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Supplemental Information

**Using a FRET Library with Multiple Probe Pairs To Drive Monte Carlo
Simulations of α -Synuclein**

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Using a Large Ensemble FRET Library with Multiple Probe Pairs to Drive Monte Carlo Simulations of Disordered α -Synuclein

Supplementary Information

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Table of Contents

General Information	S2
Protein Synthesis	S3
Protein Library Characterization	S7
FRET Data Collection and Analysis	S23
Steady-state Fluorescence Spectra and Fitting	S28
Fluorescence Lifetime Data and Fitting	S57
FCS and AFM Characterization	S66
Computational Modelling	S70
Analysis of Simulated Structure Ensembles	S88
References	S101

General Information:

Materials: *E. coli* BL21(DE3) cells were purchased from Stratagene (La Jolla, CA, USA). Milli-Q filtered (18 M Ω) water was used for all solutions (Millipore; Billerica, MA, USA). Bradford reagent assay kits were purchased from BioRAD (Hercules, CA, USA). The unnatural amino acid *p*-cyanophenylalanine was purchased from PepTech (Bedford, MA, USA; Cat. # AL240-2). Fluorescein-5-maleimide (Fam) was purchased from TCI America (Portland, OR, USA; Cat. # F0810); carboxytetramethylrhodamine-5-azide (Raz) was ordered from Lumiprobe (Hallendale Beach, FL, USA; Cat # D7130) and AlexaFluor488 maleimide, C5 isomer, was purchased from ThermoFisher Scientific (Pittsburgh, PA, USA; Cat. # A10254). *O*-Propargyltyrosine (Ppy) was synthesized as previously described(1). Amicon Ultra centrifugal filter units (3 kDa MWCO) were purchased from EMD Millipore. All other reagents and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich unless otherwise specified. DNA sequencing was performed at the University of Pennsylvania DNA sequencing facility.

Instruments: Matrix assisted laser desorption/ionization with time-of-flight detector (MALDI-TOF) mass spectra were acquired on a Bruker Ultraflex III instrument (Billerica, MA, USA). UV-Vis absorption spectra were acquired on a Hewlett-Packard 8452A diode array spectrophotometer (currently Agilent Technologies; Santa Clara, CA, USA). Fluorescence spectra were collected on a Photon Technologies International (PTI) QuantaMaster40 fluorometer (currently Horiba Scientific, Edison, NJ, USA). Details regarding the fluorescence correlation spectroscopy (FCS) equipment are contained within the FCS Measurements section of the SI. Atomic force microscopy (AFM) measurements were made using a Keysight 5500 AFM instrument (Keysight Technologies; Santa Rosa, CA, USA) equipped with a closed-loop scanner.

Protein Synthesis

DNA Oligomers Used for α S Quikchange Mutagenesis:

