Fluorescence Spectroscopy Reveals N-terminal Order in Fibrillar Forms of α-Synuclein

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Supplementary Information

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General Information. Fluorescein-5-maleimide (Fam) was purchased from TCI America (Portland, Oregon). *E. coli* BL21(DE3) cells were purchased from Stratagene (La Jolla, CA). Sequencing-grade trypsin was purchased from Promega (Madison, WI). QuikChange® site-directed mutagenesis kits were purchased from Stratagene (currently Agilent Technologies; Santa Clara, CA). DNA oligomers were purchased from Integrated DNA Technologies, Inc (Coralville, IA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) and used as received unless otherwise specified. MilliQ filtered (18 MΩ) water was used for all solutions (Millipore; Billerica, MA). 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). Preparation of the pTXB1-αS-intein-H_6 plasmid containing αS with a C-terminal fusion to the *Mycobacterium xenopi* GyrA intein and C-terminal His_6 tag was described previously; this plasmid was used as a starting point for introduction of mutations in intein constructs.[1-2] Matrix-assisted laser desorption/ionization (MALDI) mass spectra were collected with a Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer (Billerica, MA). UV/vis absorbance spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer (currently Agilent Technologies). Gel images were obtained with a Typhoon FLA 7000 (GE Lifesciences; Princeton, NJ). Circular dichroism spectra were collected on an Aviv model 410 circular dichroism spectrometer in 1 mm path length quartz cuvettes. Congo Red absorbance spectra were collected on a Tecan M1000 plate reader (Mannedorf, Switzerland). Fluorescence polarization data were collected with a Tecan F200 plate reader. Transmission electron microscopy (TEM) images were collected on a FEI Tecnai 12 electron microscope (Hillsboro, OR).

Construction of αS Cys Mutant Expression Plasmids. A previously described pTXB1-αS_intein-H_6 plasmid containing the human wild-type αS gene with a C-terminal *Mycobacterium xenopi* GyrA intein and His_6 fusion has been previously described.[3] Prior publications have utilized the αS-Cys mutants and majority of the αS-CysPpY proteins described herein.[4-5] QuikChange mutagenesis was used to generate

pTXBI-αS-TAG_{35}_intein-H_6 plasmids using QuikChange® mutagenesis (Note: subscript denotes the position of the TAG codon within the αS sequence). The construct encoding for αS-C_9PpY_{136} was generated by introducing the S9→C mutation in the previously described αS-TAG_{136}_$MxeHis_6$ sequence using previously published primers.[4-5] The resulting sequence was confirmed by DNA sequencing analysis. DNA primers for generating mutations are shown in Figure S1.

DNA Oligomers Used for αS QuikChange® Mutagenesis.

i. Mutation E35→TAG
Forward: 5' – AGAGAACACCCTATTTTGTCTTTCCTGCTGCTTCTG – 3'
Reverse: 5' – CAGAAGCAGCAGGAAAGACAAAATAGGGTGTTCTCT – 3'

Figure S1. DNA oligomers used for αS Quikchange® mutagenesis.

Overexpression and Purification of Full-length WT αS. pRK172-αS was transformed into competent *E. coli* BL21(DE3) cells and plated on LB agar plates supplemented with ampicillin overnight at 37 °C. 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 mg/L) which was then grown overnight at 37 °C with shaking at 250 rpm. The cells were harvested by centrifugation at 4000 rpm for 20 min, and the resulting pellet was resuspended 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). The cells were lysed by sonication, boiled for 15 minutes at 100 °C, and centrifuged for 20 minutes at 14,000 rpm at 4 °C. The cleared supernatant was dialyzed against purification buffer (20 mM Tris, 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 using 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 at 4 °C against αS buffer (20 mM Tris, 100 mM NaCl, pH 7.5) overnight. Following dialysis, αS in buffer was stored at -80 °C in 1.5 mL aliquots and thawed once for aggregation experiments.

Protein Expression. Proteins containing propargyl tyrosine at residues 94 or 136, as well as all αS-CFamX constructs have been previously described.[4-5] Doubly-labeled αS-$C^{Fam}{}_X PpY^{Tmr}{}_Y$ proteins, as well as the corresponding donor- or acceptor-only proteins were expressed, labelled, and purified as previously described.[4] αS-$C^{Pyr}{}_X$ proteins were prepared by transformation of the αS-Cys_X-MxeHis6 plasmid into BL21(DE3) cells via heat shock and plated on LB/Ampicillin plates overnight at 37 °C. Single colonies (3-5 per mutant) were selected and grown in LB media containing 100 μg/mL ampicillin at 37 °C, 250 rpm shaking for 5-6 hours. Following this time, a single culture was inoculated into 1 L LB media containing 100 mg/L ampicillin and grown at 37 °C, 250 rpm shaking until OD600 reached 0.7-1.0. The incubator temperature was then lowered to 18 C and protein expression was induced by addition of isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated at 18 °C overnight, then cells were harvested by centrifugation using a Sorvall SC5 centrifuge equipped with a GS3 rotor operated at 4000 rpm for 20 min. The supernatant was decanted and the resulting cell pellet resuspended in 20 mL 40 mM Tris pH 8.3 supplemented with a protease inhibitor cocktail pill (Roche cOmplete, catalogue #04693159001) and 1 mM phenylmethanesulfonyl fluoride (PMSF). The resuspended cells were lysed by sonication, then centrifuged in a Sorvall SC5 centrifuge equipped with an SS34 rotor operated at 14000 rpm for 25 min. Following this time, the supernatant was decanted and incubated with a 3 mL bed volume of Ni-NTA resin (Gold Bio, catalogue #H-350-50) for 1 hour on ice. The liquid was then allowed to flow through, and the resin was washed with 15 mL 50 mM HEPES pH 7.5, then with 20 mL 50 mM HEPES, 5 mM imidazole pH 7.5. Following these wash steps, protein was eluted by three washes consisting of 4 mL each of 50 mM HEPES, 300 mM imidazole pH 7.5. The fractions were combined and treated with β-mercaptoethanol (200 mM final concentration) overnight at room temperature. Following

this treatment, the protein was dialyzed into 20 mM Tris pH 8 at 4 °C for 6-8 hours. After dialysis, the protein solution was incubated with a 3 mL bed volume of Ni-NTA resin for 1 hour on ice, then the flow through collected and dialyzed overnight at 4 °C into 20 mM Tris pH 8. The protein was subsequently labelled as described below.

Protein Labeling. Labeling of Fam and Tmr proteins has been previously described.[1, 5-6] αS-C^{Pyr}_{X} proteins were labelled by determination of protein concentration using UV-Vis (ε_{280} = 5120 M^{-1} cm^{-1} except for αS-Cys_{136}, ε_{280} = 3840 M^{-1} cm^{-1}).[7] The protein was then treated with BondBreaker TCEP solution (ThermoFisher, catalogue #77720; 1 mM final concentration) at room temperature for 10-15 minutes. Following this time, pyrene-1-maleimide (5 molar equivalents) was added from a 25 mM stock in DMSO and the reaction incubated at 37 °C until completion as determined by MALDI-TOF MS. The protein was then centrifuged for 10 minutes at 4000 rpm to pellet insoluble dye, then dialyzed overnight into 20 mM Tris pH 8 at 4 °C. Following dialysis, labelled proteins were purified by FPLC using an AKTA FPLC system and GE HiTrap Q HP column (catalogue #17-1154-01) and gradients between 20 mM Tris pH 8 and 20 mM Tris, 1 M NaCl pH 8. Fractions containing the desired protein were identified by MALDI-TOF MS, pooled, and concentrated using Amicon Ultra 3 kDa cutoff filters (EMD catalogue #UFC900324) to a final volume of ~1 mL. This material was further purified by RP-HPLC on a Varian ProStar system equipped with a Vydac C4 semi-preparative column using gradients between 0.1% TFA in water and 0.1% TFA in acetonitrile. Fractions containing the desired protein were pooled, diluted ~5-fold with αS buffer (20 mM Tris, 100 mM NaCl pH 7.5) and concentrated using Amicon Ultra 3 kDa cutoff filters; following the first concentration, protein was re-diluted and concentrated twice more to remove residual acetonitrile. Following concentration to ~0.5 mL total volume, proteins were stored at -80 °C and thawed only once per assay.

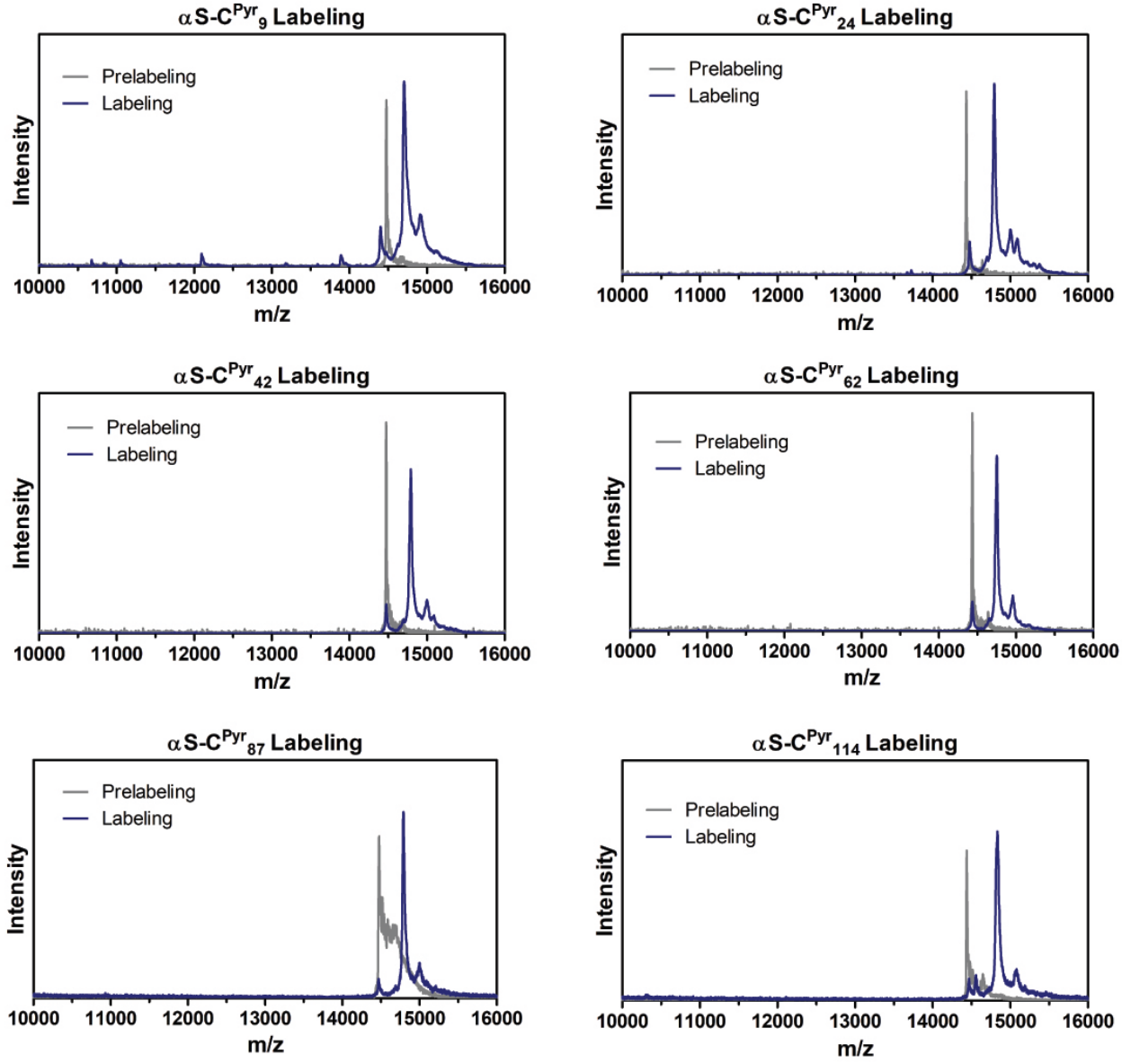

Figure S2a. MALDI-TOF MS of Protein Labeling Reactions. In each case the identity of the protein is indicated above the graph. Gray: Protein prior to labeling. Blue: Crude labeling reaction.

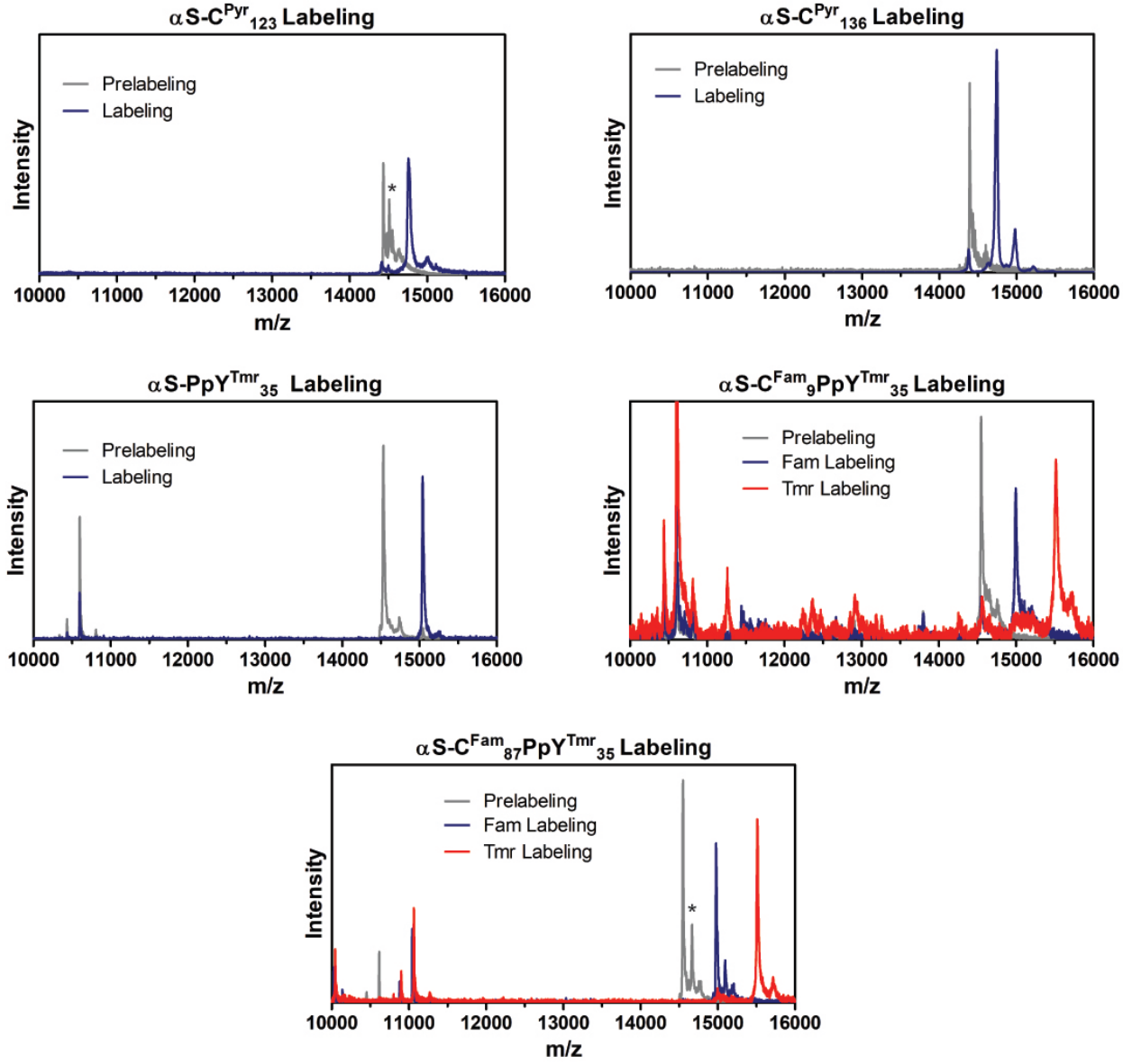

Figure S2b. MALDI-TOF MS of Protein Labeling Reactions. In each case the identity of the protein is indicated above the graph. Gray: Protein prior to labeling. Blue or red: Crude labeling reaction. Asterisk (*) indicates the presence of the βME adduct (M+78 Da), if observed.

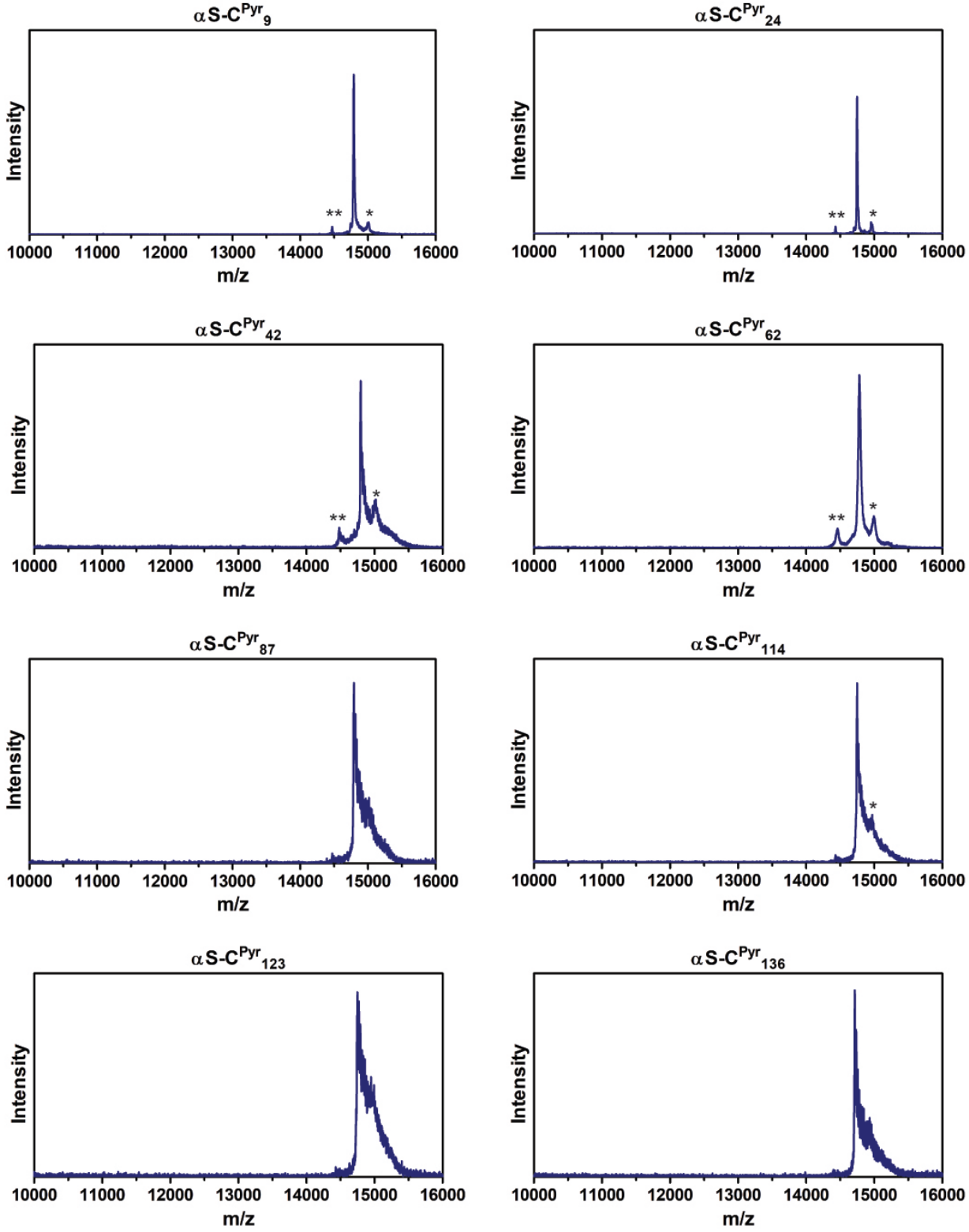

Figure S3a. MALDI-TOF MS Spectra of Full Length Labeled Proteins. The identity of the protein is indicated above each plot. Note: Asterisk (*) indicates a known matrix adduct of αS (+207 Da); double asterisk (**) indicates unlabeled protein.

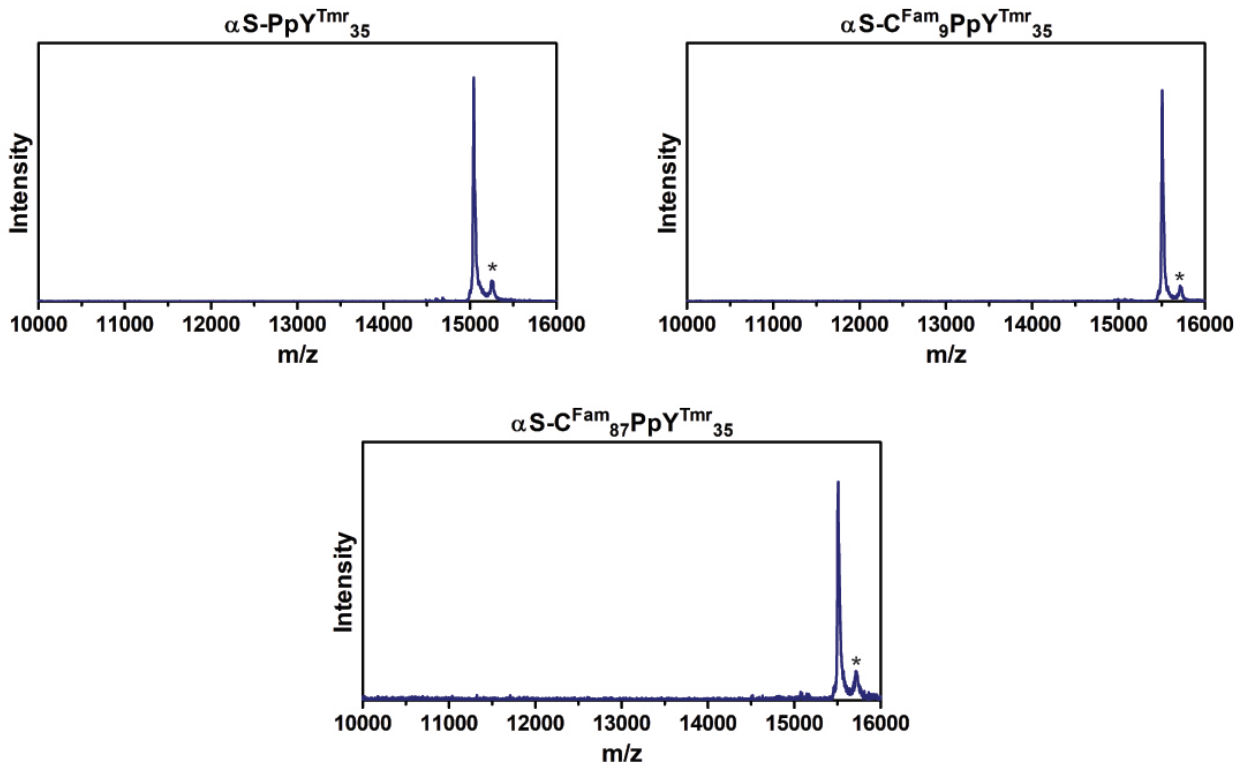

Figure S3b. MALDI-TOF MS Spectra of Full Length Labeled Proteins. The identity of the protein is indicated above each plot. Note: Asterisk (*) indicates a known matrix adduct of αS (+207 Da).

