleigh, NC, USA). All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the

University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Dissociated hippocampal neurons were plated onto poly-D-lysine coated 13mm coverslips in a 24 well plate at 100,000 cells/coverslip and allowed to mature for 10 d. Pre-formed fibrils (PFFs) were generated by diluting recombinant α-syn to 2.5 mg/ml in PBS and incubating at 37°C with constant agitation at 1,000 rpm for 7 days in an Eppendorf Thermomixer C^{TM} with ThermoTop^{TM}. PFF transduction was performed at 10 d *in vitro*, whereby αS PFFs formed from WT, 5% αS-C$^{FAM}_{114}$ + WT, or 25% αS-C$^{FAM}_{114}$ + WT, were diluted to 2.3 μM or 0.23 μM (33.3 μg/ml or 3.3 μg/ml) in PBS and sonicated for 5 min on the high setting with a Diagenode Biorupter™ (30s on, 30s off, 10°C bath temp). In total, 3 samples of each dilution were independently prepared. Neurons were then treated with PBS or sonicated PFFs to give final αS concentrations of 0, 9.4, 17.3, 34.6, 69.2, 138, and 277 nM. Three coverslips were transduced at each concentration. Every 4 d post-transduction, ~30% of the media was gently aspirated from each well and replaced with ~200 uL of freshly prepared, pre-warmed neuronal media. Transduced neurons were harvested for immunocytochemistry at 10 or 18 d post transduction (20 or 28 DIV).

Immunocytochemistry of p-syn Inclusions Resulting from Fibril Transduction. For comparison of aggregation seeding potencies of fibrils formed from 5% αS-C$^{Fam}_{114}$ + WT with fibrils formed from WT αS, neuronal medium was aspirated form each coverslip and neurons were incubated with pre-warmed 4% paraformaldehyde, 4% sucrose, and 1% Triton X-100 to extract all soluble proteins 18 d after PFF transduction. For comparison of 25% αS-C$^{Fam}_{114}$ + WT PFFs with WT αS PFFs, neurons were fixed as described 10 d after transduction. Coverslips were washed 5 times with PBS and blocked in 3% BSA, 3% FBS in PBS for 1 h. Neurons were then incubated in blocking buffer containing primary antibodies for phosphoserine129 αS (81A,

1:5000, Mse mAb)[6] and total synuclein (SynA, Rab, 1:1000, pAb)[5] overnight, followed by washing with PBS (5x) and 2 h incubation with blocking buffer containing Alexa-Fluor conjugated secondary antibodies (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA, A-11032 and A-11034, 1:1000). Coverslips were then mounted on to glass slides with Fluoromount G™ with DAPI and scanned on a Lamina™ slide scanner (PerkinElmer, Inc., Santa Clara, CA, USA). Quantification was performed using HALO™ software (Indica Labs, Inc., Corrales, NM, USA) ((area occupied x average intensity)/DAPI count) and data is reported as an average of three coverslips. Graphing was performed with Prism 4™ (GraphPad Software, Inc., La Jolla, CA, USA). Curve-fitting of the data in PRISM generated EC_{50} values of 13.1 nM for WT fibrils and 14.6 nM for 5% αS-C$^{Fam}_{114}$ fibrils 18 days post transduction. For comparison of WT fibrils and 25% αS-C$^{Fam}_{114}$ fibrils 10 days pot-transduction, curve-fitting of the data in PRISM generated EC_{50} values of 55.6 nM for WT fibrils and 45.6 nM for 25% αS-C$^{Fam}_{114}$ fibrils. Data and images for insoluble, hyperphosphorylated αS induced by PFFs composed of WT and 5% αS-C$^{FAM}_{114}$ + WT are shown in Figure S36. Data and images for insoluble, hyperphosphorylated αS induced by PFFs composed of WT and 25% αS-C$^{FAM}_{114}$ + WT are shown in Figure S37.

Determination of aggregation seeding capacity of internalized 25% αS-C$^{Fam}_{114}$ PFFs. To determine the aggregation seeding capacity of 25% αS-C$^{Fam}_{114}$ PFFs in comparison to hWT αS in primary neurons, hippocampal CD1 neurons were transduced 10 d *in vitro* with 3 independently prepared fibril suspensions as above (0.5 μg αS per coverslip, 69.2 nM). Cells were fixed 14 d after treatment with a pre-warmed solution of 4% paraformaldehyde and 4% sucrose in DPBS without calcium and magnesium before immunocytochemical analysis was

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performed as described above. Data and images for insoluble, hyperphosphorylated αS induced