Mutation	Direction	Primer Sequence
Y39F	Forward	5'-GGA AAG ACA AAA GAG GGT GTT CTC TTT GTA GGC TCC AAA-3'
	Reverse	5'-TTT GGA GCC TAC AAA GAG AAC ACC CTC TTT TGT CTT TCC-3'
Y125F	Forward	5'-GGA TCC TGA CAA TGA GGC TTT TGA AAT GCC TTC TGA-3'
	Reverse	5'-TCA GAA GGC ATT TCA AAA GCC TCA TTG TCA GGA TCC-3'
Y133F-Y136F	Forward	5'-CCT TCT GGA GAA GGG TTT CAA GAC TTC GAA CCT GAA GCC-3'
	Reverse	5'-GGC TTC AAG TTC GAA GTC TTG AAA CCC TTC CTC AGA AGG-3'
Y24W	Forward	5'-TCT GCC ACA CCC CAT TTG GTT TTC TCA GCA GCA GCC A-3'
	Reverse	5'-TGG CTG CTG CTG AGA AAA CCA AAT GGG GTG TGG CAG A-3'
Y39W	Forward	5'-ACA AAA GAG GGT GTT CTC TGG GTA GGC TCC AAA ACC AAG G-3'
	Reverse	5'-CCT TGG TTT TGG AGC CTA CCC AGA GAA CAC CCT CTT TTG T-3'
Q62W	Forward	5'-TGC TCC TCC AAC ATT TGT CAC CCA CTC TTT GGT CTT CTC AGC CAC-3'
	Reverse	5'-GTG GCT GAG AAG ACC AAA GAG TGG GTG ACA AAT GTT GGA GGA GCA-3'
Q79W	Forward	5'-CTC CCT CCA CTG TCT TCC AGG CTA CTG CTG TCA CAC-3'
	Reverse	5'-GTG TGA CAG CAG TAG CCT GGA AGA CAG TGG AGG GAG-3'
F94W	Forward	5'-TGC AGC AGC CAC TGG CTG GGT CAA AAA GGA CCA GTT C-3'
	Reverse	5'-CAA CTG GTC CTT TTT GAC CCA GCC AGT GGC TGC TGC A-3'
Y125W	Forward	5'-GAT CCT GAC AAT GAG GCT TGG GAA ATG CCT TCT GAG GAA G-3'
	Reverse	5'-CTT CCT CAG AGG GCA TTT CCC AAG CCT CAT TGT CAG GAT C-3'
Y39TAG	Forward	5'-AAA AGA GGG TGT TCT CTA GGT AGG TCT CAA AAC CAA G-3'
	Reverse	5'-CTT GGT TTT GGA GCC TAC CTA GAG AAC ACC CTC TTT T-3'
F94TAG	Forward	5'-GCA TTG CAG CAG CCA CTG GCT AGG TCA AAA AGG ACC AGT TGG G-3'
	Reverse	5'-CCC AAC TGG TCC TTT TTG ACC TAG CCA GTG GCT GCA ATG C-3'
Y125TAG	Forward	5'-CCT GAC AAT GAG GCT TAG GAA ATG CCT TCT GAG GA-3'
	Reverse	5'-TCC TCA GAA GGC ATT TCC TAA GCC TCA TTG TCA GG -3'
Y133F- Y136TAG	Forward	5'-TGC CTT CTG AGG AAG GGT TTC AAG ACT AGG AAC C-3'
	Reverse	5'-GGT TCC TAG TCT TGA AAC CCT TCC TCA GAA GGC A-3'

All of constructs and primers required for production of the Fam-Raz library have been previously reported. The construct containing the Y136TAG mutation, primers for all of Cys mutation primers have also been previously reported (2-4).

General Expression Protocol for α S-intein- H_6 Fusion Proteins: A pTXB1 plasmid encoding for the α S-intein- H_6 fusion protein was transformed into *E. coli* BL21-Gold (DE3) cells. The DNA GyrA intein from Mxe fusion construct has been previously reported (4). Transformed cells were selected based on ampicillin (Amp) (100 mg/L) resistance, encoded on the pTXB1 plasmid containing the protein of interest. Single colonies were selected and used to inoculate 5 mL primary cultures of LB media, and were grown at 37 °C, shaking at 250 rpm in the presence of 100 μ g/mL Amp. A secondary culture of LB media containing 100 mg/L Amp was inoculated with a single saturated primary culture, and was grown at 37 °C while shaking at 250 rpm. After reaching an OD₆₀₀ of 0.7-1.0, 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to the culture to induce expression. Following induction the culture continued to grow overnight at 18 °C overnight.

Expression of α S-intein- H_6 Proteins Containing Unnatural Amino Acids: The procedure for the expression of mutants containing Cnf or Ppy was identical to the general protocol above with the following noted exceptions. The pTXB1 plasmid coding for the protein of interest contained an amber stop codon (TAG) at the intended site for unnatural amino acid introduction was co-transformed with a plasmid encoding a pDULE2-pFX plasmid containing an orthogonal aminoacyl synthetase/tRNA pair (4). pDULE2-pFX encodes streptomycin (Strep) resistance, and cells containing both plasmids were selected for based on Strep (100mg/L) resistance in addition to Amp resistance. Primary cultures were also grown in the presence of both Strep (100 μ g/mL) and Amp (100 μ g/mL). In place of LB, secondary cultures were grown in M9 minimal media containing 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl and 1 mL of 2 M MgSO₄, 1 mL of 1mg/mL FeCl₂ (in 1.0 M HCl), 1 mL of 15 mg/mL, 2 mL of 10% Yeast Extract, 12.5 mL 40% glucose (w/v) in 1L of autoclaved water, along with 100 mg/L Amp and 100 mg/L Strep. Lastly, once the secondary culture reached an OD₆₀₀ of 0.7-1.0, 0.8 mM of the unnatural amino acid was added to culture prior to induction with IPTG. Expression was performed overnight at 18 °C.