Table S1. Calculated and Observed Masses of Full Length Proteins.

Protein	$[M+H]^+$ Calc.	$[M+H]^+$ Obsd.
αS-C$^{Pyr}_{9}$	14791	14790
αS-C$^{Pyr}_{24}$	14750	14748
αS-C$^{Pyr}_{42}$	14791	14796
αS-C$^{Pyr}_{62}$	14750	14753
αS-C$^{Pyr}_{87}$	14791	14793
αS-C$^{Pyr}_{114}$	14749	14749
αS-C$^{Pyr}_{123}$	14749	14749
αS-C$^{Pyr}_{136}$	14715	14715
αS-PpY$^{Tmr}_{35}$	15042	15043
αS-C$^{Fam}_{9}$PpY$^{Tmr}_{35}$	15503	15507
αS-C$^{Fam}_{87}$PpY$^{Tmr}_{35}$	15503	15510

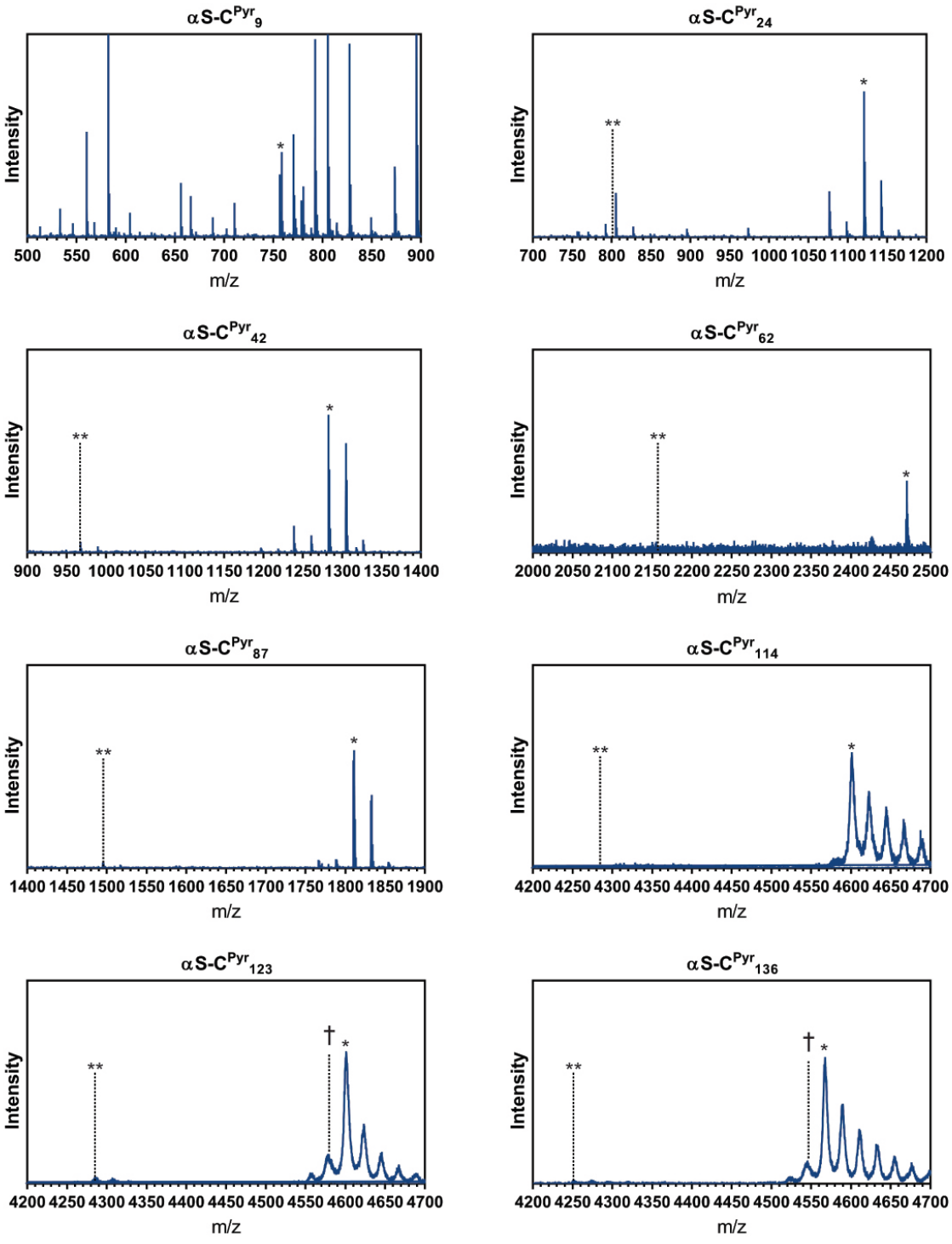

Figure S4a. MALDI-TOF MS of Trypsin Digests of Labeled Proteins. In each case the identity of the protein is indicated above the plot. Asterisk (*) indicates the position of the expected mass for the labeled fragment; double asterisk (**) indicates the position of the unlabeled fragment. Dagger (†) indicates the position of the $[M+H]^+$ mass.

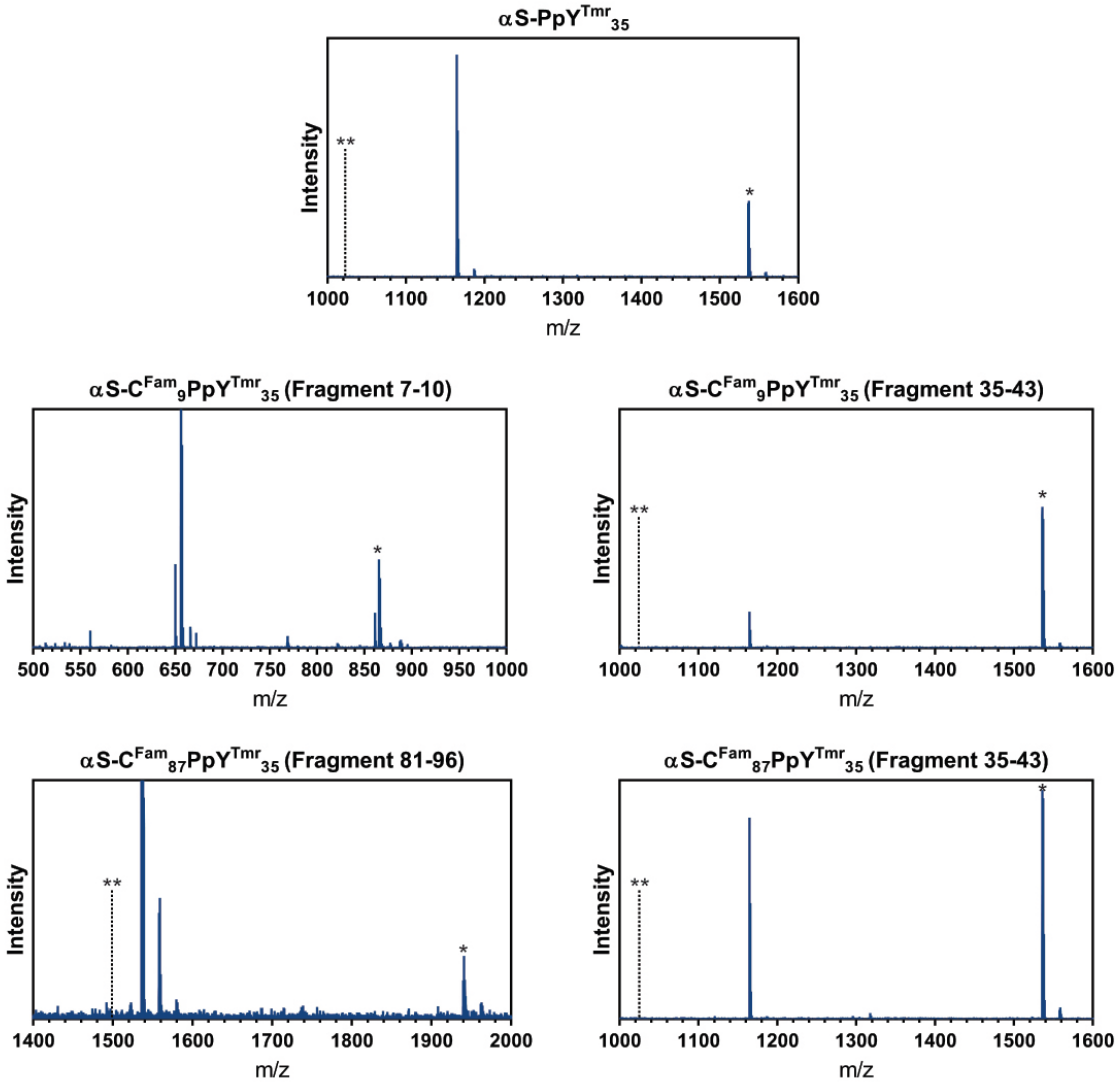

Figure S4b. MALDI-TOF MS of Trypsin Digests of Labeled Proteins. In each case the identity of the protein is indicated above the plot. Asterisk (*) indicates the position of the expected mass for the labeled fragment; double asterisk (**) indicates the position of the unlabeled fragment.

Table S2. Calculated and Observed Tryptic Digest Fragments.

Construct	Trypsin Digest Fragment	$[M+H]^+$ Calc.	$[M+H]^+$ Obs'd
αS-C^{Pyr}_{9}	7-10	758.3*	758.4*
αS-C^{Pyr}_{24}	24-32	1121.5	1120.5
αS-C^{Pyr}_{42}	35-43	1283.6	1282.6
αS-C^{Pyr}_{62}	59-80	2470.2‡	2469.2‡
αS-C^{Pyr}_{87}	81-96	1810.9	1810.0
αS-C^{Pyr}_{114}	103-140	4601.7**	4600.9
αS-C^{Pyr}_{123}	103-140	4601.7**	4601.0
αS-C^{Pyr}_{136}	103-140	4567.6**	4567.2
αS-PpY$^{Tmr}_{35}$	35-43	1536.1	1535.8
αS-C^{Fam}_{9}PpY$^{Tmr}_{35}$	7-10	865.3	865.3
αS-C^{Fam}_{9}PpY$^{Tmr}_{35}$	35-43	1536.1	1535.8
αS-C^{Fam}_{87}PpY$^{Tmr}_{35}$	81-96	1939.8	1939.9
αS-C^{Fam}_{87}PpY$^{Tmr}_{35}$	35-43	1536.1	1535.8

Note: Asterisk (*) indicates calculated and observed masses for $[M+Na]^+$ adducts. Double asterisk (**) indicates calculated and observed masses for $[M+Na]^+$ average masses, rather than monoisotopic. Double dagger (‡) indicates the presence of a single missed tryptic cut site observed as the $[M+Na]^+$ adduct.

Circular Dichroism (CD) Spectroscopy of αS WT and αS-PpY$^{Tmr}_{Y}$ Constructs. The concentration of purified WT αS (ε_{280} = 5120 M^{-1} cm^{-1}) or HPLC purified Tmr-labeled proteins in buffer (20 mM Tris, 100 mM NaCl pH 7.5) were determined by UV-Vis absorbance (ε_{555} = 87000 M^{-1} cm^{-1}). Samples for CD data acquisition were then prepared by dilution of the protein stock solutions using 20 mM Tris, 100 mM NaCl pH 7.5, and 100 mM sodium dodecylsulfate (SDS) in 20 mM Tris, 100 mM NaCl pH 7.5 to yield samples composed of 5 μM protein (WT αS or αS-PpY$^{Tmr}_{Y}$) with 10 mM SDS in 20 mM Tris, 100 mM NaCl pH 7.5. CD spectra were acquired on an Aviv Model 410 spectrometer in quartz cuvettes with a path length of

1 mM at 25 °C. Spectra were collected over the range of 190-260 nm using a 1 nm step size, 2 nm bandwidth, and 10 second integration time at each wavelength. The spectra of WT αS and each αS-PpY$^{Tmr}_{X}$ mutant were background subtracted for buffer blank and corrected for concentration, path length, and number of residues as described by Equation 1, where θ_{sample} and θ_{blank} refer to raw elipticity values for the sample and buffer blank, respectively, ε is the path length of the cuvette, *c* is the protein concentration, and *n* is the number of residues.

Equation 1: $$[\theta] = \frac{((\theta_{sample} - \theta_{blank}) - (\theta_{sample,260\,nm} - \theta_{blank,260\,nm}))}{10 \times \epsilon \times c \times n} \times 10^{-3}$$

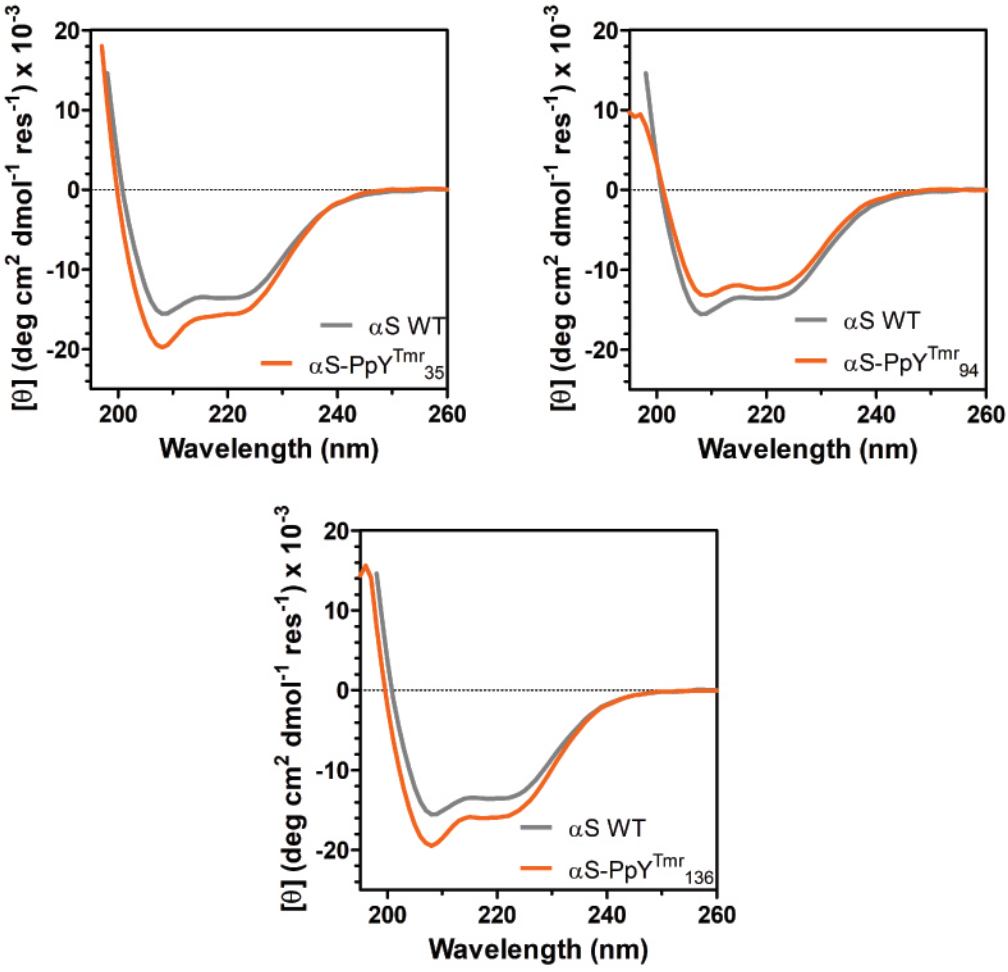

Figure S5. Circular Dichroism Spectra of Labeled Proteins. CD spectra were collected in the presence of 10 mM SDS in 20 mM Tris, 100 mM NaCl pH 7.5 for αS-PpY$^{Tmr}_{35}$ (top left), αS-PpY$^{Tmr}_{94}$ (top right) and αS-PpY$^{Tmr}_{136}$ (bottom) in orange as compared to WT αS shown in gray.

Aggregation Kinetics. Aggregation reactions were carried out by diluting each labeled construct into a mixture of WT αS in a 1:99 molar ratio to a final concentration of 100 μM in 20 mM Tris, 100 mM NaCl pH 7.5. Aggregation samples were prepared in triplicate. Aggregation was initiated by shaking the solution at 37 °C and 1500 rpm on an IKA MS3 digital orbital shaker in parafilm-sealed 1.7 mL Eppendorf tubes. At each time point, aliquots were removed from the aggregation reaction and assessed by fluorescence polarization (FP) and Congo Red absorbance in separate assays. Fluorescence polarization measurements were obtained by dilution of 10 μL of the aggregation solution into 90 μL αS buffer (100 μL total volume). The samples were gently vortexed and transferred to an untreated Corning® Costar black nonsterile 96-well plate and analyzed on a Tecan F200 plate reader. Congo Red absorbance measurements were obtained by dilution of 10 μL of the aggregation solution into 140 μL 20 μM CR dissolved in αS buffer (20 mM Tris, 100 mM NaCl pH 7.5). The samples were allowed to sit at room temperature for 15 minutes prior to being transferred to an untreated Corning® Costar black, nonsterile 96-well plate and absorbance measurements acquired on a Tecan M1000 plate reader using a wavelength range of 230-700 nm and 1 nm step size. Following completion of the aggregation assay, the samples were split into two separate aliquots; fibrils in each aliquot were then pelleted by centrifugation (13,200 rpm for 90 minutes 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. The second aliquot was frozen at – 20 °C until resuspension and analysis by TEM. Note that other singly-labeled constructs (αS-C^{Fam}_X constructs) have previously been assayed for perturbations to aggregation kinetics.[5]

The FP and CR measurements made during the aggregation procedure described above were fitted using sigmoidal equations to determine an estimated $T_{1/2}$ of aggregation for each labeled construct according to Equation 2 below:

Equation 2: $y(x) = Min + \frac{Max-Min}{1+\left(\frac{T_{\frac{1}{2}}}{x}\right)^{Z}}$

The maximum value is the upper asymptote (Y at x = 48 hours) and the minimum is the lower asymptote (Y at x = 0 hours) and Z is a scaling parameter. The $T_{1/2}$ is determined when the data is fit as a parameter in Equation 2. No constraints were used for fitting with Equation 2 and only the mean Y values at each time point were considered. Error values derived from fitting with Equation 2 are errors from the fit and parameters for the exponential component (Z) are shown in Table S3.

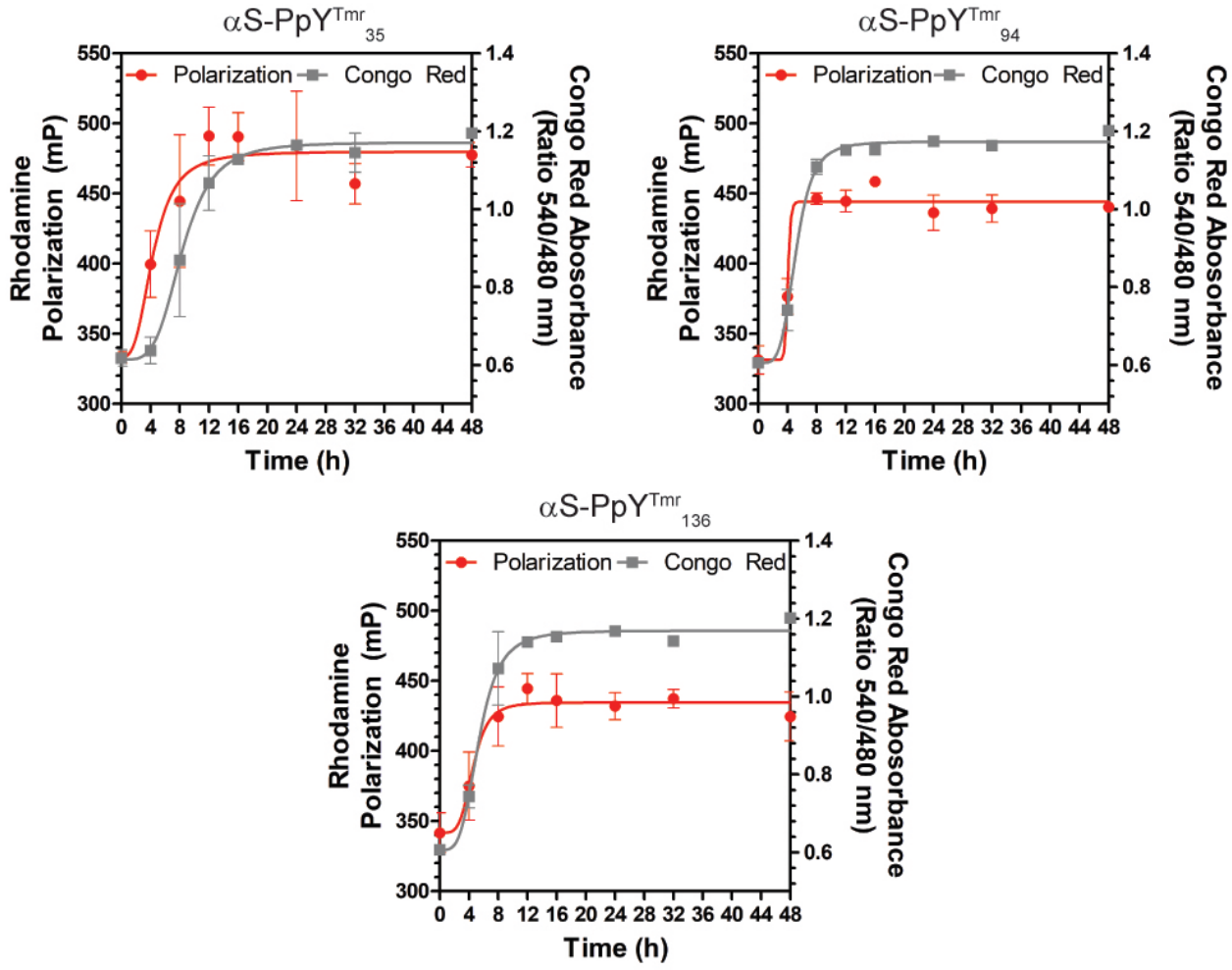

Figure S6. Labeled Protein Aggregation Kinetics. Aggregation kinetics for αS-PpY$^{Tmr}_{35}$ (top left), αS-PpY$^{Tmr}_{94}$ (top right) or αS-PpY$^{Tmr}_{136}$ via fluorescence polarization (left Y-axis) or Congo Red binding (right Y-axis).