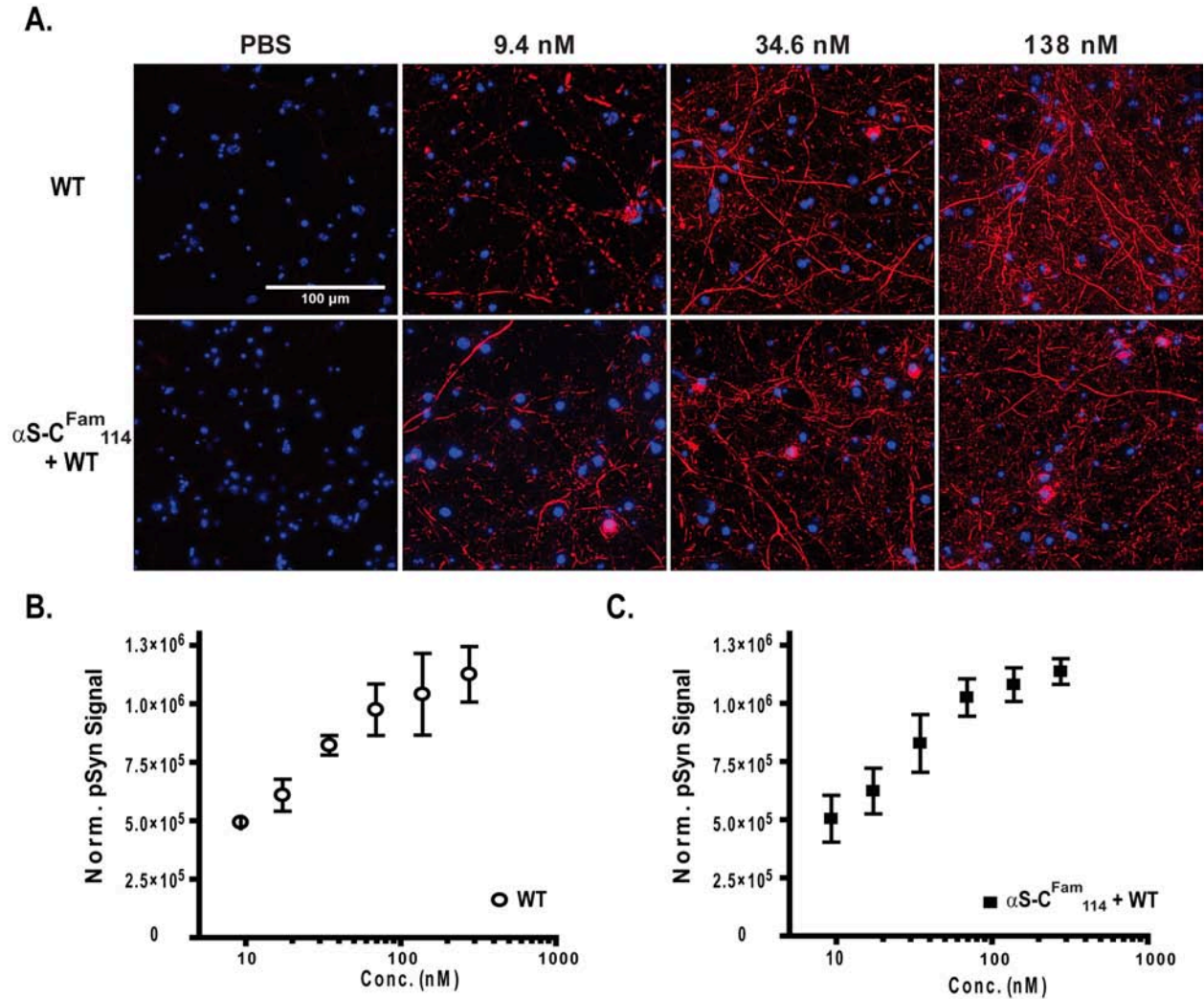by PFFs composed of WT and 25% αS -C$^{Fam}_{114}$ + WT are shown in Figure S37.