General Purification Protocol for Cnf-Trp containing α S-intein- H_6 Fusion Proteins: Cells were harvested via centrifugation at 5000 rpm for 20 minutes at 4 °C with a GS3 rotor on a Sorvall RC-5 centrifuge. The supernatant was decanted away from the cell pellet, and the pellet was resuspended in 20 mL resuspension buffer containing 40 mM Tris, 5 mM EDTA, pH 8.3 and a protease inhibitor tablet (Roche cOmplete mini tabs, EDTA free). Cells were lysed by sonication

at an amplitude of 30 for 5 minutes with 1 second on 1 second off. Lysate was subsequently centrifuged at 14000 rpm for 20 minutes using an SS-34 rotor on a Sorvall RC-5 centrifuge. His tagged protein was isolated from the supernatant via nickel affinity. Ni-NTA resin (3 mL CV) was incubated with the supernatant on ice for 1 hour. Following incubation the mixture was loaded into a fritted column, and the lysate was drained. The resin was initially washed with 15 mL of 50 mM HEPES, pH 7.5 followed by 20 mL of 50 mM HEPES, 5 mM imidazole, pH 7.5, prior to elution of the protein of interest with 12 mL of 50 mM HEPES, 300 mM imidazole, pH 7.5. Cleavage of the intein was performed via addition of β -mecaptoethanol (β ME) to a final concentration of 200 mM and incubation at room temperature for 18 hours on a rotisserie. Following cleavage of the intein, removal of imidazole and β ME was facilitated by dialysis into 20 mM tris base pH 8.0 at 4 °C overnight. The undesired intein was removed by a second Ni column (3 mL CV). The flow though containing the protein of interest was collected after 1 hour incubation with the Ni resin on ice.

General Labeling Protocol for Fam-Raz containing α S-intein-H₆ Fusion Proteins: α S variants containing Cys and/or Ppy were expressed as described above and purified by Ni-NTA affinity. Following the second purification subsequent to intein cleavage, the desired protein was dialyzed into 20 mM Tris, pH 8.0 overnight at 4 °C. The protein was then labeled in this semi-crude state in one (donor-only or acceptor-only) or two (doubly-labeled) labeling steps as described below. Proteins lacking an unnatural amino acid (α S-Cys) were quantified by UV-Vis spectroscopy using $\epsilon_{280} = 5120 \text{ M}^{-1} \text{ cm}^{-1}$ with the exception of α S-Cys₁₃₆, which was quantified using $\epsilon_{280} = 3840 \text{ M}^{-1} \text{ cm}^{-1}$ (5). Protein variants containing Ppy were quantified using the BCA assay with bovine serum albumin (BSA) standards generated by two-fold serial dilution in water from 2 mg/mL to 0.125 mg/mL. Labeling reactions of proteins containing Cys were carried out by addition of BondBreaker® TCEP solution to a final concentration of 1 mM and incubation at room temperature for 10-15 minutes. Following this time, fluorescein-5-maleimide (Fam) was added from a 25 mM stock in DMSO to a final concentration equal to five-fold excess relative to protein (typical dye concentration was \sim 500-800 μ M). The labeling reaction was shielded from light by wrapping in aluminum foil and incubated at 37 °C. The labeling reaction was monitored by MALDI-TOF MS following 5-fold dilution of the reaction into water. Following complete disappearance of unlabeled protein as determined by MALDI, the labeled product was dialyzed