Table S3. Aggregation Kinetic Fit Parameters.

Protein	CR $T_{1/2}$ (hr)	FP $T_{1/2}$ (hr)	R^2 (CR)	R^2 (FP)	Z (CR)	Z (FP)
WT αS	7.5	-	0.998	-	2.6	-
αS-PpY$^{Tmr}_{35}$	8.4	4.4	0.949	0.807	4.0	3.0
αS-PpY$^{Tmr}_{94}$	5.2	4.1	0.991	0.948	4.5	4.1
αS-PpY$^{Tmr}_{136}$	5.4	4.6	0.976	0.843	3.8	4.6

αS-C^{Pyr} Excimer Data Acquisition. Aliquots of αS WT (ε_{280} = 5120 M^{-1} cm^{-1}) and αS-C$^{Pyr}_X$ were quantified by UV-Vis (ε_{343} = 28000 M^{-1} cm^{-1}).[8] Following quantification, samples were prepared by dilution of αS WT and αS-C$^{Pyr}_X$ to 75 and 25 μM, respectively, in 20 mM Tris, 100 mM NaCl pH 7.5 to a final volume of 410 μL. A 50 μL aliquot was removed and stored at -20 C to be used as a UV-Vis quantification standard at the end of the assay. The remaining volume was split into 3 equivalent 1.7 mL Eppendorf tubes for aggregation. At the start and end of aggregation, 10 μL of aggregation reaction was diluted into 140 μL 20 μM Congo Red solution in αS buffer and incubated for 5-10 minutes at room temperature, then transferred to a 96-well plate and absorbance measurements acquired on a Tecan M1000 plate reader. At the start and end of aggregation, and following centrifugation and resuspension of the fibrils, a separate 10 μL aliquot was removed and diluted into 140 μL αS buffer for fluorescence measurements. Fluorescence spectra were acquired on a PTI QuantaMaster fluorometer using quartz cuvettes with a 1 cm path length; all measurements were acquired at 20 °C. Emission spectra were acquired using λ_{ex} = 343 nm, λ_{em} = 350-650 nm with 3 nm excitation and emission slit widths, 1 nm step size, and 0.25 second integration time. Excitation scans were acquired using λ_{em} = 383 nm, λ_{ex} = 250-375 nm, using 3 nm excitation and emission slit widths, 1 nm step size, and 0.25 second integration time. Additional excitation spectra were acquired based on the observation of excimer fluorescence using λ_{em} = 460 nm, λ_{ex} = 250-450 nm with the parameters otherwise identical to those above. Following completion of the aggregation reaction (48 hours) fibrils were pelleted by centrifugation, the supernatant removed, and the pellet resuspended in an equivalent volume of

αS buffer. Standards saved from the start of aggregation were thawed at room temperature. Each standard or fibril sample (25 μL) was transferred to a 0.6 mL Eppendorf tube and 5 μL 150 mM SDS was added; following addition of SDS, samples were boiled for 10-15 minutes, then cooled on ice. Following this time, 100 μL αS buffer was added and UV-Vis absorbance acquired over a range of 200-800 nm with baseline subtraction from 700-800 nm. Further pelleted samples were stored for subsequent analysis by TEM (see Fig. S21).

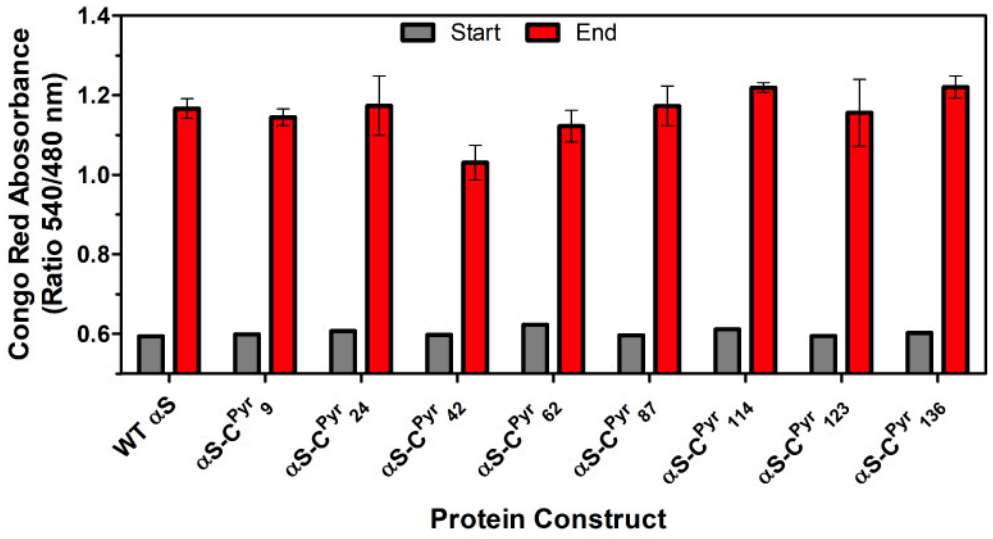

Figure S7. Congo Red Results from αS-C^{Pyr} Aggregations. The ratio of Congo Red absorbance at 540 nm/480 nm demonstrates aggregation reactions containing labeled protein proceed to completion similar to WT αS.

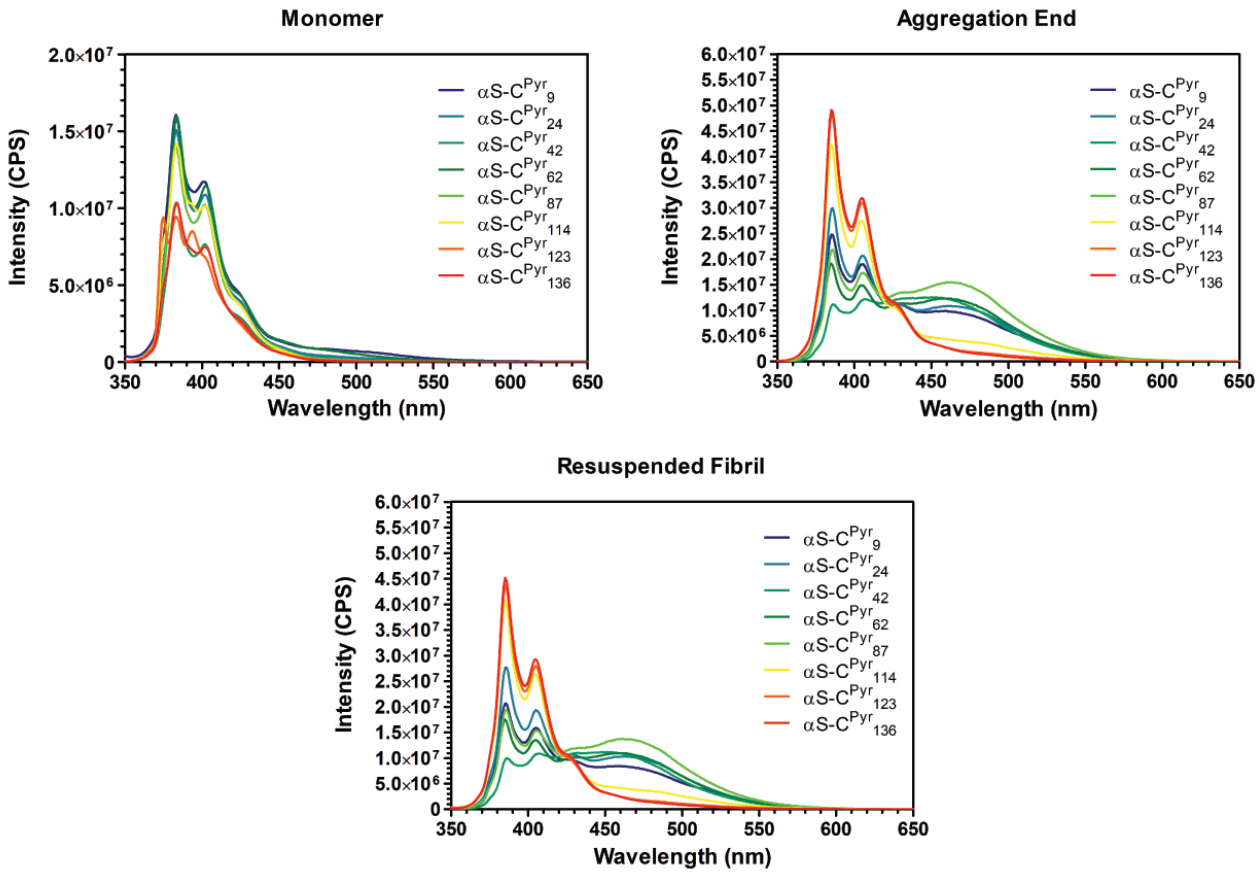

Figure S8. Emission Spectra of αS-C^{Pyr} Proteins. Top left: Monomeric protein prior to aggregation. Top right: Samples from the end of aggregation (48 hours). Bottom: Resuspended fibril emission spectra. All spectra are average of triplicate samples, color coded from N-terminus (blue) to C-terminus (red).

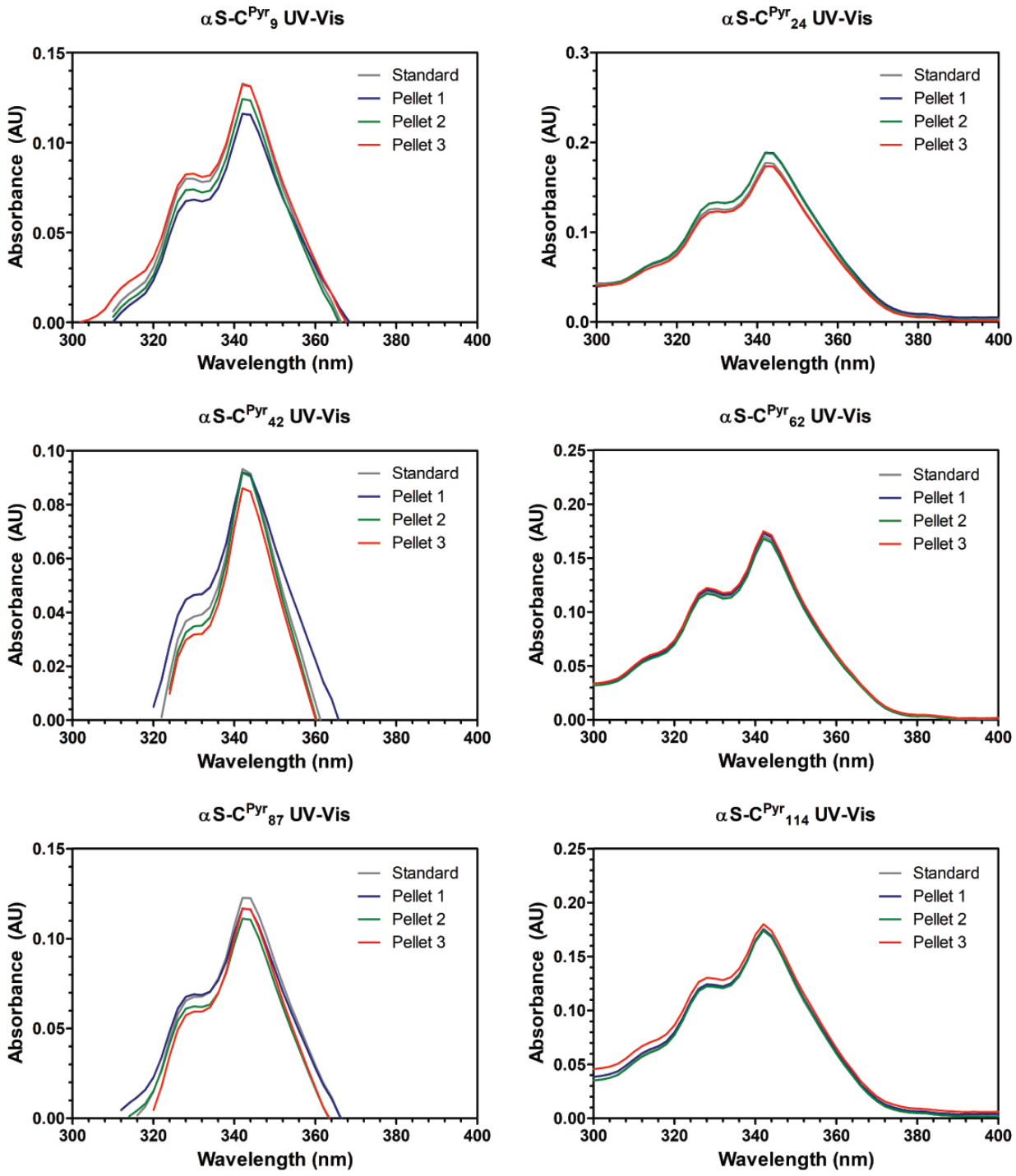

Figure S9a. UV-Vis Spectra of Resuspended Fibrils of Pyrene Labeled Proteins. The identity of each protein is indicated above the corresponding plot. The absorbance at 343 nm from each of the triplicate samples is compared to a monomeric standard (gray) saved from the start of aggregation, indicating high levels of labeled protein incorporation in fibrils.

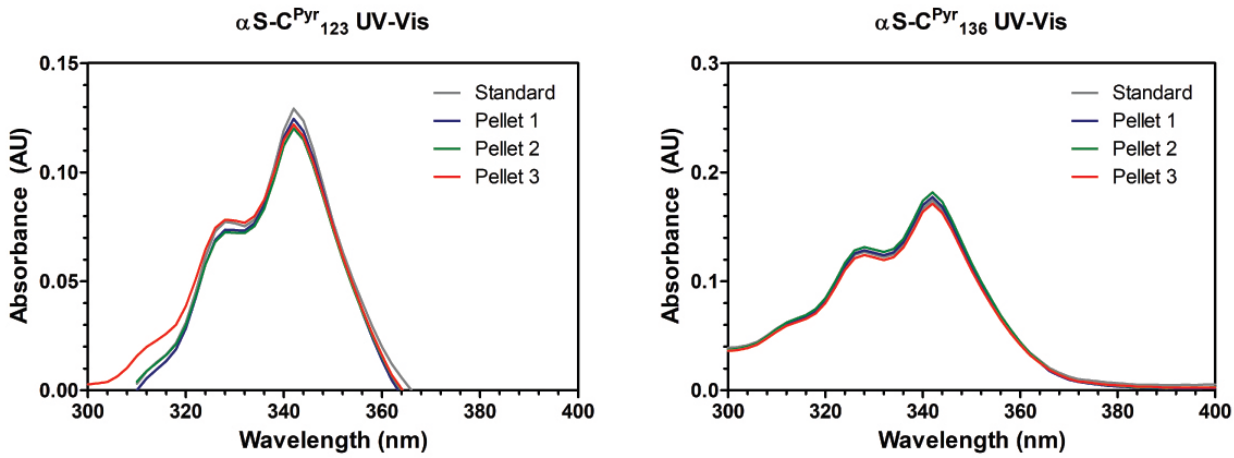

Figure S9b. UV-Vis Spectra of Resuspended Fibrils of Pyrene Labeled Proteins. The identity of each protein is indicated above the corresponding plot. The absorbance at 343 nm from each of the triplicate samples is compared to a monomeric standard (gray) saved from the start of aggregation, indicating high levels of labeled protein incorporation in fibrils.

Fluorescein Bathochromism and Thioacetamide Quenching. Aliquots of αS WT (ε_{280} = 5120 M^{-1} cm^{-1}) and αS-C^{Fam}_X were quantified by UV-Vis (ε_{494} = 68000 M^{-1} cm^{-1}). Proteins were combined to a final αS WT concentration of 99 μM with 1 μM αS-C^{Fam}_X, after which steady-state emission spectra were collected as described below in the presence and absence of 100 mM thioacetamide. Analogous samples were prepared by aggregation of monomeric protein by agitation at 1500 rpm, 37 °C for 48 hours. Following aggregation, fibrils were pelleted by centrifugation (Eppendorf bench top centrifuge, 13200 rpm, 4 °C, 90 min). The supernatant was removed and an equivalent volume of buffer was added, then samples were vortexed repeatedly to resuspend fibrillar αS. Monomeric samples or fibrils following resuspension were diluted (15 μL) into αS buffer (135 μL) or into αS buffer supplemented with thioacetamide (111.1 mM thioacetamide from a 500 mM stock in αS buffer; final thioacetamide concentration of 100 mM). Fluorescence spectra were acquired on a PTI QuantaMaster fluorometer using λ_{ex} = 485 nm, λ_{em} = 495-700 nm, 1 nm step size, 0.25 sec integration time at 20 °C in a 1 cm path length cuvette. Quenching efficiency from triplicate samples acquired in the presence of thioacetamide was calculated using Equation 3, where F_{Thio} is the maximum fluorescence intensity in the presence of thioacetamide and F_{Buffer} is the maximum fluorescence intensity in the absence of thioacetamide (Fig. S10). Comparison of maximum emission wavelength (for

monomeric versus fibrillar samples) are plotted below as the maximum emission wavelength from triplicate samples (± standard deviation, Fig. S11). TCSPC measurements of fluorescence lifetime decays for fibrils in the presence and absence of thioacetamide were collected using a pulsed LED with a maximum emission at 486 nm with a 500 nm short pass filter (ThorLabs catalogue #FES0500) in the excitation beam path. Fluorescence was collected at 515 nm with 15 nm slit widths. The instrument response function (IRF) was collected under identical conditions. The resulting fluorescence decays were fit using the Exponential Series Method (ESM) in PowerFit 10 (Photon Technologies, Inc) to obtain lifetime distributions representative of the decay.[9]

Equation 3: $$E_{Quenching} = 1 - \frac{F_{Thio}}{F_{Buffer}}$$

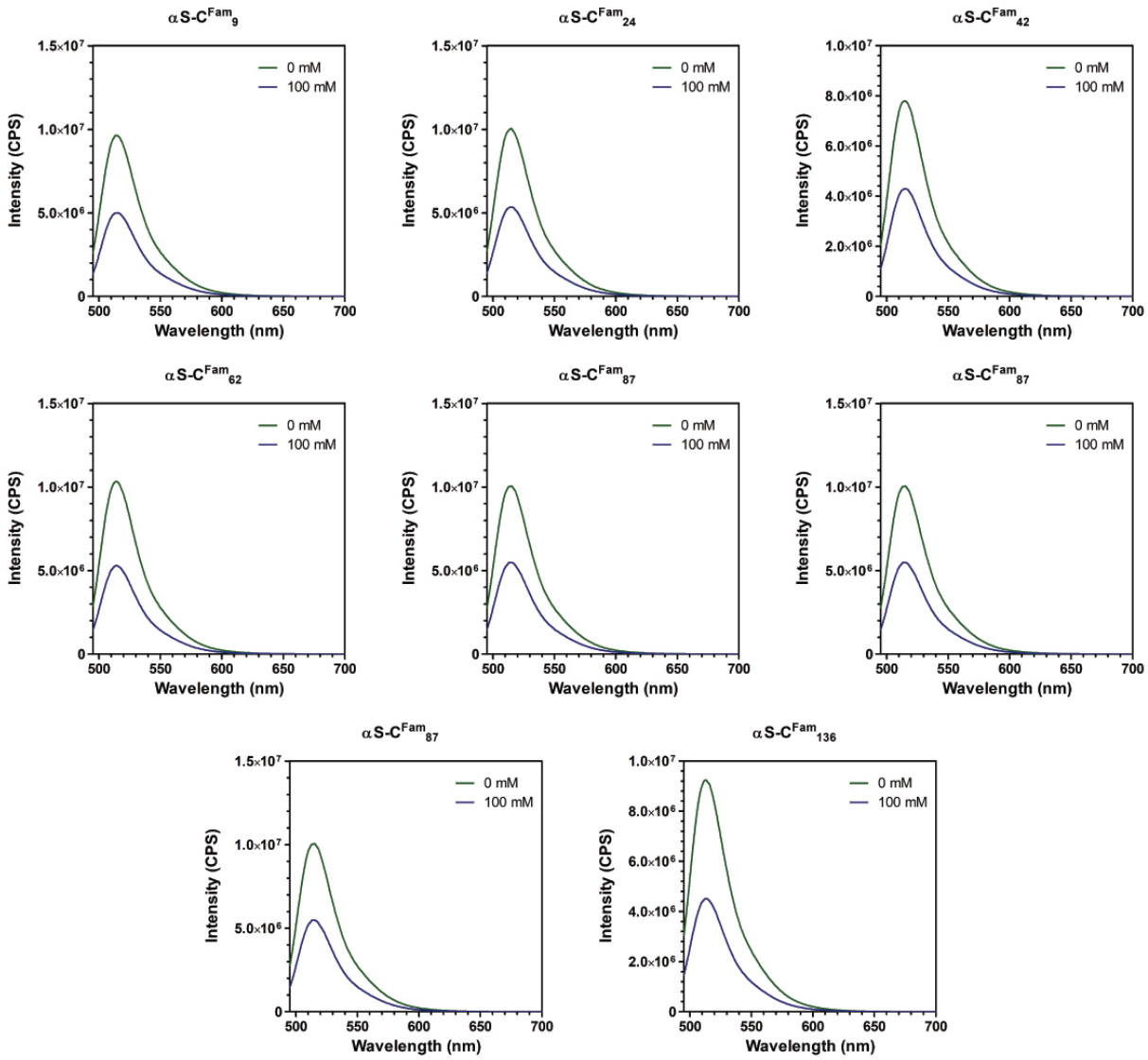

Figure S10a. Thioacetamide Quenching Efficiency in αS-C$^{Fam}_{X}$ Monomer. Steady state fluorescence emission spectra are shown for each αS-C$^{Fam}_{X}$ construct, with the identity of the labeled protein indicated above the plot; green: buffer emission spectra, blue: emission spectra obtained in the presence of 100 mM thioacetamide.