Figure S36. Determination of Aggregation Seeding Potency of Internalized 5% αS-C$^{Fam}_{114}$ PFFs. A. Treatment of non-transgenic mouse primary hippocampal neurons with αS or 5% αS-C$^{Fam}_{114}$ + WT PFFs results in progressive accumulation of Lewy body-like insoluble, phosphorylated αS (pSyn) protein, as detected by immunostaining with phosphorylated Ser129 specific (81A, red) antibody. Soluble proteins were removed during fixation. Nuclei were stained with DAPI (blue). Scale bar = 100 μm. B. Quantitation of coverslips treated with increasing amounts of WT αS PFFs reveals a dose-dependent increase in insoluble, pSyn. A sigmoidal fit of these data gave an EC$_{50}$ of 13.1 nM. bar = SEM C. Quantitation of coverslips treated with increasing amounts of 5% αS-C$^{Fam}_{114}$ + WT αS PFFs reveals a dose-dependent increase in insoluble, pSyn. A sigmoidal fit of these data gave an EC$_{50}$ of 14.6 nM. bar = SEM.
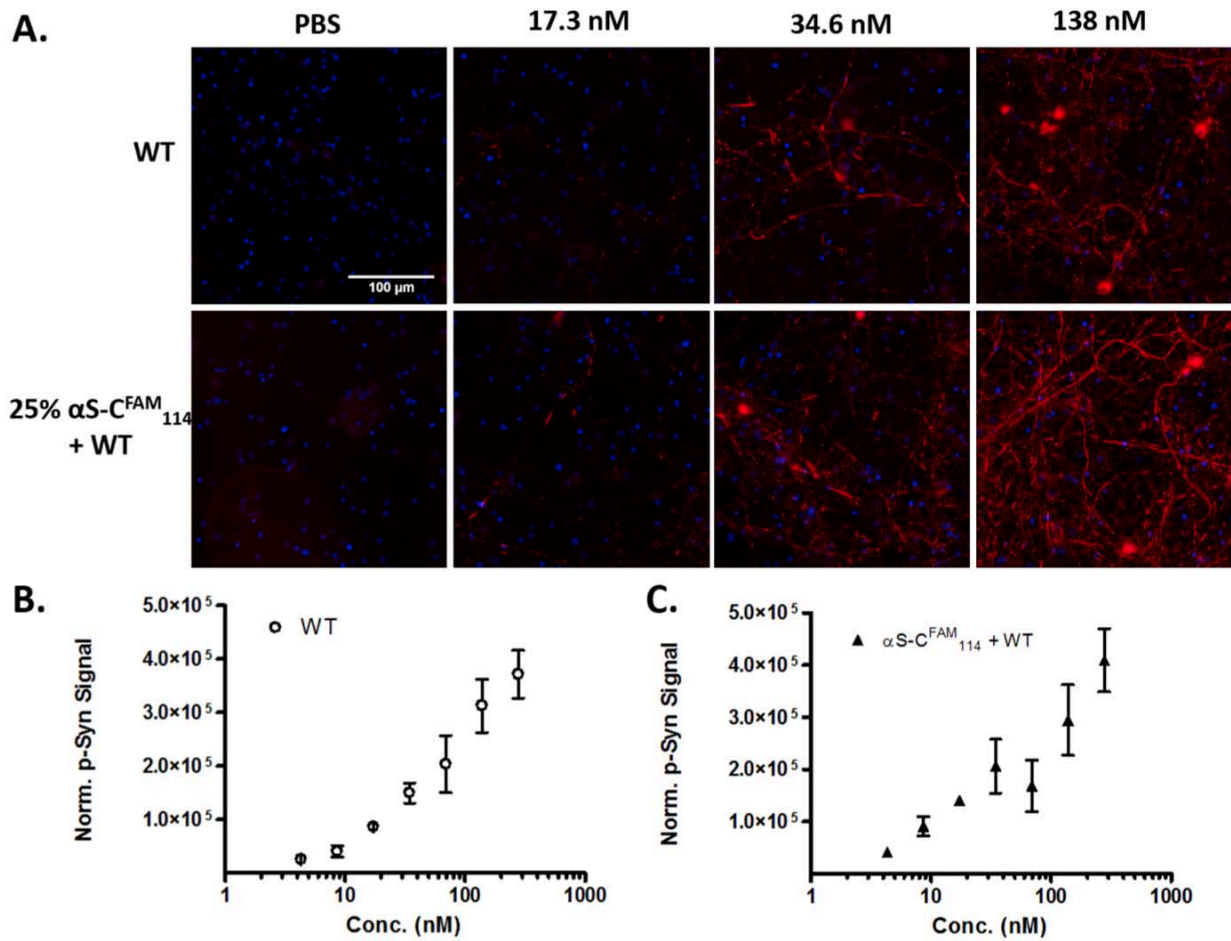
Figure S37. Comparison of Aggregation Seeding Potency of Internalized 25% αS-C^{Fam}114 PFFs and WT αS. A. Treatment of non-transgenic mouse primary hippocampal neurons with αS or 25% αS-C^{Fam}114 + WT PFFs results in progressive accumulation of Lewy body-like insoluble, phosphorylated αS (*p*Syn) protein, as detected by immunostaining with phosphorylated Ser129 specific (81A, red) antibody. Nuclei were stained with DAPI (blue). Scale bar = 100 µm. B. Quantitation of coverslips treated with increasing amounts of WT αS PFFs reveals a dose-dependent increase in insoluble, *p*Syn. A sigmoidal fit of these data gave an EC$_{50}$ of 55.6 nM. bar = SEM C. Quantitation of coverslips treated with increasing amounts of 25% αS-C^{Fam}114 + WT αS PFFs reveals a dose-dependent increase in insoluble, *p*Syn. A sigmoidal fit of these data gave an EC$_{50}$ of 45.6 nM. bar = SEM.

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