overnight at 4 °C in 20 mM Tris pH 8.0. Proteins containing Ppy were labeled via copper(I) catalyzed azide-alkyne cycloaddition as described below; doubly-labeled proteins were first labeled with Fam as described above and subsequently by Raz. Azide-alkyne labeling reactions were performed by preparation of copper(I)-tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) catalyst mixture by using 80 mM CuSO₄ in water (1.25 μL/mL protein solution) to which was added 50 mM THPTA in water (30 μL/mL protein solution) and then 100 mM sodium ascorbate in water (30 μL/mL protein solution). This solution was incubated at room temperature for 10-15 minutes prior to addition to the protein solution. Protein was labeled by addition of five equivalents of Raz from a 50 mM stock in DMSO (typical dye concentrations were ~300-500 μM), followed by addition of the Cu(I) catalyst mixture. Labeling reactions were then shielded from light and incubated at 37 °C and reaction progress monitored by MALDI-TOF MS following 5-fold dilution of the reaction in water. Upon completion of the reaction, the labeled product was dialyzed into 20 mM Tris pH 8.0 overnight at 4 °C to remove excess dye.

Purification Protocol for All αS Proteins: All proteins were then purified by anion-exchange chromatography on an ÄKTA FPLC system using HiTrap Q HP columns and elution between 20 mM Tris pH 8.0 (buffer A) and 20 mM Tris, 1 M NaCl pH 8.0 (buffer B). Fractions containing the desired protein were identified by MALDI-TOF MS and were pooled. All proteins from the Fam-Raz library as well as poorly expressing member of the Cnf-Trp library were concentrated using Amicon Ultra 3 kDa filters to a total volume of ≤ 1 mL. Further purification was then performed using a Varian HPLC system and a Vydac C4 TP reverse-phase semi-preparative column with a flow rate of 4 mL/min and gradient between 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Fractions containing the desired product were identified by MALDI-TOF MS and judged to be of high purity were combined and diluted a minimum of 5-fold by addition of 20 mM Tris, 100 mM NaCl pH 7.5 and subsequently concentrated via Amicon μLtra 3 kDa centrifugal filters to a total volume of ≤ 5 mL, diluted 3-5 fold and re-concentrated; the final dilution and concentration was repeated twice. All proteins were then assessed for purity and integrity of the fluorescent label by MALDI-TOF analysis of whole protein and tryptic fragments.