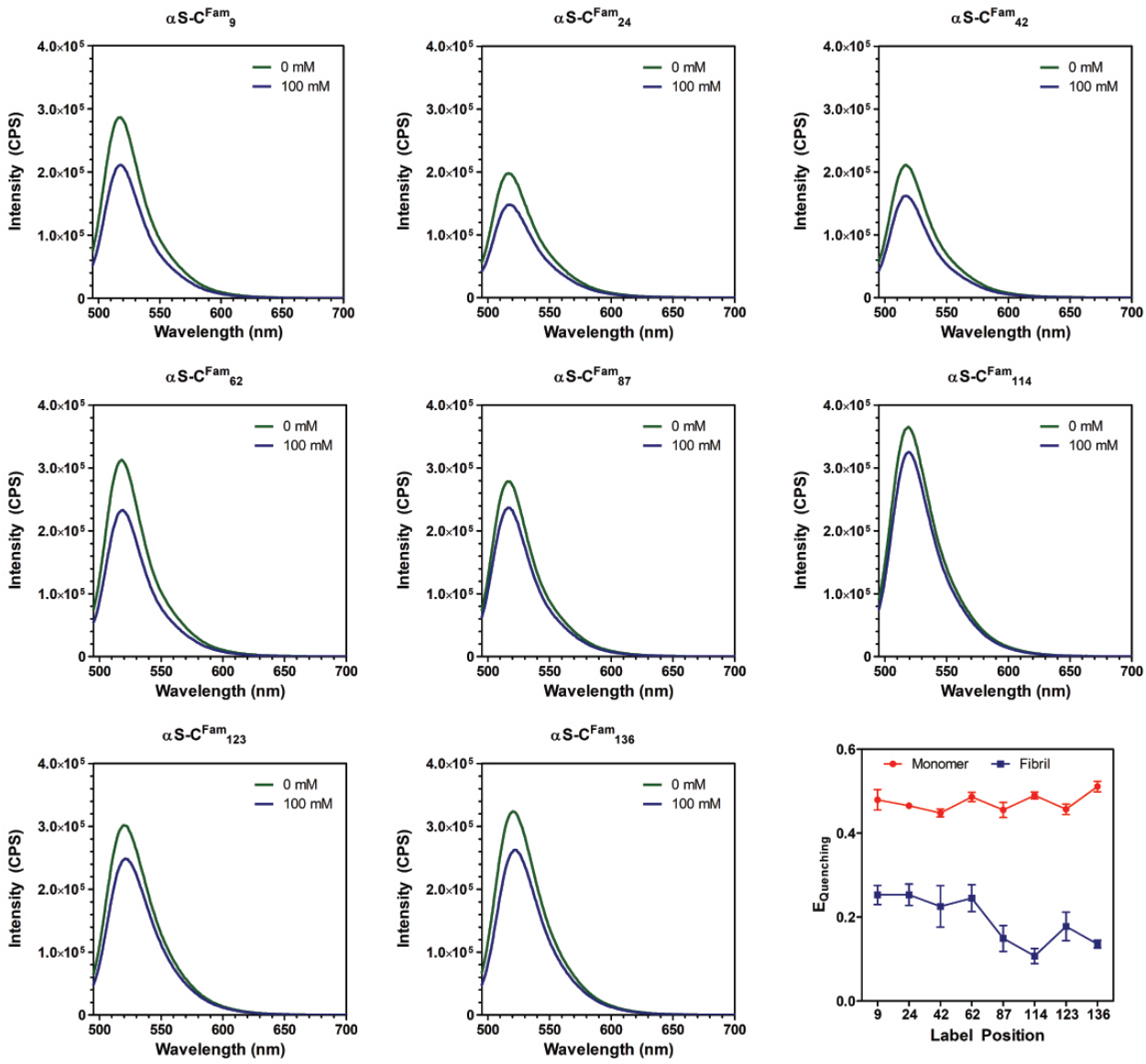

Figure S10b. Thioacetamide Quenching Efficiency in Fibrils. Steady state fluorescence emission spectra are shown for each αS-C$^{Fam}_{X}$ construct, with the identity of the labeled protein indicated above the plot; green: buffer emission spectra, blue: emission spectra obtained in the presence of 100 mM thioacetamide. Bottom right: quenching efficiency (see Equation 3) for each position in the presence of 100 mM thioacetamide; points represent the mean of triplicate samples and error bars represent the standard deviation of the mean.

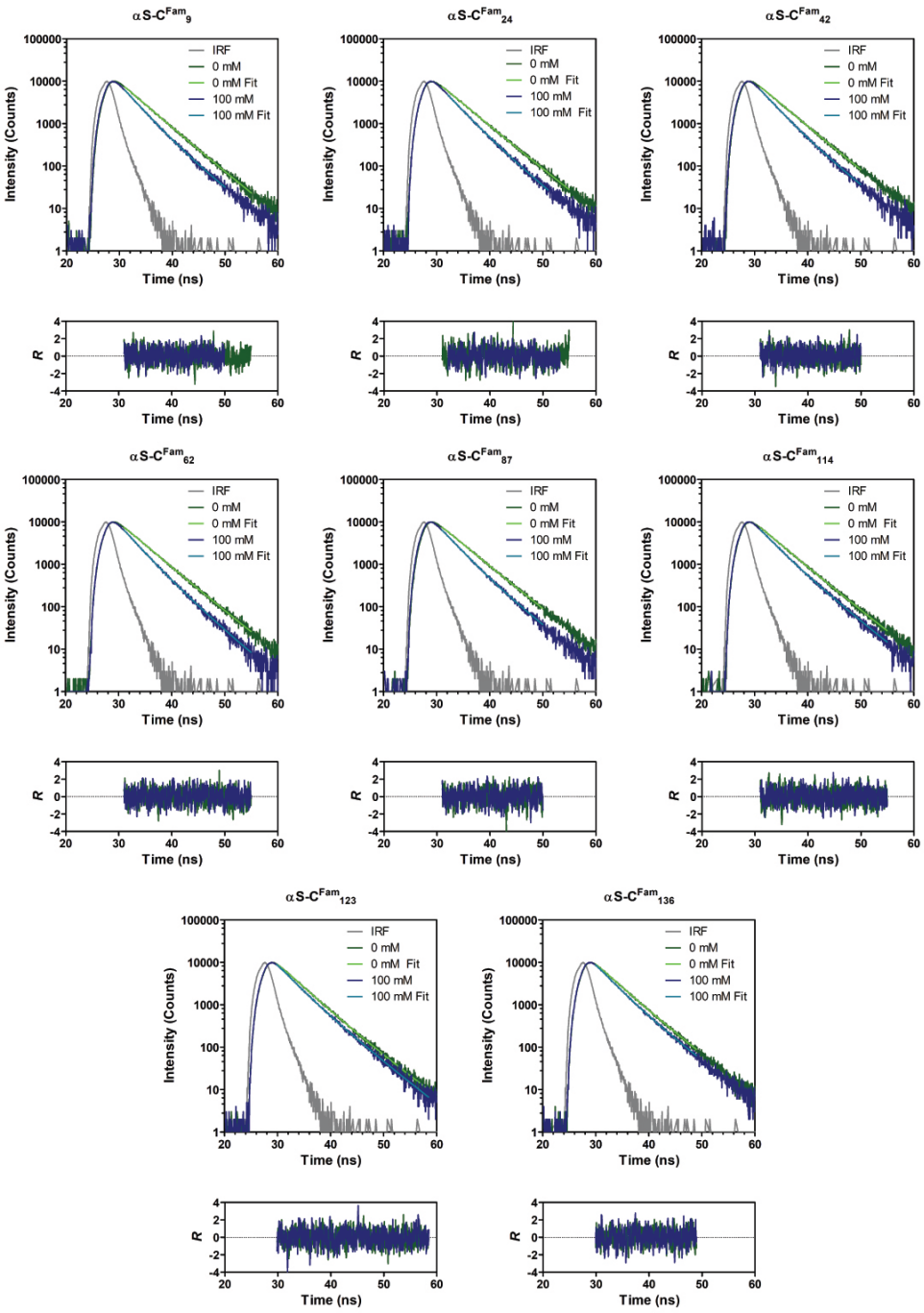

Figure S11a. Time Correlated Single Photon Counting (TCSPC) decays of αS-C$^{Fam}_{X}$ Proteins. TCSPC decays were collected for fibrils containing each αS-C$^{Fam}_{X}$ construct in the presence or absence of 100 mM thioacetamide. The identity of the labeled protein is indicated above the corresponding TCSPC decay. Below each decay are the residuals of fitting the decay to an exponential series.

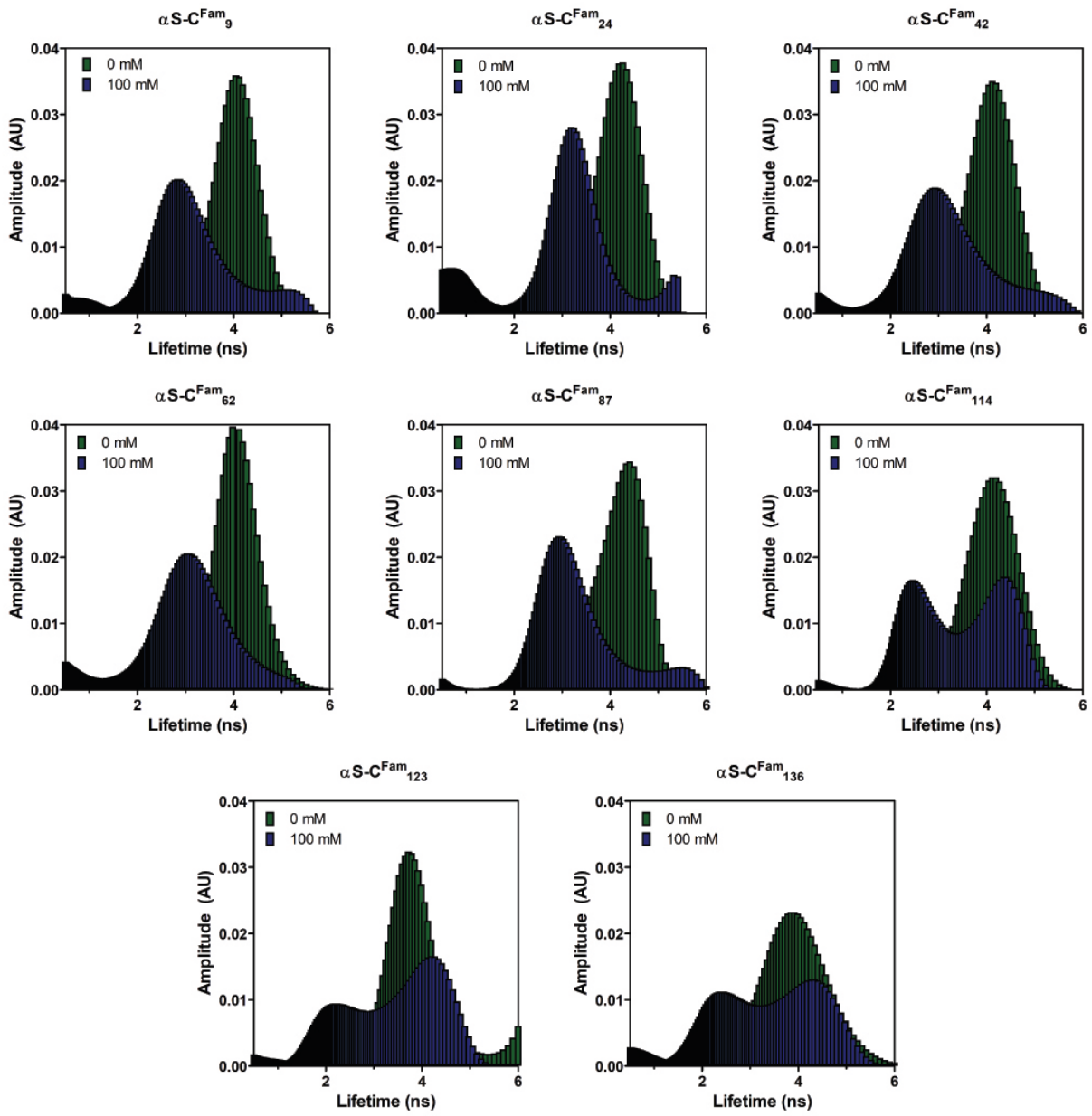

Figure S11b. Exponential Series Distributions of αS-C$^{Fam}_{X}$ Proteins. Exponential series resulting from fitting the decays of each αS-C$^{Fam}_{X}$ protein in the absence or presence of 100 mM thioacetamide; the identity of the labeled protein is indicated above each distribution plot.

Fluorescein free dye (5-carboxyfluorescein) bathochromism was investigated by dissolving fluorescein in DMSO to a final concentration of 1 mM (based on mass to volume calculation). This stock was then diluted into αS buffer (20 mM Tris, 100 mM NaCl pH 7.5) containing dimethylsulfoxide (DMSO) at concentrations ranging from 0% to 25% (v/v) with a total volume of 150 μL and a dye concentration of 1

μM. Steady state emission spectra were acquired on a PTI QuantaMaster fluorometer using λ_{ex} = 485 nm, λ_{em} = 495-700 nm, 1 nm step size, 0.25 sec integration time at 20 °C in a 1 cm path length cuvette.

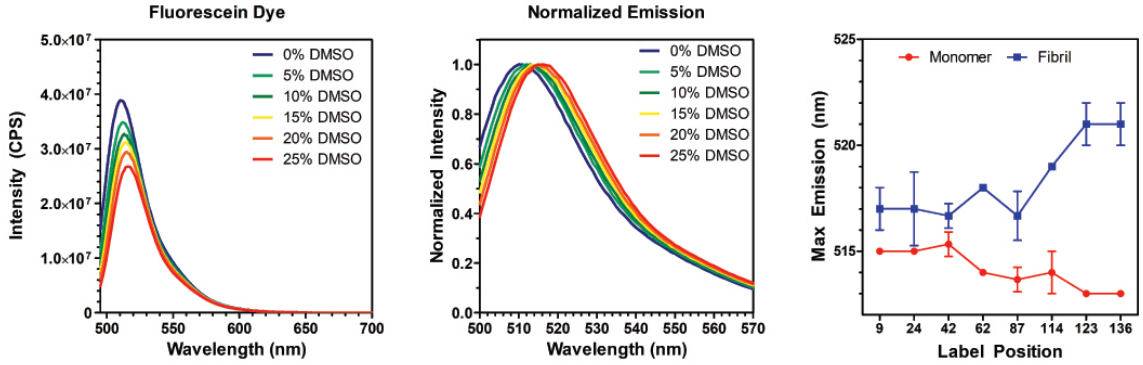

Figure S12. Fluorescein Bathochromism. Left: Steady state emission spectra of 5-carboxyfluorescein (1 μM) in increasing concentrations of DMSO. Middle: Normalized emission spectra of the data at left. Right: Observed maximum emission wavelength for each αS-C$^{Fam}_{X}$ construct in monomer or fibrils, plotted as mean with error bars representing the standard deviation of the mean.

Aggregation for FRET Data Collection and Analysis. Concentrations for singly- and doubly-labeled proteins were determined via UV-Vis absorbance using ε_{494} = 68000 M^{-1} cm^{-1} for αS-C$^{Fam}_{X}$ proteins and ε_{555} = 87000 M^{-1} cm^{-1} for αS-PpY$^{Tmr}_{Y}$ proteins and doubly-labeled proteins. αS WT was quantified by UV-Vis using ε_{280} = 5120 M^{-1} cm^{-1}. Following quantification, samples were prepared such that the concentration of αS WT was 100 μM with the labeled protein at 1 μM concentration. A small aliquot (30 μL) was then removed and stored at -20 °C to act as a standard for SDS-PAGE analysis. The remaining stock was split into triplicate samples in 1.7 mL Eppendorf tubes and sealed with parafilm. Initial measurements were acquired by dilution of 10 μL of sample into 140 μL of 20 μM Congo Red in αS buffer. A separate sample (15 μL) was diluted into 135 μL αS buffer for fluorescence polarization and fluorescence emission acquisition. Samples were then placed in Ika MS3 orbital shakers and agitated at 1500 rpm, 37 °C for 48 hours. Following this time, measurements were acquired by dilution of samples into Congo Red and buffer, respectively, as described above. Aggregated material was then isolated by centrifugation (13200 rpm, 4 °C, 90 min). Following centrifugation, the supernatant was removed and pelleted material resuspended by addition of an equivalent volume of buffer followed by vortex. Resuspended samples were diluted (15 μL)

into 135 μL buffer for fluorescence data acquisition as described below. Congo Red absorbance measurements were acquired by transferring samples to a 96 well, black CoStar plate and absorbance readings taken on a Tecan M1000 plate reader from 200-700 nm. Fluorescence polarization measurements were acquired by transferring samples in buffer to a 96 well, black CoStar plate and read on a Tecan F200 plate reader using λ_{ex} = 485 ± 20 nm, λ_{em} = 535 ± 25 nm for fluorescein and λ_{ex} = 530 ± 21 nm and λ_{em} = 574 ± 26 nm. Fluorescence spectra were acquired on a PTI QuantaMaster fluorometer equipped with Peltier temperature controller at 20 °C in 1 cm path length cuvettes. All measurements were acquired with 5 nm excitation and emission slit widths, with gain set to -6.5 V (minimum possible), 1 nm step size and 0.25 sec integration/wavelength. Fluorescein λ_{ex} = 485 nm, λ_{em} = 495-700 nm; rhodamine λ_{ex} = 555 nm, λ_{em} = 565-700 nm. Fluorescein excitation scans were acquired using instrument parameters above with λ_{em} = 515 nm and λ_{ex} = 350-505 nm. Rhodamine excitation scans acquired using instrument parameters above with λ_{em} = 580 nm and λ_{ex} = 350-575 nm. All TCSPC measurements of fluorescence lifetime decays were collected using a pulsed LED with a maximum emission at 486 nm with a 500 nm short pass filter (ThorLabs catalogue #FES0500) in the excitation beam path. Fluorescence was collected at 515 nm with 15 nm slit widths. The instrument response function (IRF) was collected under identical conditions.

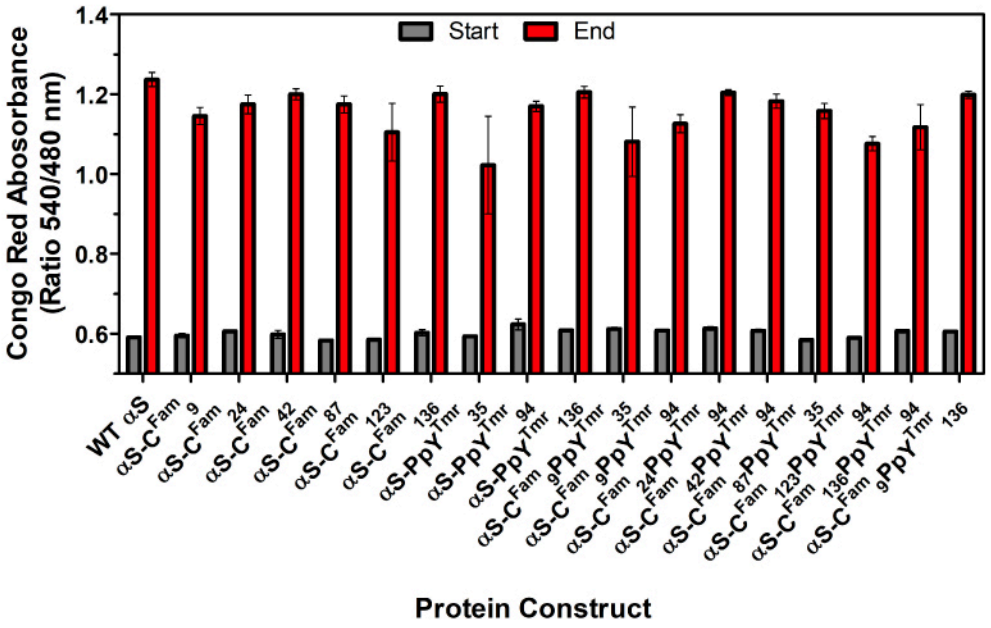

Figure S13. Congo Red from Labeled Protein Aggregation. The ratio of Congo Red absorbance at 540 nm/480 nm demonstrates aggregation reactions containing labeled protein proceed to completion similar to WT αS.

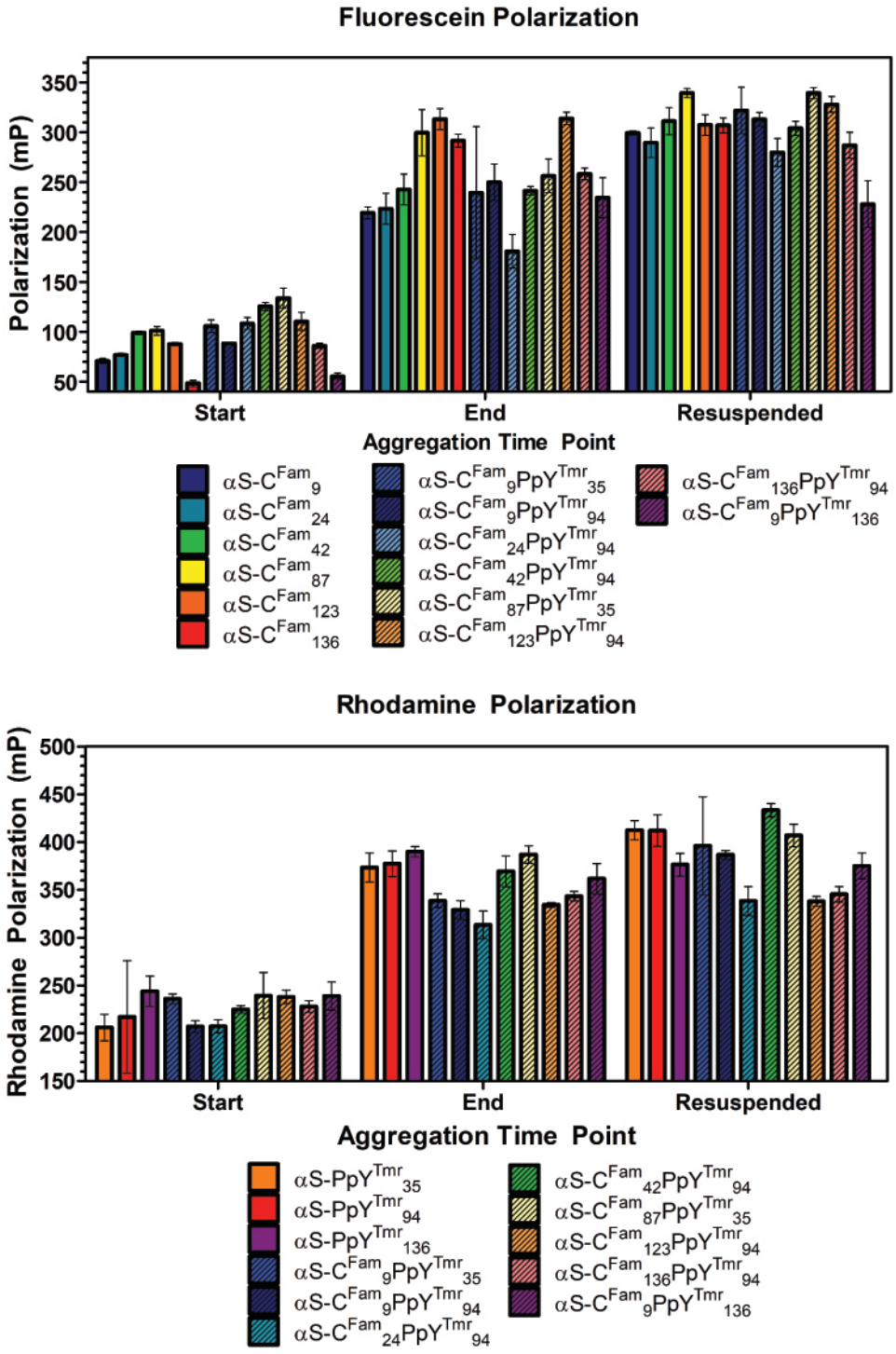

Figure S14. Fluorescence Polarization from Labeled Protein Aggregations. Top: Fluorescein polarization for αS-C$^{Fam}_{X}$ or doubly-labeled constructs. Bottom: Rhodamine polarization from αS-PpY$^{Tmr}_{Y}$ constructs or doubly-labeled constructs. All measurements are the mean of triplicate samples with error bars representing standard deviation of the mean.

FRET Data Fitting

Fitting Steady-State Data. Following data collection, the single labeled spectra were used to quantify the degree of energy transfer. The spectral overlap of the donor and acceptor were deconvoluted by fitting the double labeled spectrum with the linear sum of the individual donor and acceptor labeled spectra (Equation 4). Fitting was performed by minimizing the total least squared difference using the Excel Solver feature to minimize the constants *A* and *B*:

$$\textbf{Equation 4: } \sum_{\lambda}\left(I(\lambda)_{DA} - AI(\lambda)_{D} - BI(\lambda)_{A}\right)^2 \rightarrow \min$$

$$\textbf{Equation 5: } I(\lambda)_{DA} = AI(\lambda)_{D} + BI(\lambda)_{A}$$

Here, $I(\lambda)_{DA}$, $I(\lambda)_{D}$ and $I(\lambda)_{A}$ are the wavelength dependent fluorescence intensities of the double-labeled and single labeled protein, containing the donor and acceptor fluorophores, respectively. Solutions to Equation 5 were obtained by utilizing the Excel Solver functionality. The linear contributions of the single-labeled construct containing the donor only, *A*, and the contribution from the single-labeled construct containing the acceptor only, *B*, were both used to independently calculate the E_{FRET} through Equation 2 and Equation 5 and combined in a weighted average via Equation 4:

$$\textbf{Equation 6: } E_D = (1 - A)$$

$$\textbf{Equation 7: } E_A = (B - 1)\frac{\varepsilon_A}{\varepsilon_D}$$

$$\textbf{Equation 8: } E_{FRET} = \left(\frac{1}{S_D} + \frac{1}{S_A}\right)^{-1}\left(\frac{E_D}{S_D} + \frac{E_A}{S_A}\right)$$

In Equations 6-8, E_D and E_A are E_{FRET} values calculated from the donor and acceptor weights respectively. Additionally, Equation 7 requires ratio of the extinction coefficient for the acceptor, ε_A, to the donor, ε_D, and were calculated using the absorption spectrum of each fluorophore scaled using published extinction coefficients for Fam $\varepsilon_{494} = 68{,}000\ M^{-1}cm^{-1}$ [10] and Tmr $\varepsilon_{555} = 87{,}000\ M^{-1}cm^{-1}$ as indicated by the manufacturer

(see: https://www.lumiprobe.com/p/tamra-azide-5). The E_{FRET} values from the donor and acceptor were then used to compute a weighted average E_{FRET} value by using the inverse of the experimental error to weight each E_{FRET} value, where S_D and S_A represent the respective donor and acceptor propagated error (Equations 9-11).

Equation 9: $$S_A = A \times \sqrt{\left(S_{I_A} \times B / I_D\right)^2 + \left(S_{I_{DL}} / I_D\right)^2 + \left(S_{I_D} \times \left(I_{DL} - B \times I_A\right) / I_D^2\right)^2}$$

Equation 10: $$S_B = B \times \sqrt{\left(S_{I_D} \times A / I_A\right)^2 + \left(S_{I_{DL}} / I_A\right)^2 + \left(S_{I_A} \times \left(I_{DL} - A \times I_D\right) / I_A^2\right)^2}$$

Equation 11: $$S_{EFRET} = \sqrt{2} \Big/ \left(\frac{1}{S_A} + \frac{1}{S_B}\right)$$

Where in Equation 11, S_{EFRET} represents the propagated error of the calculated weighted average E_{FRET} value.

Accurate calculation of R_0 was required for calculation of interresidue distances from the determined E_{FRET}. R_0 was calculated using the Equation 12:

Equation 12: $$R_0^6 = \left(9 \ln(10) \kappa^2 \Phi_D J\right) / \left(128 \pi^2 N_A n^4\right)$$

Here, N_A is Avogadro's number, κ^2 is the dipole orientation factor, approximated as 2/3, Φ_D is the quantum yield of the donor, J is the spectral overlap integral between the emission of the donor and the absorbance of the acceptor and n is the refractive index. The overlap integral of the donor fluorescence and acceptor absorbance spectra for each fluorophore pair was determined empirically from the absorbance and emission spectra of the singly-labeled proteins through application of the integral:

Equation 13: $$J = \int_0^\infty f_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$$

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 14: $$f_D(\lambda) = \frac{F_D(\lambda)}{\int_0^\infty F_D(\lambda) d\lambda}$$

where $F_D(\lambda)$ is the fluorescence emission spectrum of the donor dye. In order to account for changes in donor quantum yield during fibrillization, excitation and emission spectra were used to empirically correct for slight decreases in donor quantum yield. This was achieved by taking the ratio of the area under the curve of the emission spectra from 495-600 nm over the area under the curve of the excitation spectra from 350-505 nm. In no case did the quantum yield of the donor change by greater than 10%, suggesting a minimal impact on the calculated Förster radius. Following determination of R_0 average distance values from the Förster equation, monomeric samples (Table S4a) or αS-C^{Fam}_{9}PpY$^{Tmr}_{136}$ distances were determined by utilizing a Gaussian Chain polymer scaled version of the Förster equation (Equation 15, 16) determined in Wolfram Mathematica.

Equation 15: $$E_{FRET} = \sum_r P_n(r,x) / \left(1 + (r/R_0)^6\right)$$

Equation 16: $$P_1(r,x) = 4\pi r \left(\frac{3}{2\pi x^2}\right)^{3/2} \exp\left(-\frac{3}{2}\frac{r^2}{x^2}\right) \quad \text{(Eq. 3)}$$

Fitting Lifetime Data. Lifetime data were fit using PowerFit10 distributed by Photon Technologies, Inc. Each decay was fit to a single or double exponential decay where the time regime was selected to minimize the chi-squared values and the residuals. E_{FRET} was determined from 1-(τ_{DA}/τ_D) where τ_{DA} and τ_D are the lifetimes for double-labeled and donor-only constructs respectively. For biexponential decays the both amplitude average and intensity average lifetimes were used to calculate E_{FRET} values for comparison to the E_{FRET} values extracted from steady-state measurement.

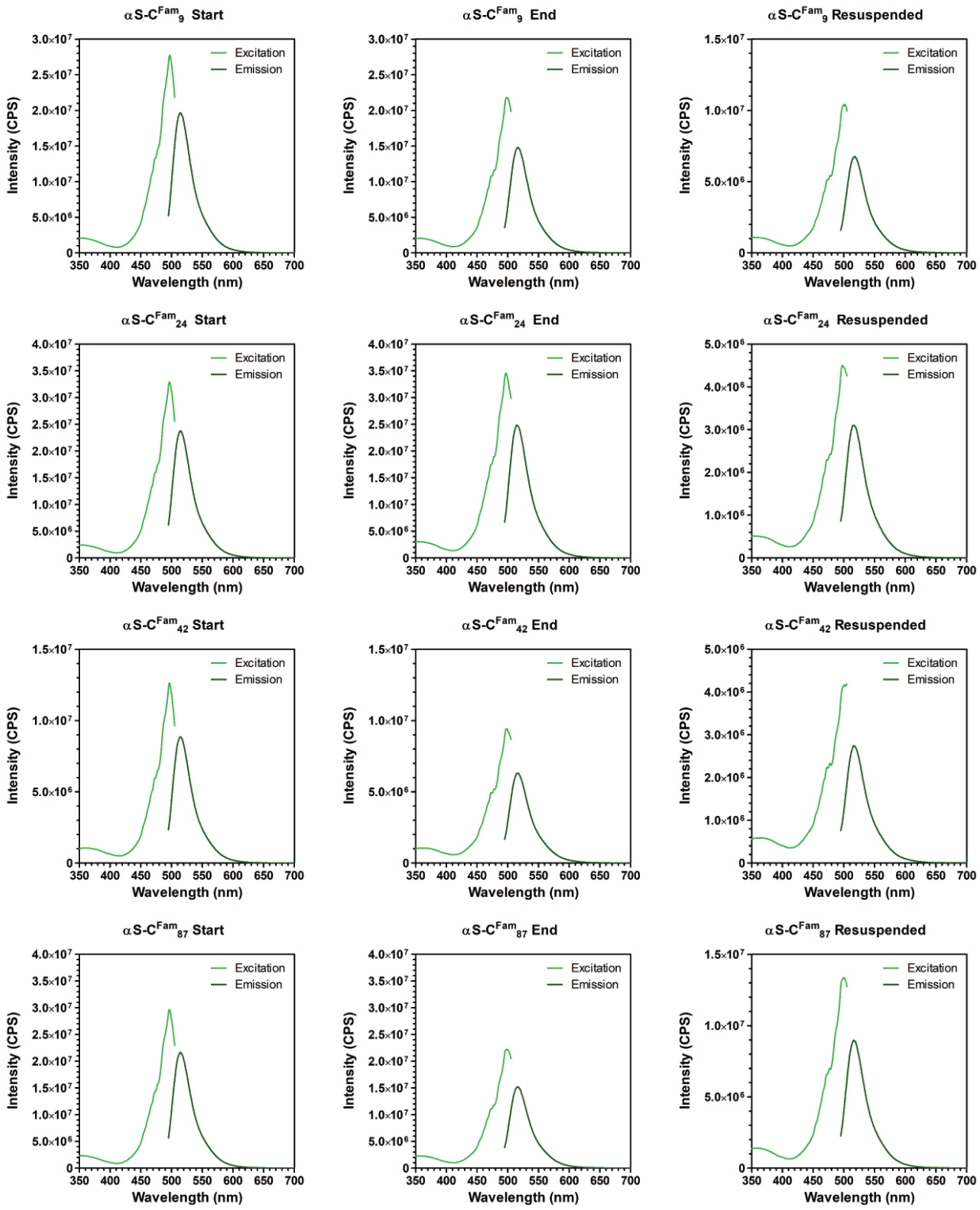

Figure S15a. Donor Excitation and Emission Spectra. The identity of each donor-labeled protein and aggregation time point is indicated above the spectra.

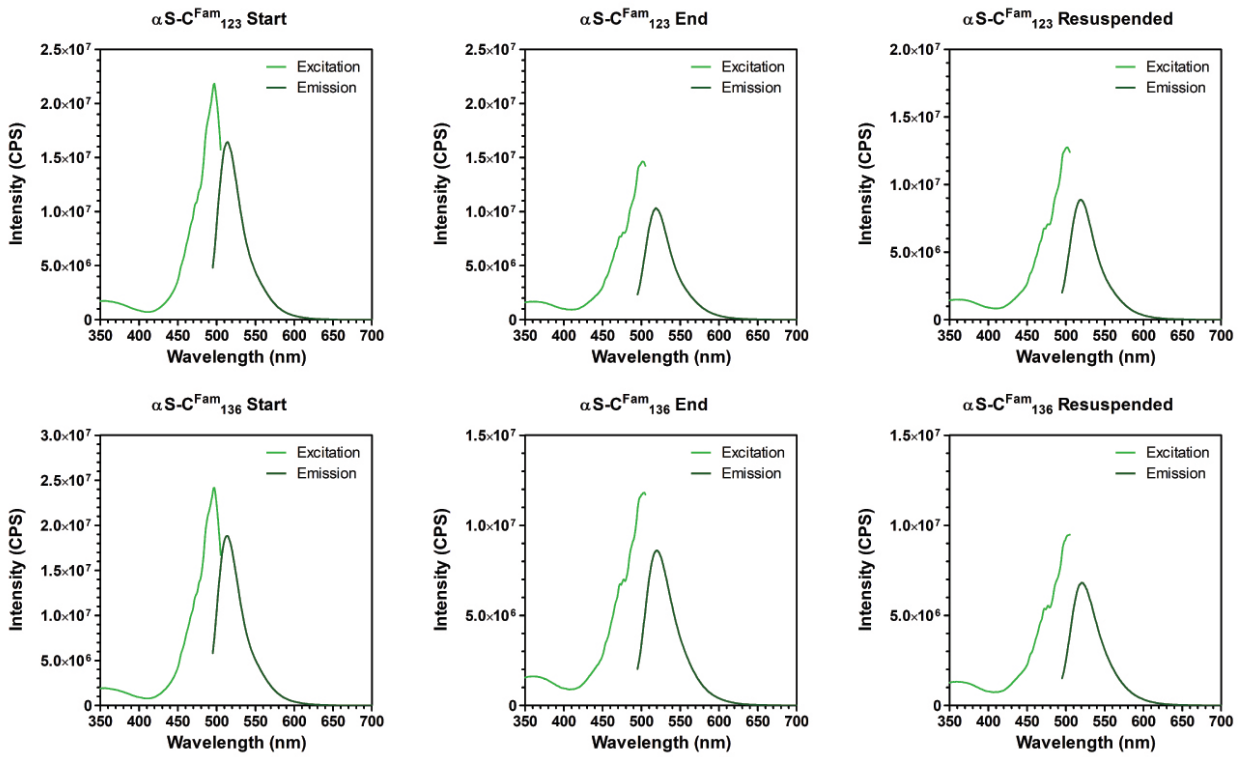

Figure S15b. Donor Excitation and Emission Spectra. The identity of each donor-labeled protein and aggregation time point is indicated above the spectra.

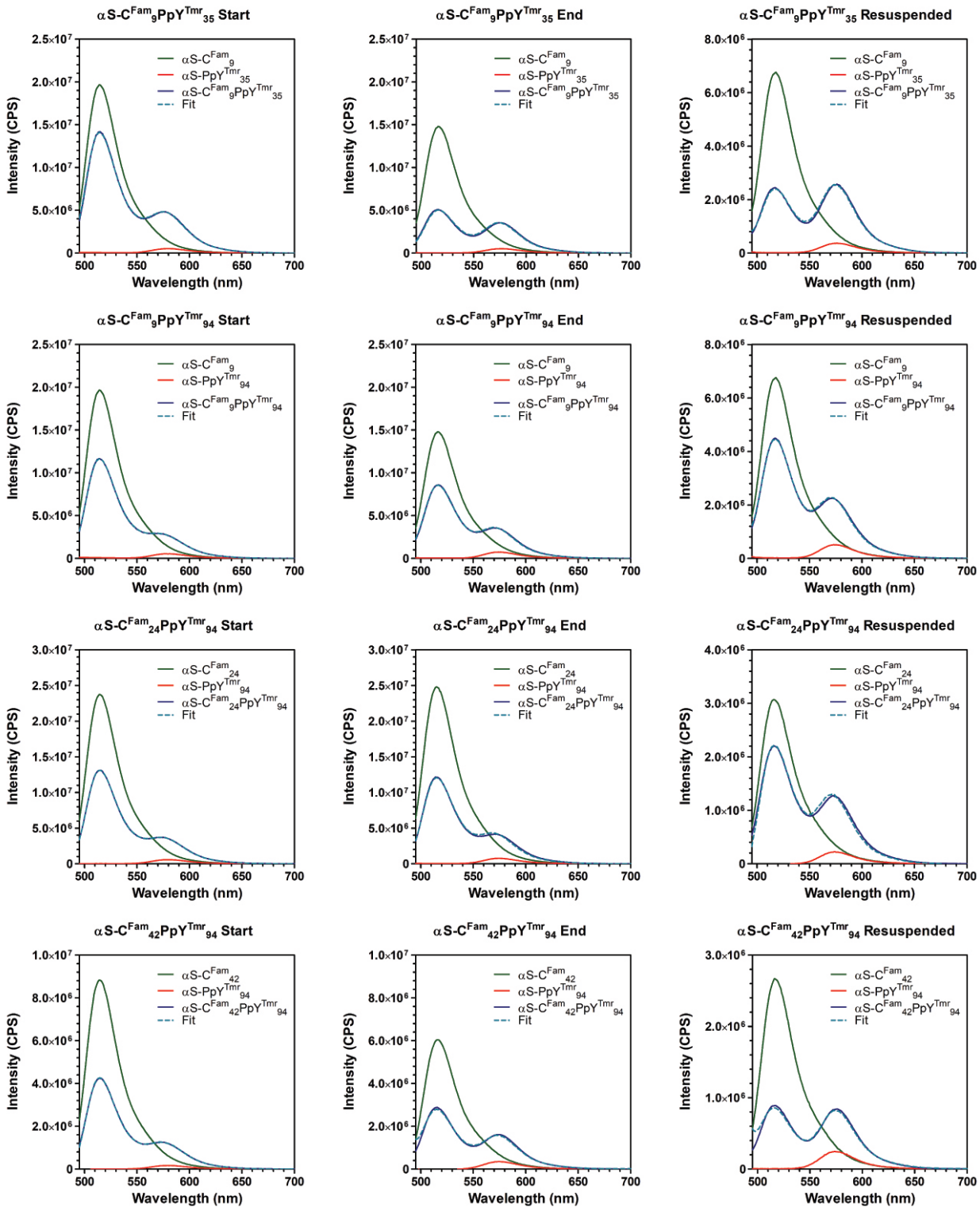

Figure S16a. Steady State Emission Spectra of Single and Doubly Labeled αS. Emission spectra collected from the start (left), end (middle) or on resuspended fibrils (right) are fit as described above (dashed blue line) to extract FRET efficiency and intramolecular distances. The identity of the labeled αS is indicated above each plot.

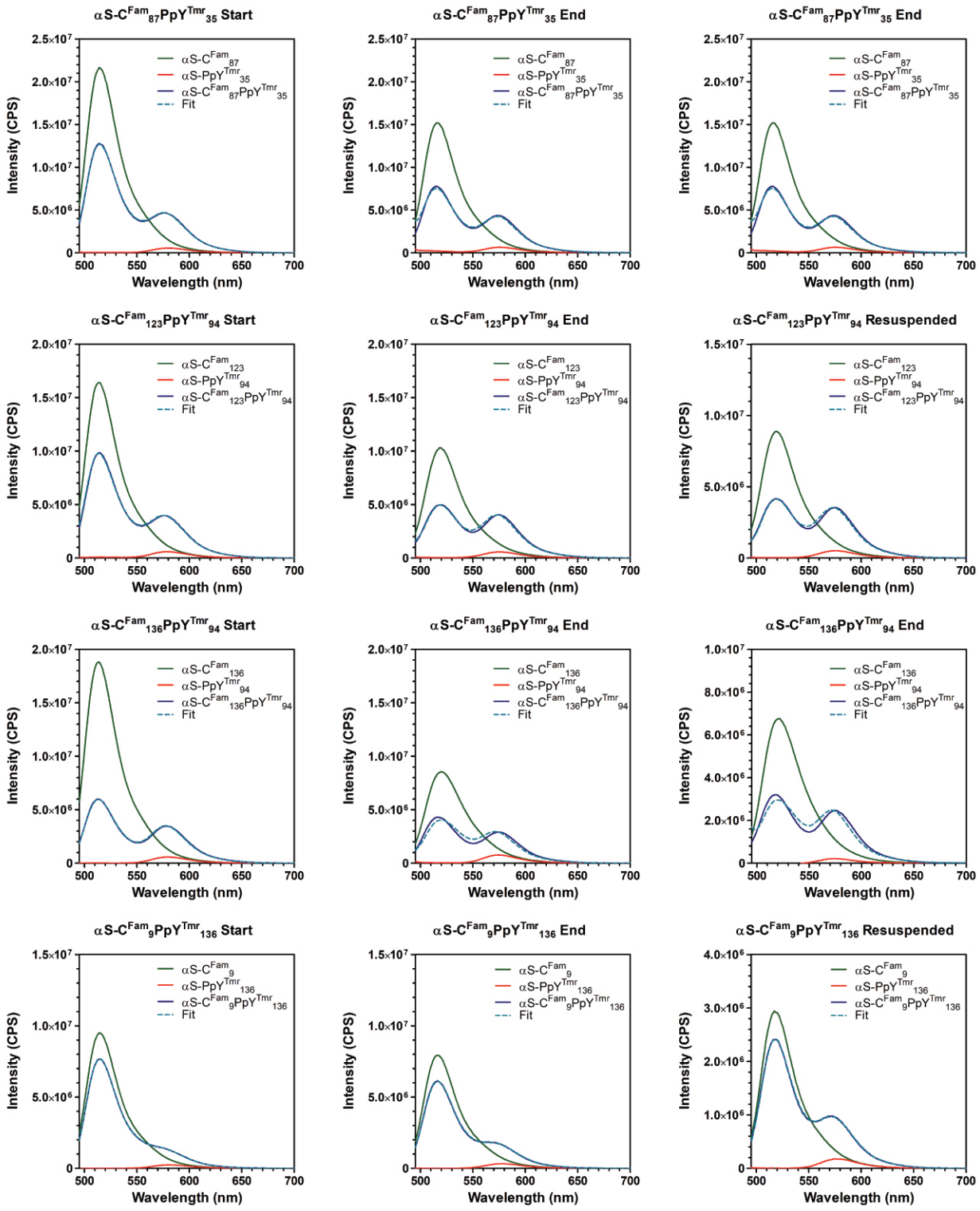

Figure S16b. Steady State Emission Spectra of Single and Doubly Labeled αS. Emission spectra collected from the start (left), end (middle) or on resuspended fibrils (right) are fit as described above (dashed blue line) to extract FRET efficiency and intramolecular distances. The identity of the labeled αS is indicated above each plot.