Protein Library Characterization

Table S1. MALDI Masses from Cnf-Trp Library

Construct	Full Length		Tyrpsin Digest				
	Exp. [M+H] ⁺	Obs. [M+H] ⁺	Fragment	Exp. [M+H] ⁺	Obs. [M+H] ⁺	Exp. [M+Na] ⁺	Obs. [M+Na] ⁺
α S-Cnf ³⁹	14421	14422.4	35-43	960.5	960.7	982.5	982.7
α S-Cnf ⁶⁴	14421	14421.4	81-96	1504.7	1504.0	1526.7	1526.0
α S-Cnf ¹²⁵	14421	14421.7	103-140	4266.4	4265.9	4288.4	4287.9
α S-Cnf ¹³⁶	14421	14421	103-140	4266.4	4265.9	4288.4	4287.9
α S-Trp ²⁴	14454	14455.6	24-32	888.5	888.6	910.5	910.6
α S-Trp ³⁹	14435	14434.7	35-43	974.5	974.8	996.5	996.8
α S-Trp ⁶²	14454	14454	61-80	1986.1	1986.5	2008.1	2008.5
α S-Trp ⁷⁹	14454	14454.3	61-80	1986.1	1986.4	2008.1	2008.4
α S-Trp ⁹⁴	14435	14435.4	81-96	1517.8	1518.1	1539.8	1540.1
α S-Trp ¹²⁵	14435	14434.8	103-140	4280.5	4280.0	4302.5	4302.0
α S-Cnf ³⁹ -Trp ²⁴	14479	14479.5	35-43	960.5	960.7	982.5	982.7
			24-32	888.5	888.7	910.5	910.7
α S-Cnf ³⁹ -Trp ⁶²	14479	14480.1	35-43	960.5	960.7	982.5	982.7
			61-80	1986.1	1986.4	2008.1	2008.4
α S-Cnf ³⁹ -Trp ⁷⁹	14479	14479.3	35-43	960.5	960.7	982.5	982.7
			61-80	1986.1	1986.4	2008.1	2008.4
α S-Cnf ³⁹ -Trp ⁹⁴	14460	14461.6	35-43	960.5	960.7	982.5	982.7
			81-96	1517.8	1518.1	1539.8	1540.1
α S-Cnf ⁶⁴ -Trp ²⁴	14479	14480.2	81-96	1504.7	1504.1	1526.7	1526.0
			24-32	888.5	888.6	910.5	910.6
α S-Cnf ⁶⁴ -Trp ³⁹	14460	14461.4	81-97	1504.7	1504.1	1526.7	1526.0
			35-43	974.5	974.7	996.5	996.7
α S-Cnf ⁶⁴ -Trp ⁶²	14479	14480.1	81-98	1504.7	1504.1	1526.7	1526.1
			61-80	1986.1	1986.4	2008.1	2008.4
α S-Cnf ⁶⁴ -Trp ⁷⁹	14479	14480.1	81-99	1504.7	1504.1	1526.7	1526.1
			61-80	1986.1	1986.4	2008.1	2008.4
α S-Cnf ¹²⁵ -Trp ²⁴	14479	14480.1	103-140	4266.4	4268.7	4288.4	4290.7
			24-32	888.5	888.6	910.5	910.6
α S-Cnf ¹²⁵ -Trp ³⁹	14460	14461.5	103-140	4266.4	4265.8	4288.4	4287.8
			35-43	974.5	974.7	996.5	996.7
α S-Cnf ¹²⁵ -Trp ⁶²	14479	14479.6	103-140	4266.4	4265.3	4288.4	4287.3
			61-80	1986.1	1986.4	2008.1	2008.4
α S-Cnf ¹²⁵ -Trp ⁷⁹	14479	14480	103-140	4266.4	4265.5	4288.4	4287.5
			61-80	1986.1	1986.4	2008.1	2008.4
α S-Cnf ¹²⁵ -Trp ⁹⁴	14460	14460	103-140	4266.4	4265.7	4288.4	4287.7
			81-96	1517.8	1518.1	1539.8	1540.0
α S-Cnf ¹³⁶ -Trp ²⁴	14479	14478.5	103-140	4266.4	4265.9	4288.4	4287.9
			24-32	888.5	888.6	910.5	910.6
α S-Cnf ¹³⁶ -Trp ³⁹	14460	14460.9	103-140	4266.4	4266.2	4288.4	4288.2
			35-43	974.5	974.7	996.5	996.7
α S-Cnf ¹³⁶ -Trp ⁹⁴	14460	14461.6	103-140	4266.4	4265.5	4288.4	4287.5
			81-96	1517.8	1518.1	1539.8	1540.1
α S-Cnf ¹³⁶ -Trp ¹²⁵	14460	14460.9	103-140	4305.5	4304.8	4327.5	4326.8