Table S4a. E_{FRET} Parameters and Distances from Monomeric αS-$C^{Fam}_{X}PpY^{Tmr}_{Y}$

Donor/Acceptor	Φ_D	R_0 (Å)	$\varepsilon_A/\varepsilon_D$	A	E_D	S_A	B	E_A	S_B	E_{FRET}	S_{EFRET}	R (Å)	σ_R
9/35	0.82	53.7	0.24	0.70	0.303	0.126	7.06	1.454	1.478	0.313	0.017	80.9	2.37
9/94	0.82	53.7	0.24	0.58	0.418	0.003	3.14	0.515	0.487	0.419	0.004	68.8	0.41
24/94	0.79	53.6	0.24	0.55	0.452	0.005	4.47	0.834	1.082	0.453	0.007	65.5	0.64
42/94	0.76	53.8	0.24	0.48	0.519	0.007	5.34	1.043	2.884	0.521	0.011	60.0	0.85
87/35	0.80	53.7	0.24	0.57	0.428	0.191	6.56	1.334	7.179	0.452	0.264	65.7	10.07
123/94	0.81	54.9	0.24	0.57	0.428	0.166	5.43	1.063	4.501	0.451	0.226	67.4	9.66
136/94	0.86	54.7	0.24	0.29	0.711	0.443	5.06	0.975	13.964	0.719	0.608	46.6	11.75
9/136	0.76	53.3	0.24	0.81	0.191	0.026	3.18	0.525	4.142	0.194	0.036	101.8	9.02

Table S4b. E_{FRET} Parameters and Distances from Aggregation Endpoint for αS-$C^{Fam}_{X}PpY^{Tmr}_{Y}$

Donor/Acceptor	Φ_D	R_0 (Å)	$\varepsilon_A/\varepsilon_D$	A	E_D	S_A	B	E_A	S_B	E_{FRET}	S_{EFRET}	R (Å)	σ_R
9/35	0.74	53.1	0.24	0.33	0.667	0.022	5.80	1.153	2.890	0.671	0.031	47.2	2.41
9/94	0.74	53.1	0.24	0.57	0.428	0.026	3.52	0.603	2.147	0.431	0.037	55.7	2.10
24/94	0.77	53.5	0.24	0.49	0.508	0.045	3.71	0.651	12.283	0.509	0.063	53.2	2.44
42/94	0.69	53.3	0.24	0.39	0.610	0.030	6.05	1.213	3.199	0.615	0.042	49.2	2.24
87/35	0.73	53.1	0.24	0.41	0.588	0.165	5.38	1.052	3.495	0.609	0.222	49.4	2.21
123/94	0.75	54.8	0.24	0.46	0.539	0.465	5.87	1.168	8.011	0.574	0.622	52.2	0.81
136/94	0.79	56.0	0.24	0.47	0.528	0.758	3.92	0.702	11.172	0.538	1.001	46.8	1.32
9/136	0.72	53.1	0.24	0.68	0.321	0.081	3.30	0.553	6.726	0.323	0.113	78.6	14.38

Table S4c. E_{FRET} Parameters and Distances from Resuspended Fibrils of αS-$C^{Fam}{}_X PpY^{Tmr}{}_Y$

Donor/Acceptor	Φ_D	R_0 (Å)	$\varepsilon_A/\varepsilon_D$	A	E_D	S_A	B	E_A	S_B	E_{FRET}	S_{EFRET}	R (Å)	σ_R
9/35	0.71	53.0	0.24	0.35	0.655	0.007	6.24	1.257	2.677	0.656	0.009	47.6	0.92
9/94	0.71	52.9	0.24	0.65	0.349	0.051	3.42	0.581	2.077	0.354	0.070	58.5	0.75
24/94	0.73	53.1	0.24	0.73	0.273	0.007	4.57	0.858	0.299	0.285	0.009	61.9	0.08
42/94	0.65	52.8	0.24	0.32	0.678	0.055	5.81	1.154	1.108	0.702	0.073	45.8	0.13
87/35	0.72	53.1	0.24	0.28	0.720	0.018	5.16	0.998	0.351	0.733	0.025	44.8	0.07
123/94	0.75	54.8	0.24	0.45	0.547	0.546	5.77	1.145	9.057	0.581	0.728	51.9	0.91
136/94	0.78	55.8	0.24	0.43	0.568	0.578	3.80	0.672	7.819	0.573	0.759	53.2	4.73
9/136	0.68	52.7	0.24	0.82	0.183	0.076	3.82	0.677	3.003	0.196	0.106	100.2	25.24

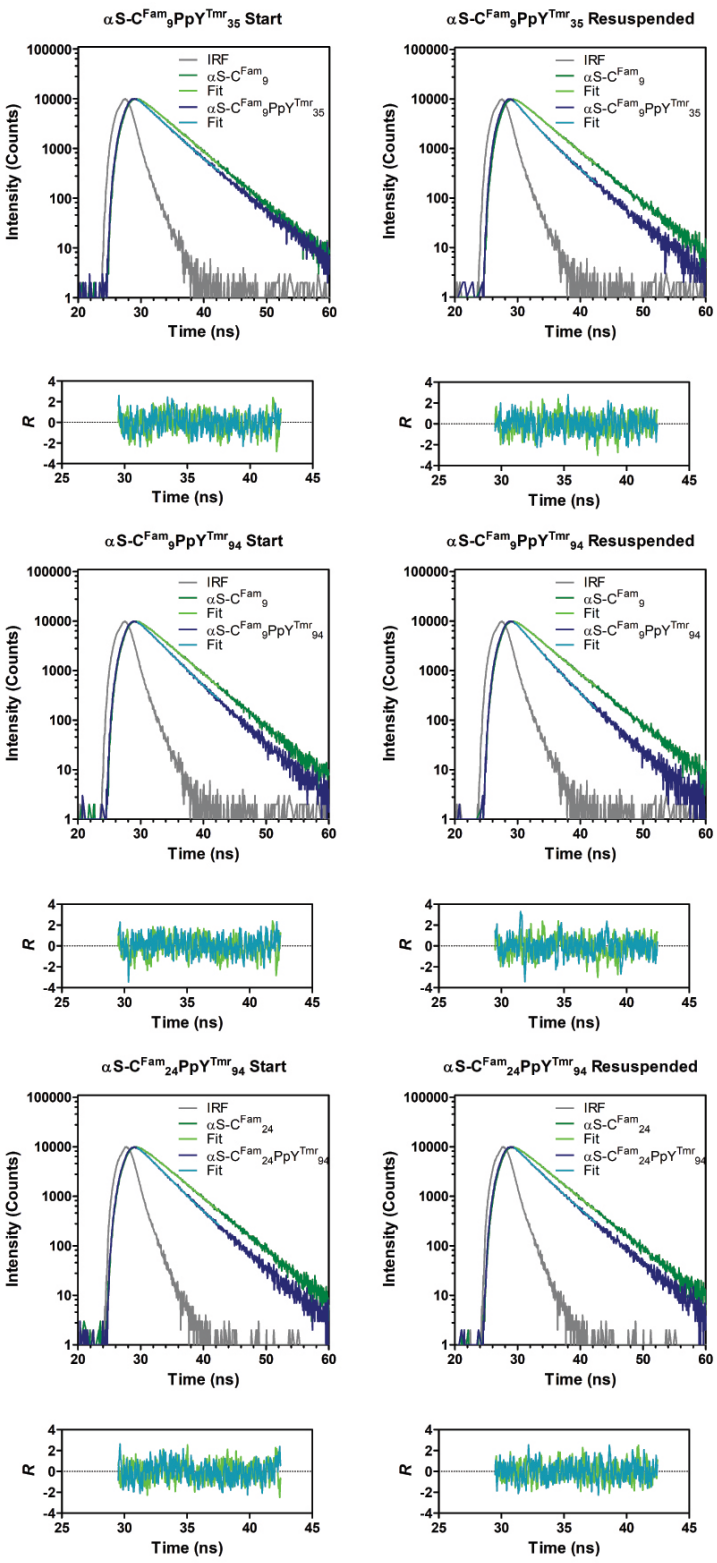

Figure S17a. Lifetime Data for Labeled αS. The identity and time point associated with each plot is indicated above the TCSPC decay. Residual values from the fit are plotted below the corresponding decay data.

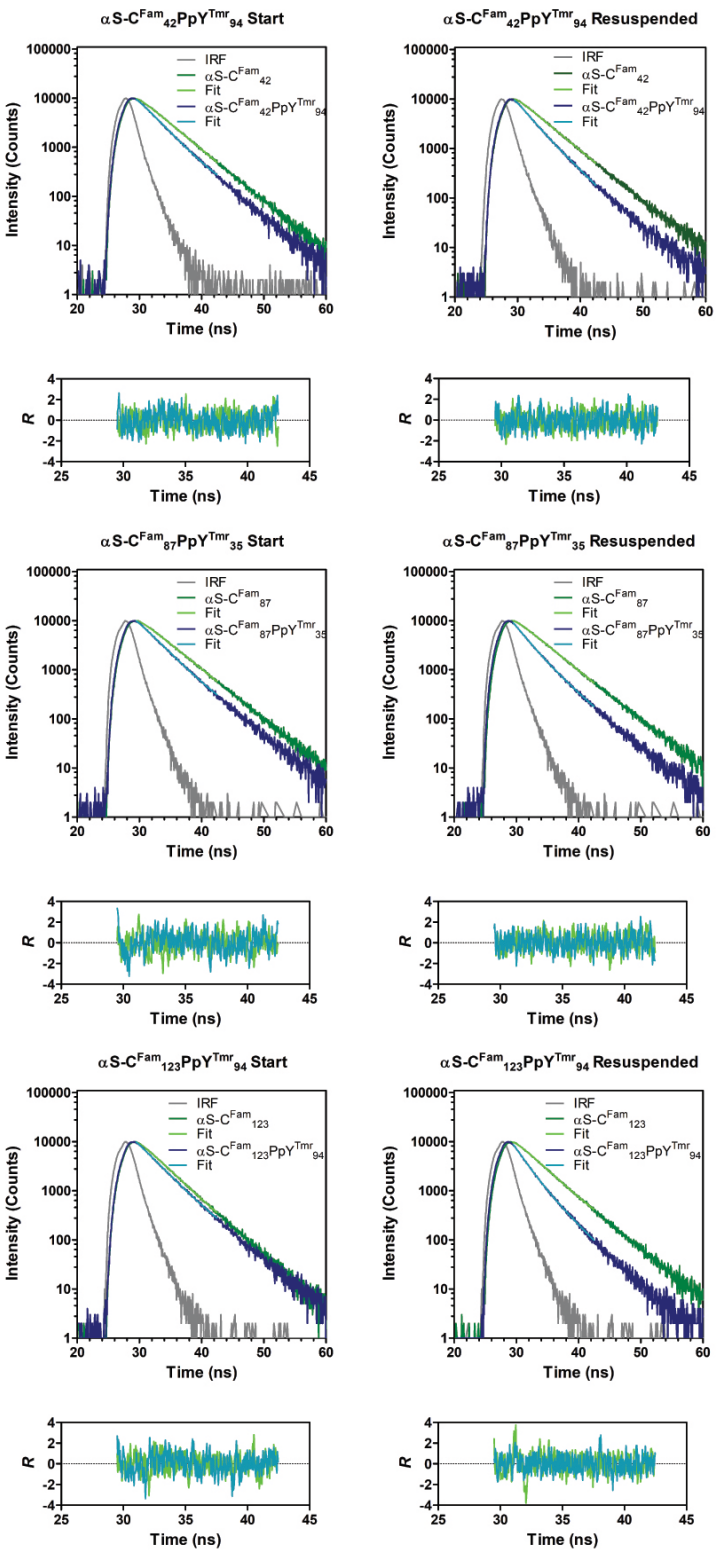

Figure S17b. Lifetime Data for Labeled αS. The identity and time point associated with each plot is indicated above the TCSPC decay. Residual values from the fit are plotted below the corresponding decay data.

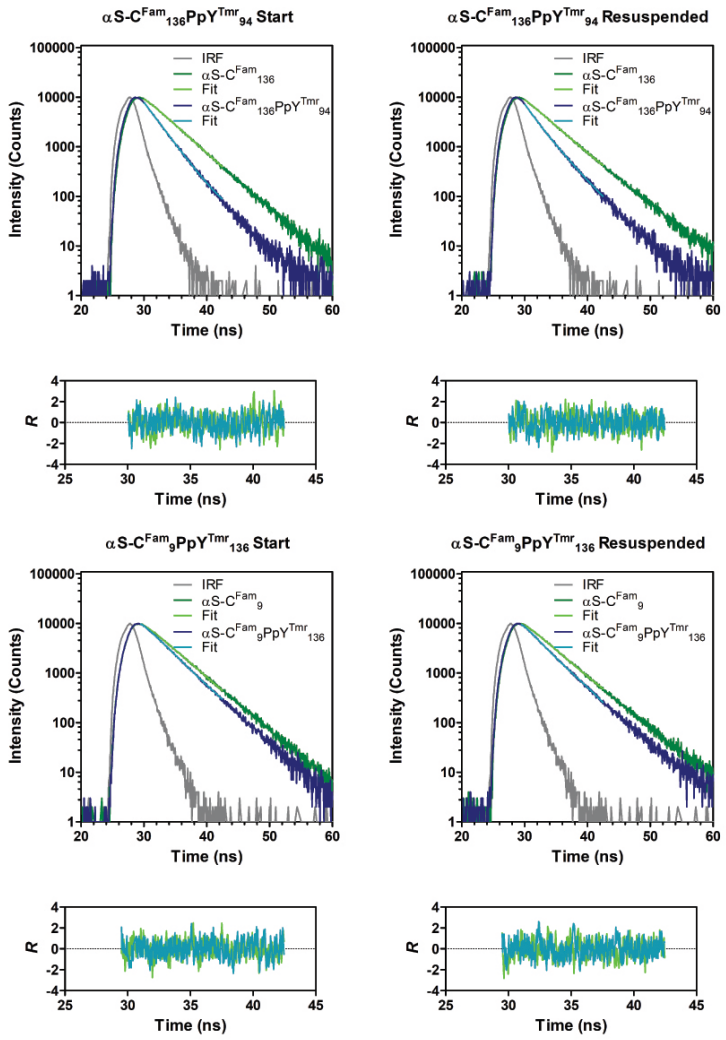

Figure S17c. Lifetime Data for Labeled αS. The identity and time point associated with each plot is indicated above the TCSPC decay. Residual values from the fit are plotted below the corresponding decay data.

Table S5. Fluorescence Lifetimes of Donor- and Doubly-Labeled Proteins.

Start						Resuspended Fibrils					
Protein	τ1 (ns)	τ2 (ns)	τ_{avg} (ns)	χ^2	E_{FRET}	Protein	τ1 (ns)	τ2 (ns)	τ_{avg} (ns)	χ^2	E_{FRET}
αS-C^{Fam}_{9}	3.959			1.03		αS-C^{Fam}_{9}	3.88			1.05	
αS-$C^{Fam}_{9}PpY^{Tmr}_{35}$	3.573			0.97	0.10	αS-$C^{Fam}_{9}PpY^{Tmr}_{35}$	1.33	3.62	2.31	0.97	0.41
αS-$C^{Fam}_{9}PpY^{Tmr}_{94}$	3.257			0.92	0.18	αS-$C^{Fam}_{9}PpY^{Tmr}_{94}$	1.90	4.01	2.59	1.11	0.33
αS-C^{Fam}_{24}	4.08			0.93		αS-C^{Fam}_{24}	4.06			0.86	
αS-$C^{Fam}_{24}PpY^{Tmr}_{94}$	3.26			1.00	0.20	αS-$C^{Fam}_{24}PpY^{Tmr}_{94}$	1.07	3.60	2.73	0.95	0.33
αS-C^{Fam}_{42}	4.06			1.05		αS-C^{Fam}_{42}	3.97			0.84	
αS-$C^{Fam}_{42}PpY^{Tmr}_{94}$	3.00			1.13	0.26	αS-$C^{Fam}_{42}PpY^{Tmr}_{94}$	1.82	3.97	2.54	0.97	0.36
αS-C^{Fam}_{87}	4.26			1.06		αS-C^{Fam}_{87}	4.15			0.95	
αS-$C^{Fam}_{87}PpY^{Tmr}_{35}$	3.39			1.23	0.20	αS-$C^{Fam}_{87}PpY^{Tmr}_{35}$	1.58	3.58	2.37	0.87	0.43
αS-C^{Fam}_{123}	3.56			1.00		αS-C^{Fam}_{123}	3.69			1.12	
αS-$C^{Fam}_{123}PpY^{Tmr}_{94}$	3.35			1.16	0.06	αS-$C^{Fam}_{123}PpY^{Tmr}_{94}$	1.62	3.55	2.05	0.86	0.44
αS-C^{Fam}_{136}	3.79			1.03		αS-C^{Fam}_{136}	3.84			0.90	
αS-$C^{Fam}_{136}PpY^{Tmr}_{94}$	2.52			0.98	0.34	αS-$C^{Fam}_{136}PpY^{Tmr}_{94}$	1.16	3.02	1.86	0.93	0.51
αS-$C^{Fam}_{9}PpY^{Tmr}_{136}$	3.41			0.93	0.15	αS-$C^{Fam}_{9}PpY^{Tmr}_{136}$	2.29	5.34	3.09	0.98	0.21

Cryogen Treatment of αS Fibrils. Samples comprised of 1% αS-$C^{Fam}_{9}PpY^{Tmr}_{94}$, αS-$C^{Fam}_{87}PpY^{Tmr}_{35}$ or αS-$C^{Fam}_{123}PpY^{Tmr}_{94}$ were prepared and aggregated as described above. Following aggregation, the fibrils were pelleted by centrifugation (13,200 rpm, 90 min, 4 °C). After centrifugation, the supernatant was removed and the pellet resuspended in an equivalent volume of αS buffer (20 mM Tris, 100 mM NaCl pH 7.5). Each sample was then diluted ten fold (15 μL resuspended fibril, 135 μL buffer) and frozen in liquid nitrogen for 5-10 minutes. Following this treatment, samples were thawed at room temperature and fluorescence spectra

collected as described above. The resulting data are plotted (Fig. S18a) as the raw spectra and normalized to the area under the curve of the resulting doubly-labeled protein spectra.

Similarly, samples comprised of 25% αS-C^{Pyr}_{9}, αS-C^{Pyr}_{42} or αS-C^{Pyr}_{123} were prepared as described above. Following aggregation, the fibrils were pelleted by centrifugation (13,200 rpm, 90 min, 4 °C). After centrifugation, the supernatant was removed and the pellet resuspended in an equivalent volume of αS buffer (20 mM Tris, 100 mM NaCl pH 7.5). Each sample was then diluted ten fold (15 μL resuspended fibril, 135 μL buffer) and frozen in liquid nitrogen for 5-10 minutes. Following this treatment, samples were thawed at room temperature and fluorescence spectra collected as described above. The resulting data are plotted as normalized spectra to the max monomeric emission peak (385 nm) (Fig. S18b).

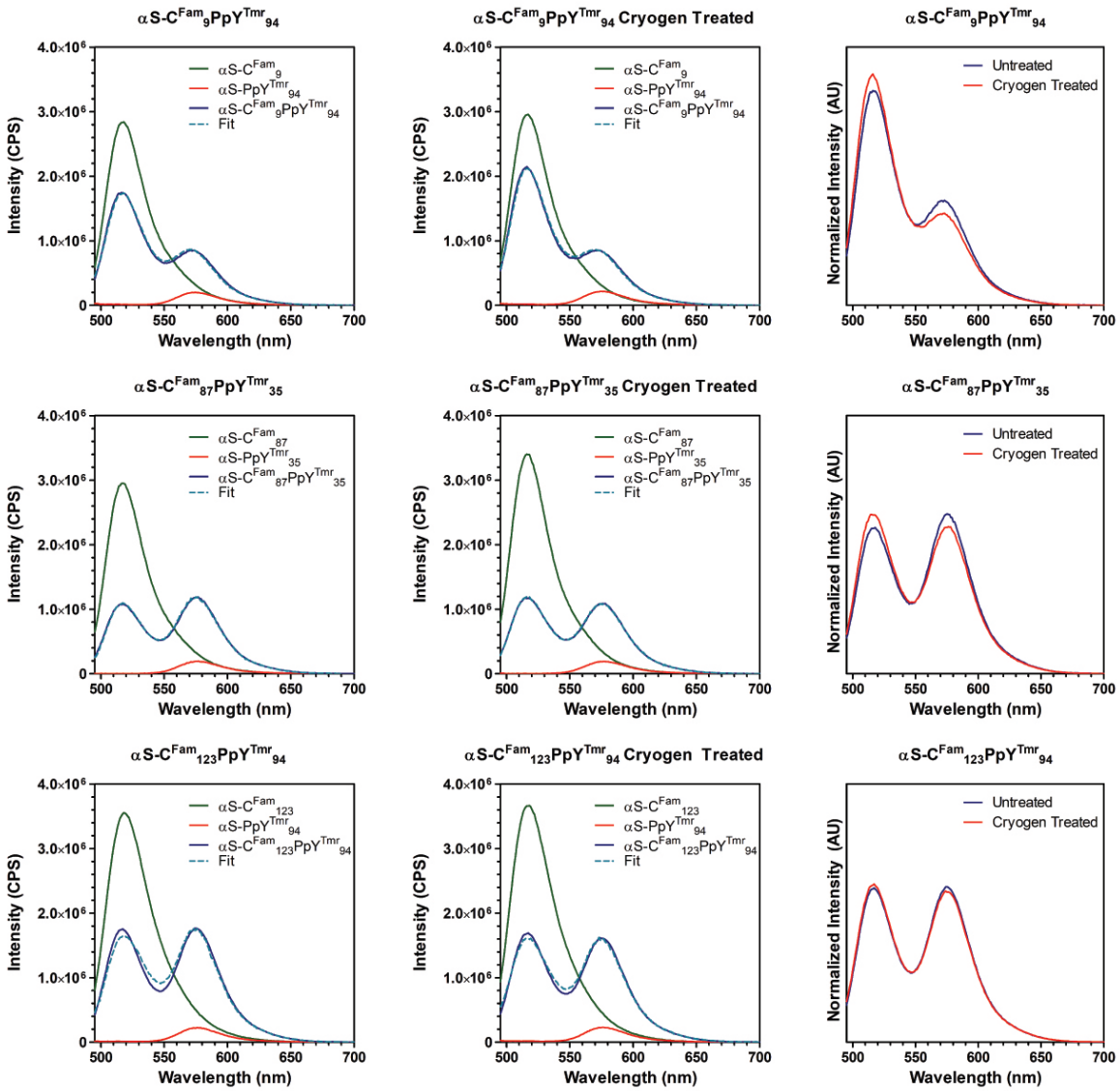

Figure S18a. Steady-state Fluorescence Spectra of Cryogen Treated Samples. Steady-state emission spectra of αS-$C^{Fam}_{x}PpY^{Tmr}_{Y}$ labeled proteins either without (left) or with (middle) cryogenic freezing. At right: doubly-labeled protein emission spectra normalized to the area under the emission spectrum.

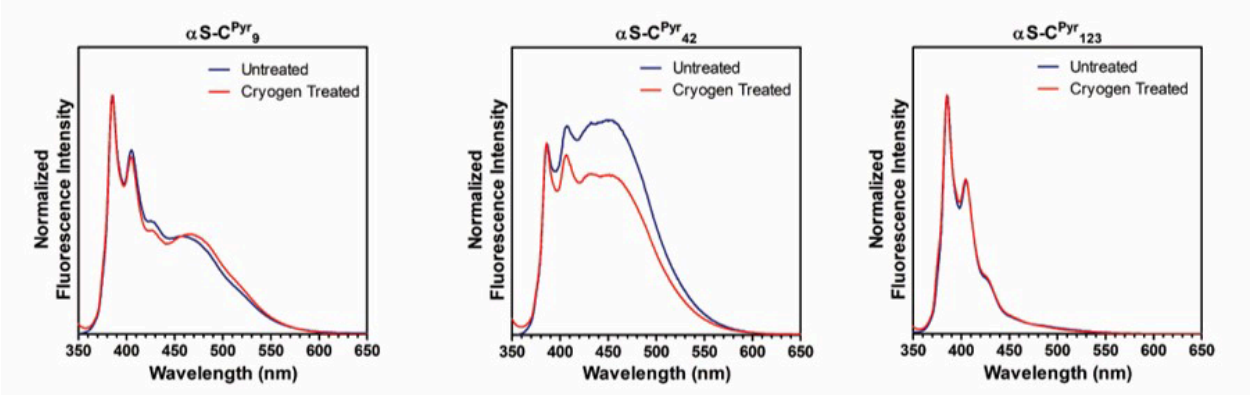

Figure S18b. Emission Spectra of Cryogen Treated αS-C$^{Pyr}_{X}$ Proteins. Emission spectra collected without (blue) or with (red) cryogenic freezing and thawing and normalized relative to the maximum monomeric emission peak (385 nm).

Table S6. E_{FRET} Parameters and Distances from Cryogenically Treated Fibrils of αS-C$^{Fam}_{X}$PpY$^{Tmr}_{Y}$

Donor/Acceptor	Φ_D	R_0 (Å)	$\varepsilon_A/\varepsilon_D$	A	E_D	S_A	B	E_A	S_B	E_{FRET}	S_{EFRET}	R (Å)	σ_R
9/94	0.71	53.6	0.24	0.59	0.407	0.032	3.31	0.555	0.683	0.413	0.044	56.8	0.90
9/94 Cryo	0.73	53.7	0.24	0.70	0.299	0.029	2.88	0.452	2.100	0.302	0.041	61.9	2.54
87/35	0.70	54.2	0.24	0.41	0.630	0.027	5.54	1.090	2.078	0.636	0.037	48.6	2.86
87/35 Cryo	0.74	53.9	0.24	0.35	0.649	0.014	5.09	0.981	2.921	0.650	0.019	48.5	2.74
123/94	0.74	54.2	0.24	0.44	0.561	0.008	6.90	1.416	0.521	0.576	0.012	51.5	0.39
123/94 Cryo	0.76	54.4	0.24	0.41	0.588	0.009	6.20	1.247	5.430	0.589	0.012	51.2	1.28

SDS-PAGE Analysis of Labeled Protein Incorporation. Triplicate samples of WT αS, or doubly-labeled protein in 99% WT αS were prepared and aggregated as described above. A 30 μL aliquot was removed and stored at -20 °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 minutes, 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 SDS (2 μL from a 150 mM stock in water; 25 mM final concentration) and boiled for 15-20 minutes. The samples were then cooled on ice for 10-15 minutes, then 3 μL 4X loading dye added. Monomeric samples for calibration were prepared by serial 2-fold dilution of 10 μL with water (standard lanes correspond to 100, 50, 25, and 12.5

μM total protein from left to right with 1% αS-$C^{Fam}_{X}PpY^{Tmr}_{Y}$); 2 μL water was then added to each standard sample, followed by 3 μL loading dye. Standard and pellet samples were then analyzed by SDS-PAGE (15% acrylamide gel run at 150 V for 1.5hours). Fluorescence images were acquired using a Typhoon FLA7000, and each gel stained with Coomassie Brilliant Blue then destained at room temperature and imaged (Figure S18). 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. S17).

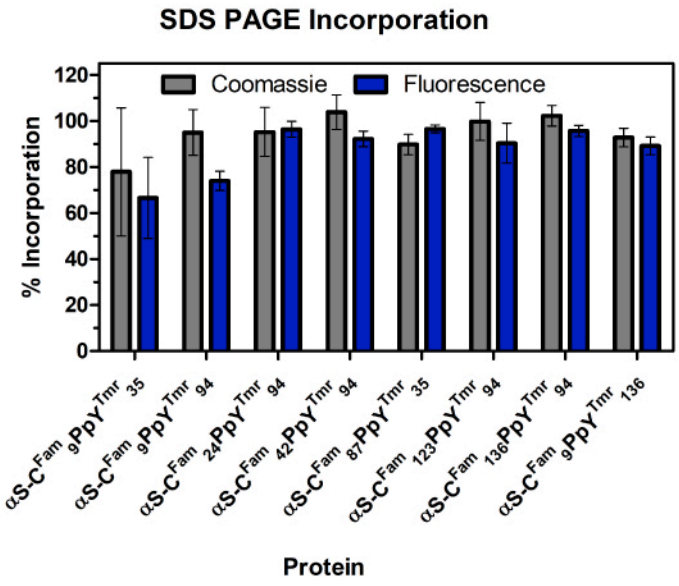

Figure 19. Protein Incorporation into Fibrils by SDS-PAGE. Gel densitometry relative to monomeric standards was used to quantify protein incorporated into fibrils by Coomassie Brilliant Blue staining (grey bars) or in gel fluorescence (blue bars). Bars represent the average of triplicate samples with standard deviation as the error bars.

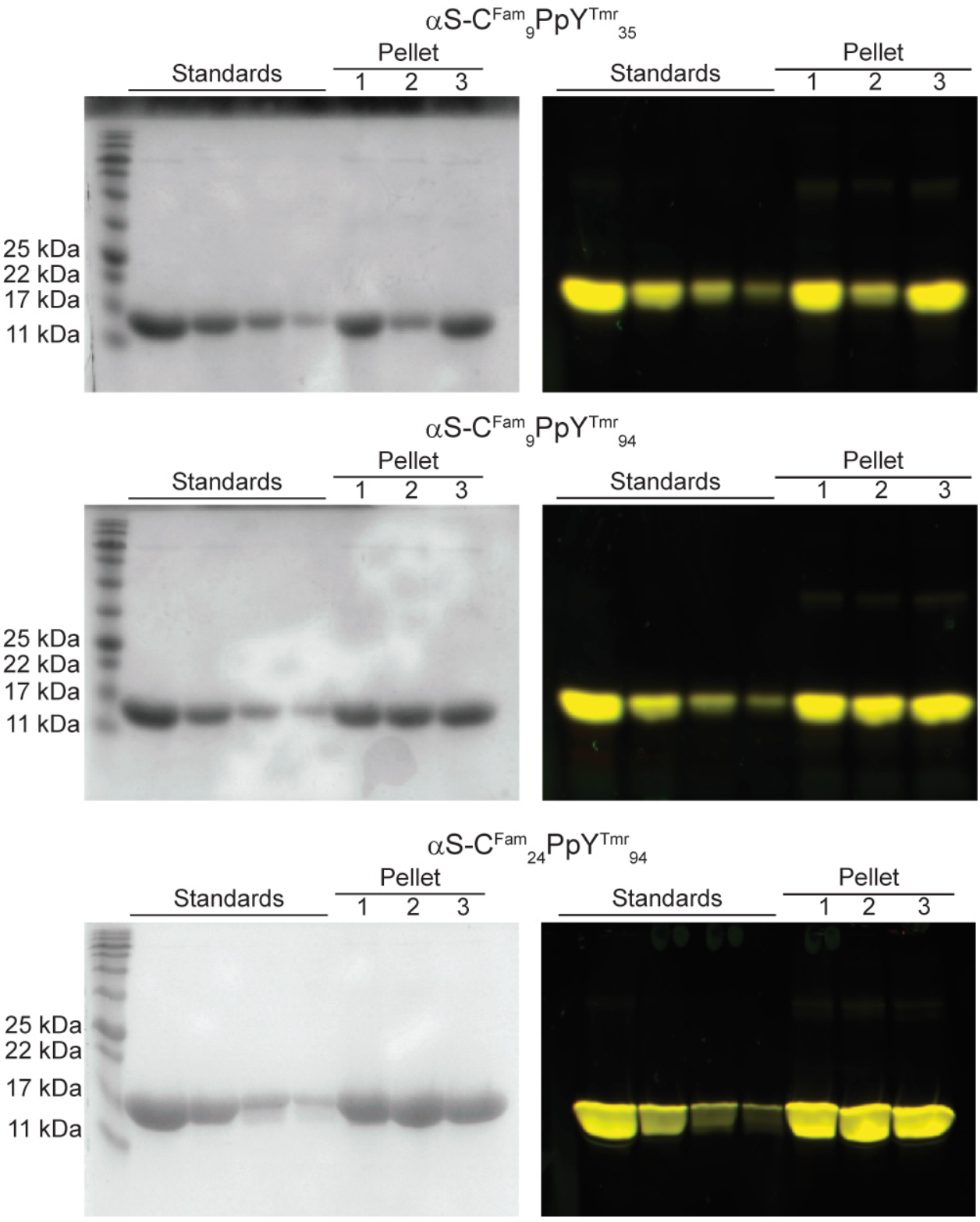

Figure S20a. SDS-PAGE Gels. The identity of each protein is indicated above the corresponding gels. Left: Coomassie stained gel images. Right: In-gel fluorescence, merged fluorescein and rhodamine emission.

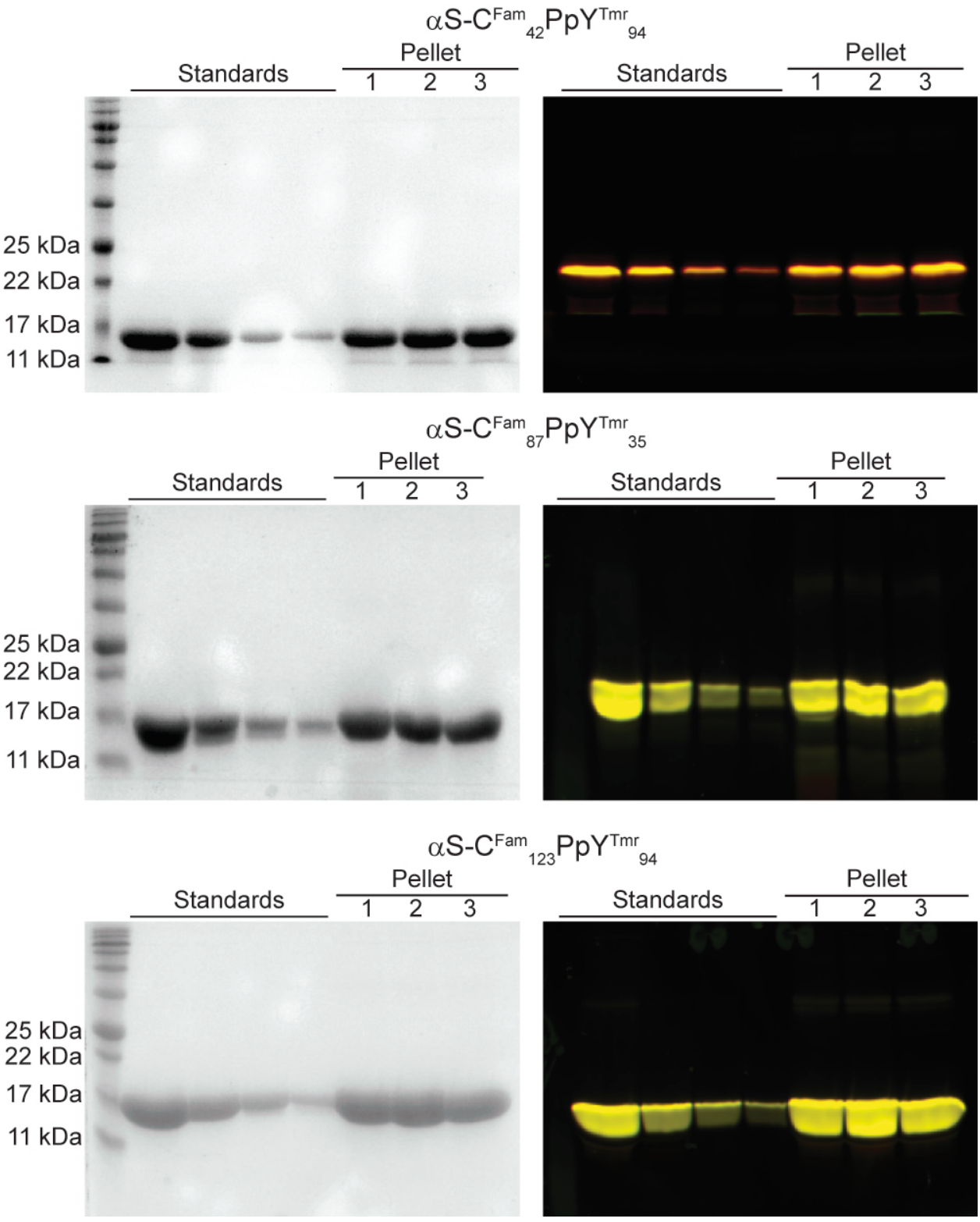

Figure S20b. SDS-PAGE Gels. The identity of each protein is indicated above the corresponding gels. Left: Coomassie stained gel images. Right: In-gel fluorescence, merged fluorescein and rhodamine emission.

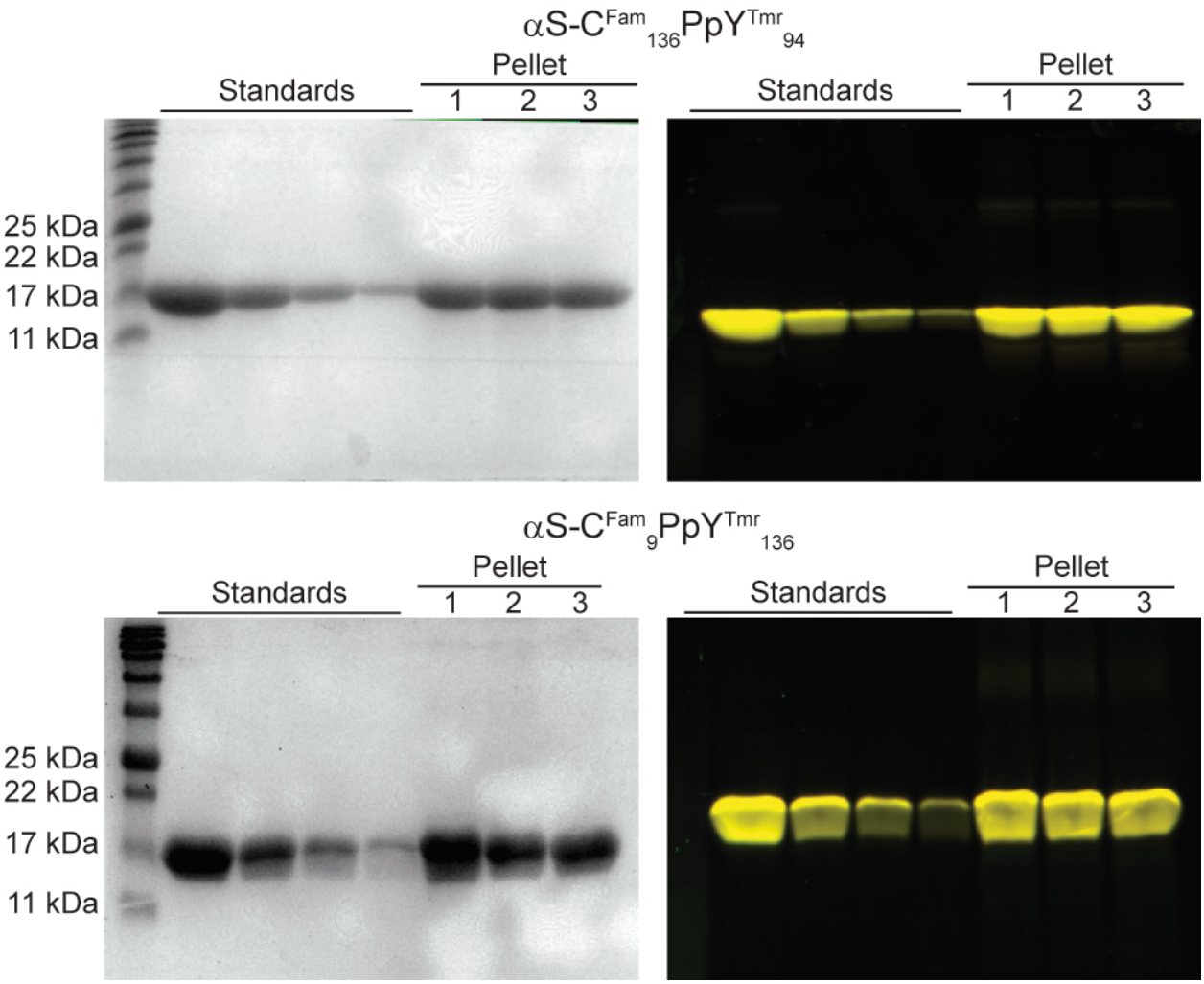

Figure S20c. The identity of each protein is indicated above the corresponding gels. Left: Coomassie stained gel images. Right: In-gel fluorescence, merged fluorescein and rhodamine emission.

Transmission Electron Microscopy (TEM). TEM was carried out on an FEI Tecnai T12 instrument with an accelerating voltage of 120 kV. Fibril samples obtained from aggregation and centrifugation and stored at – 20 °C as dry pellets were resuspended in 20 mM Tris, 100 mM NaCl pH 7.5. Glow discharged 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 dried at room temperature for 2 minutes and then were

imaged. Images were collected at magnifications ranging from 6500 x to 42000 x. Fibril images were examined for qualitative deviations from WT αS fibrils.

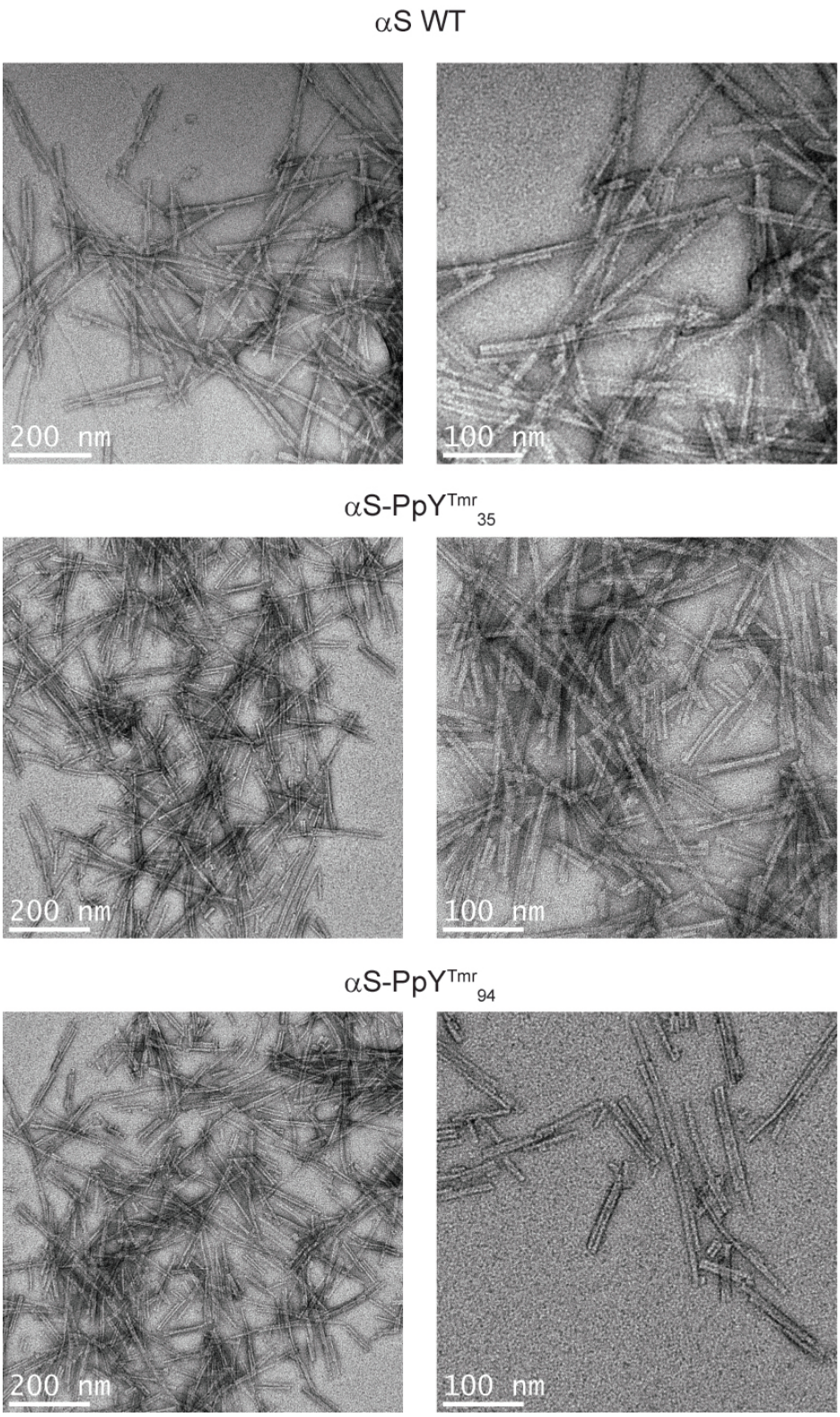

Figure S21a. Transmission Electron Micrographs. Representative images of fibrils containing the protein indicated above the images, acquired at 21000 x (left) or 42000 x (right) magnification.