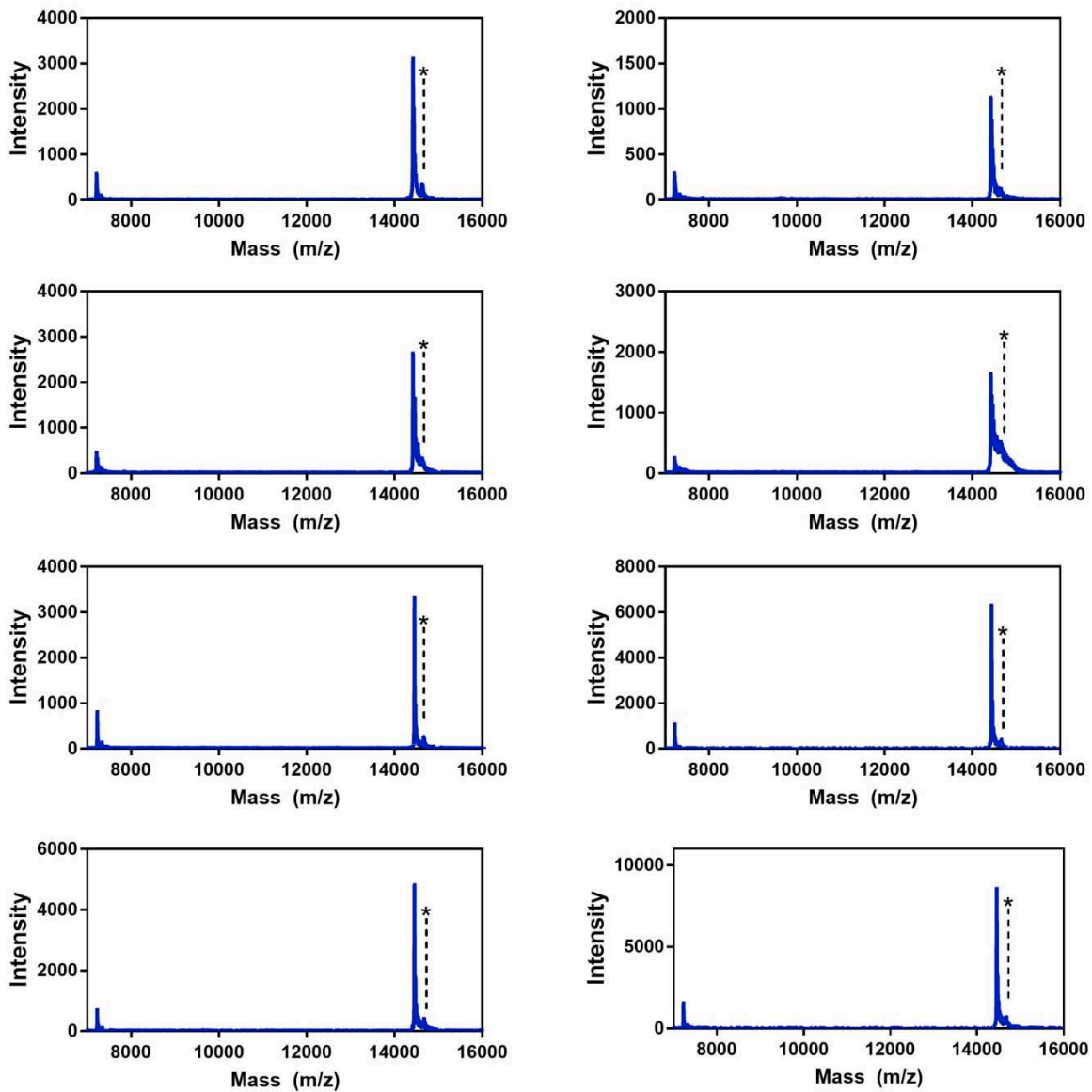


Figure S1: MALDI Spectra of Full-Length Cnf-Trp Constructs. Plots show α S-Cnf³⁹ (top left), α S-Cnf⁹⁴ (top right), α S-Cnf¹²⁵ (upper middle left), α S-Cnf¹³⁶ (upper middle right), α S-Trp²⁴ (lower middle left), α S-Trp³⁹ (lower middle right), α S-Trp⁶² (bottom left) and α S-Trp⁷⁹ (bottom right). On each plot the matrix adduct is noted with *.