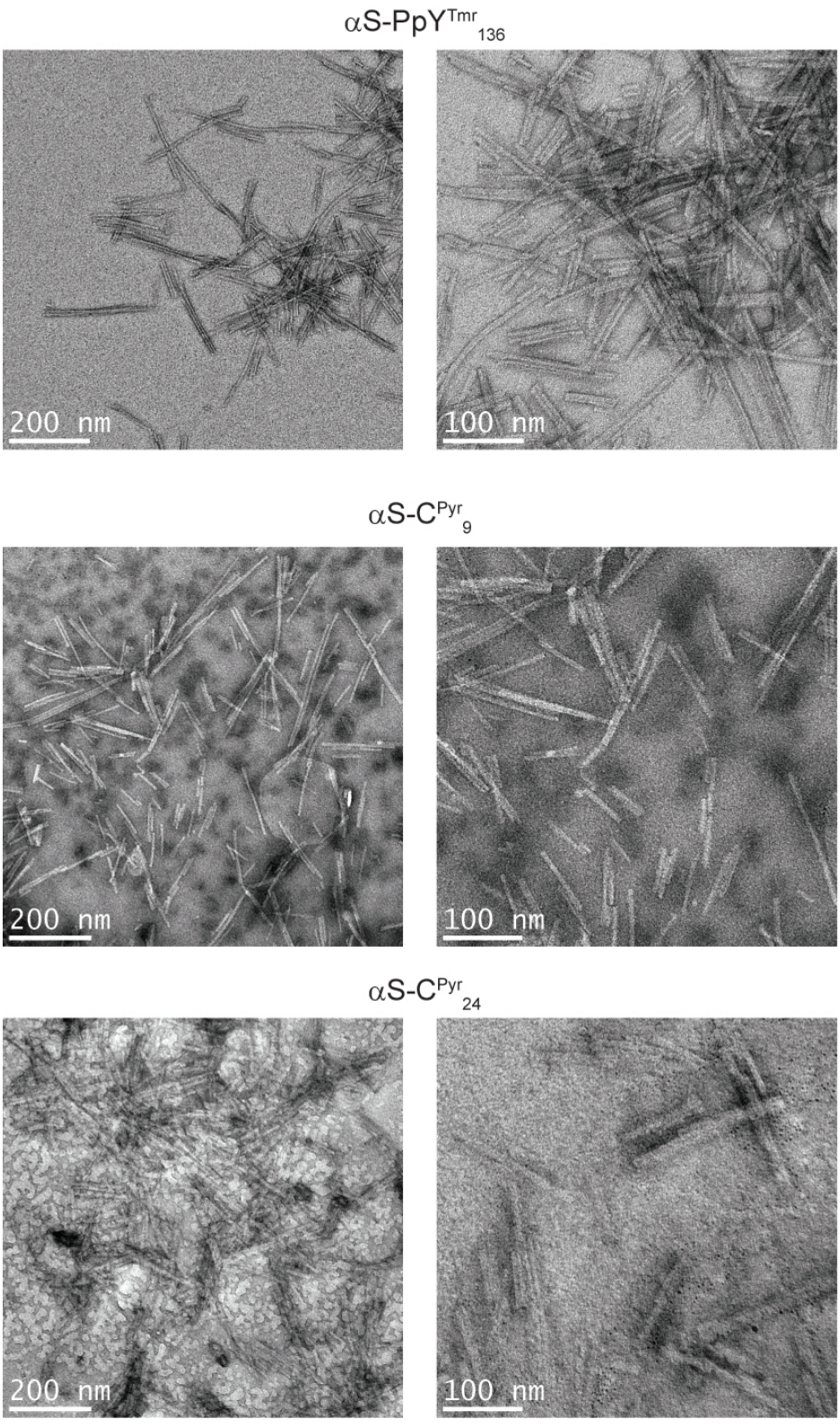

Figure S21b. Transmission Electron Micrographs. Representative images of fibrils containing the protein indicated above the images, acquired at 21000 x (left) or 42000 x (right) magnification.

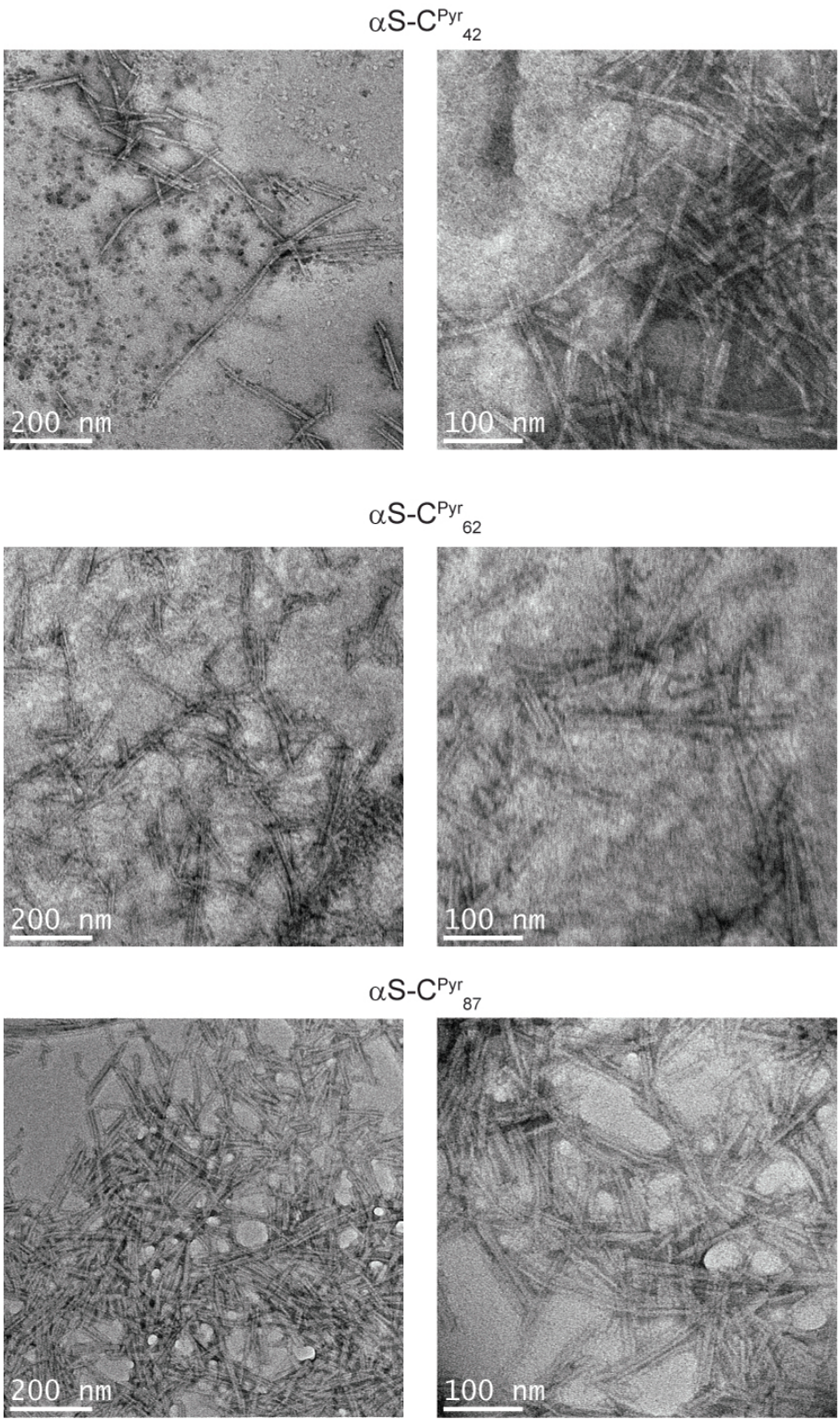

Figure S21c. Transmission Electron Micrographs. Representative images of fibrils containing the protein indicated above the images, acquired at 21000 x (left) or 42000 x (right) magnification.

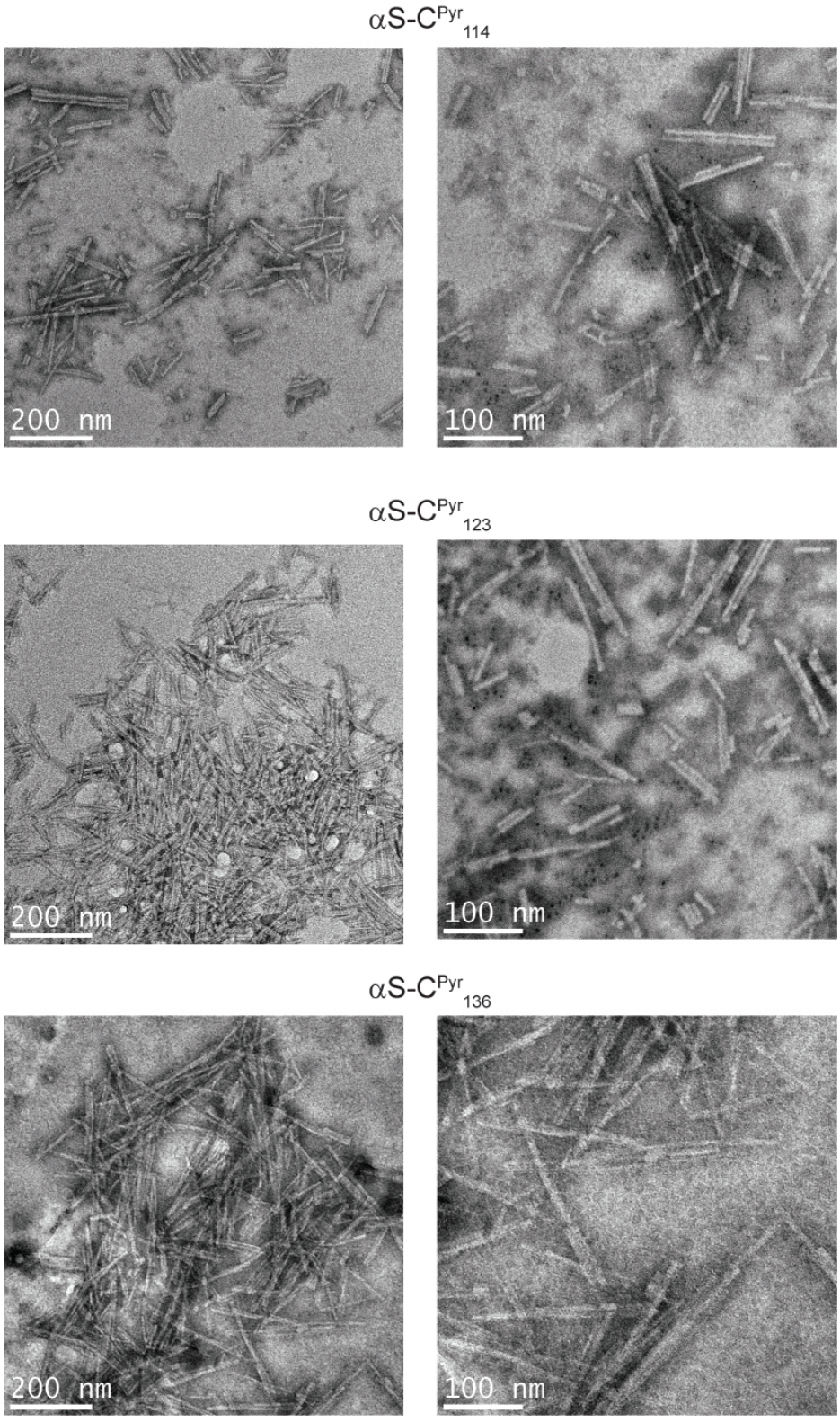

Figure S21d. Transmission Electron Micrographs. Representative images of fibrils containing the protein indicated above the images, acquired at 21000 x (left) or 42000 x (right) magnification.

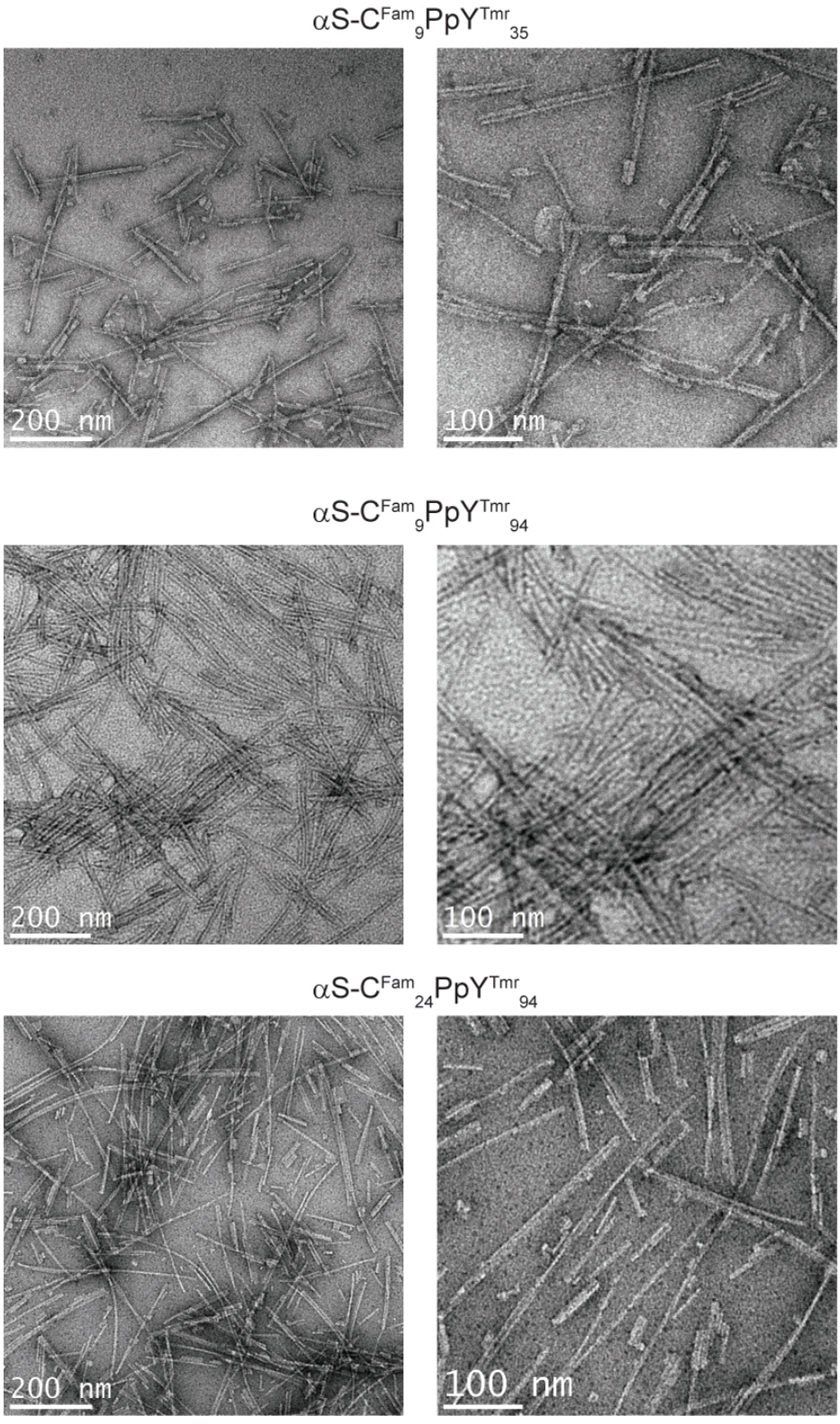

Figure S21e. Transmission Electron Micrographs. Representative images of fibrils containing the protein indicated above the images, acquired at 21000 x (left) or 42000 x (right) magnification.

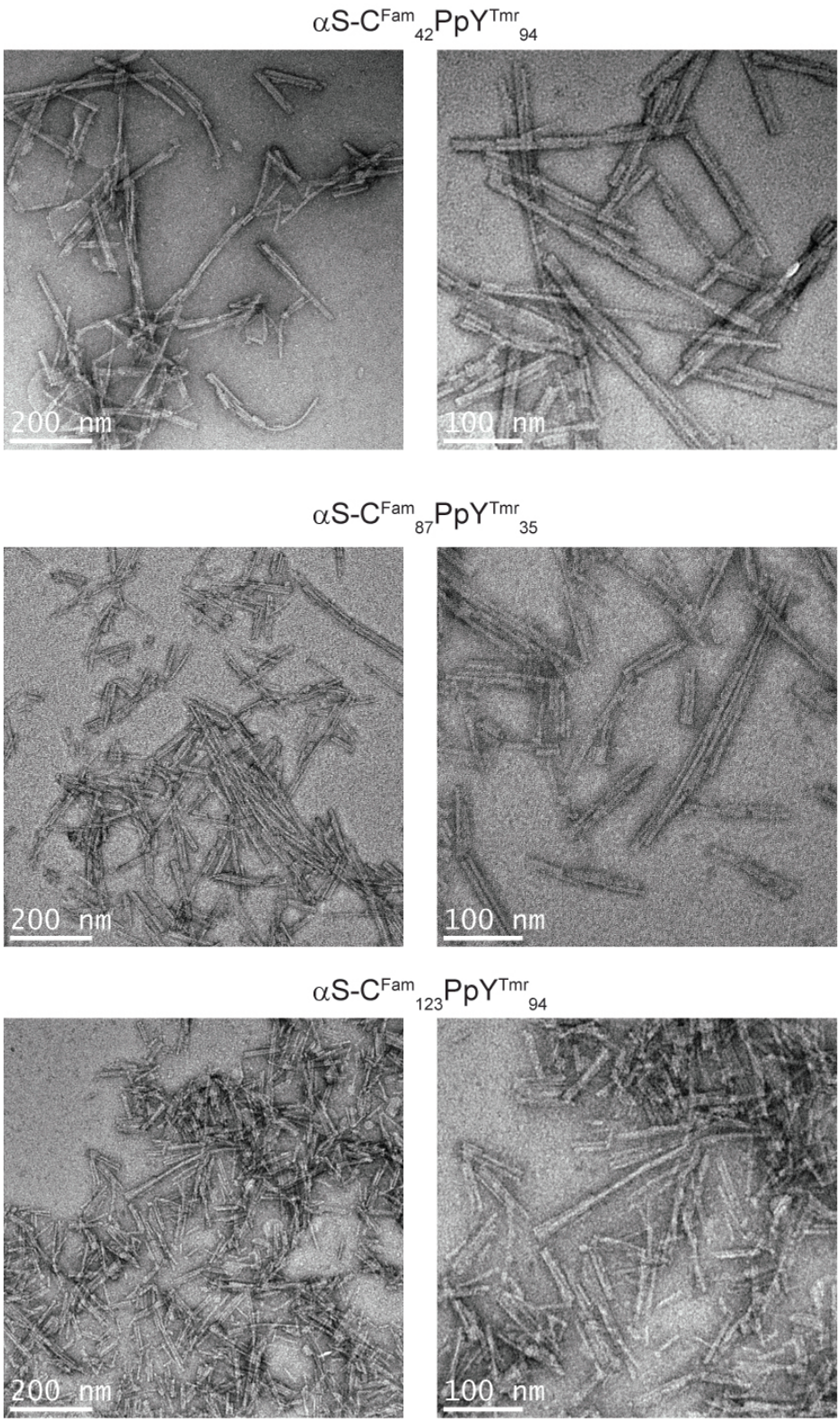

Figure S21f. Transmission Electron Micrographs. Representative images of fibrils containing the protein indicated above the images, acquired at 21000 x (left) or 42000 x (right) magnification.

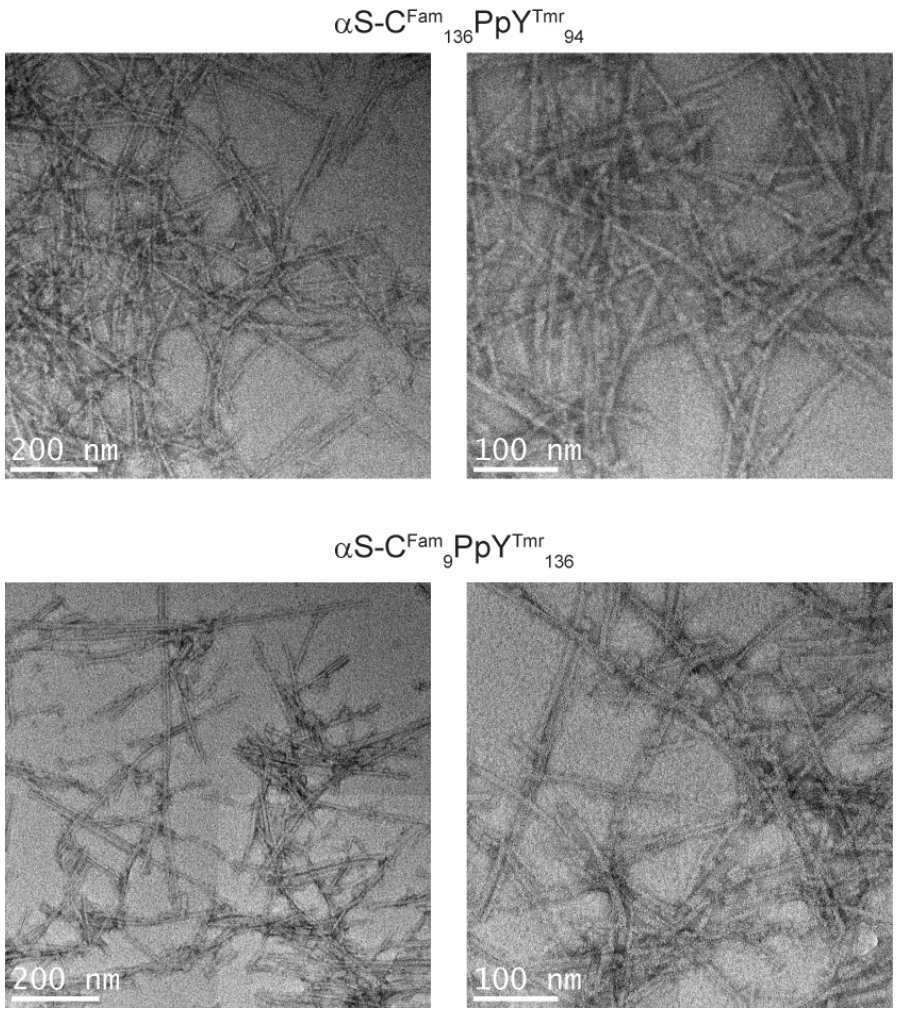

Figure S21g. Transmission Electron Micrographs. Representative images of fibrils containing the protein indicated above the images, acquired at 21000 x (left) or 42000 x (right) magnification.

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