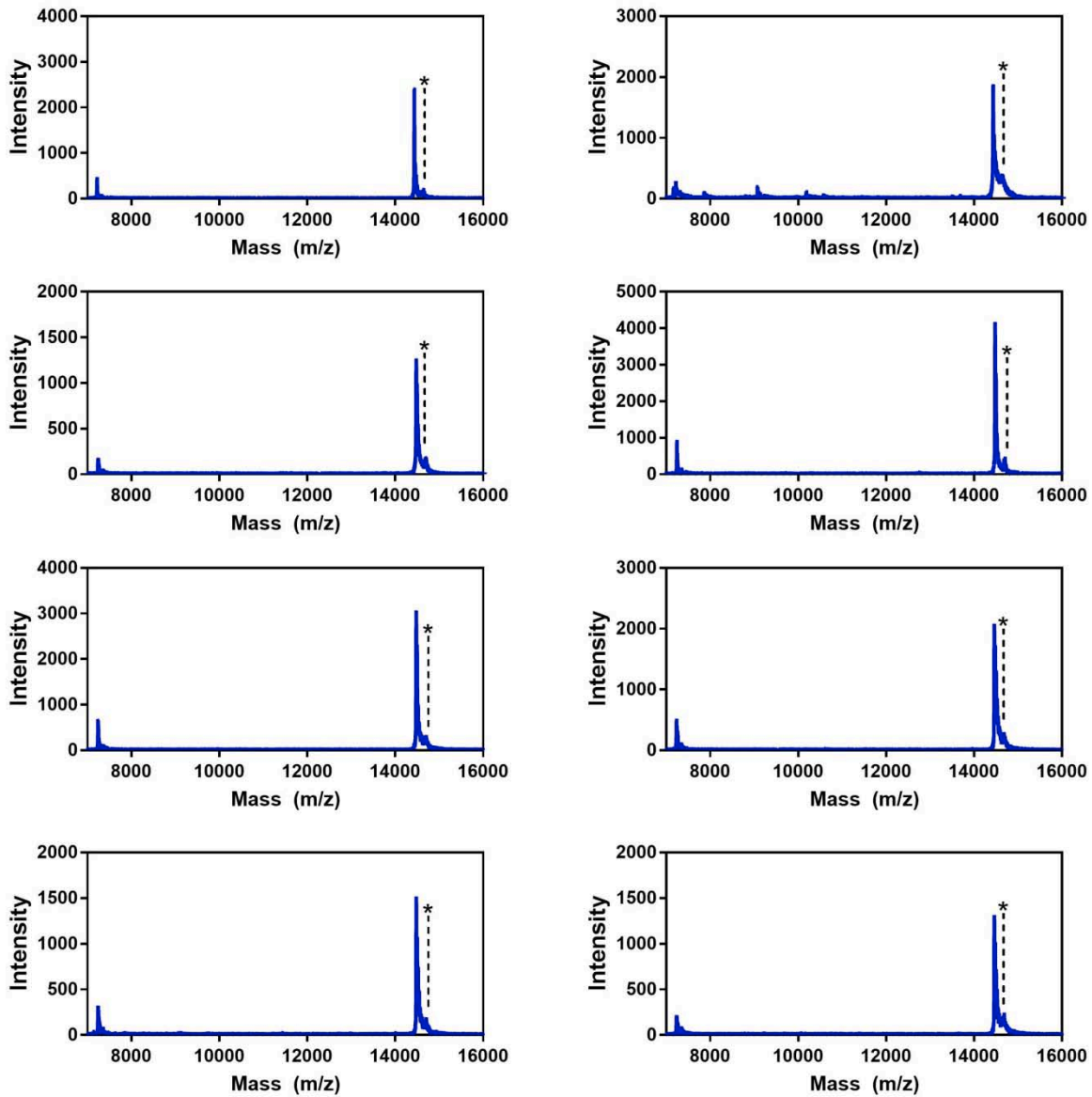


Figure S2: MALDI Spectra of Full-Length Cnf-Trp Constructs. Plots show α S-Trp⁹⁴ (top left), α S-Trp¹²⁵ (top right), α S-Cnf³⁹-Trp²⁴ (upper middle left), α S-Cnf³⁹-Trp⁶² (upper middle right), α S-Cnf³⁹-Trp⁷⁹ (lower middle left), α S-Cnf³⁹-Trp⁹⁴ (lower middle right), α S-Cnf⁹⁴-Trp²⁴ (bottom left) and α S-Cnf⁹⁴-Trp³⁹ (bottom right). On each plot the matrix adduct is noted with *.

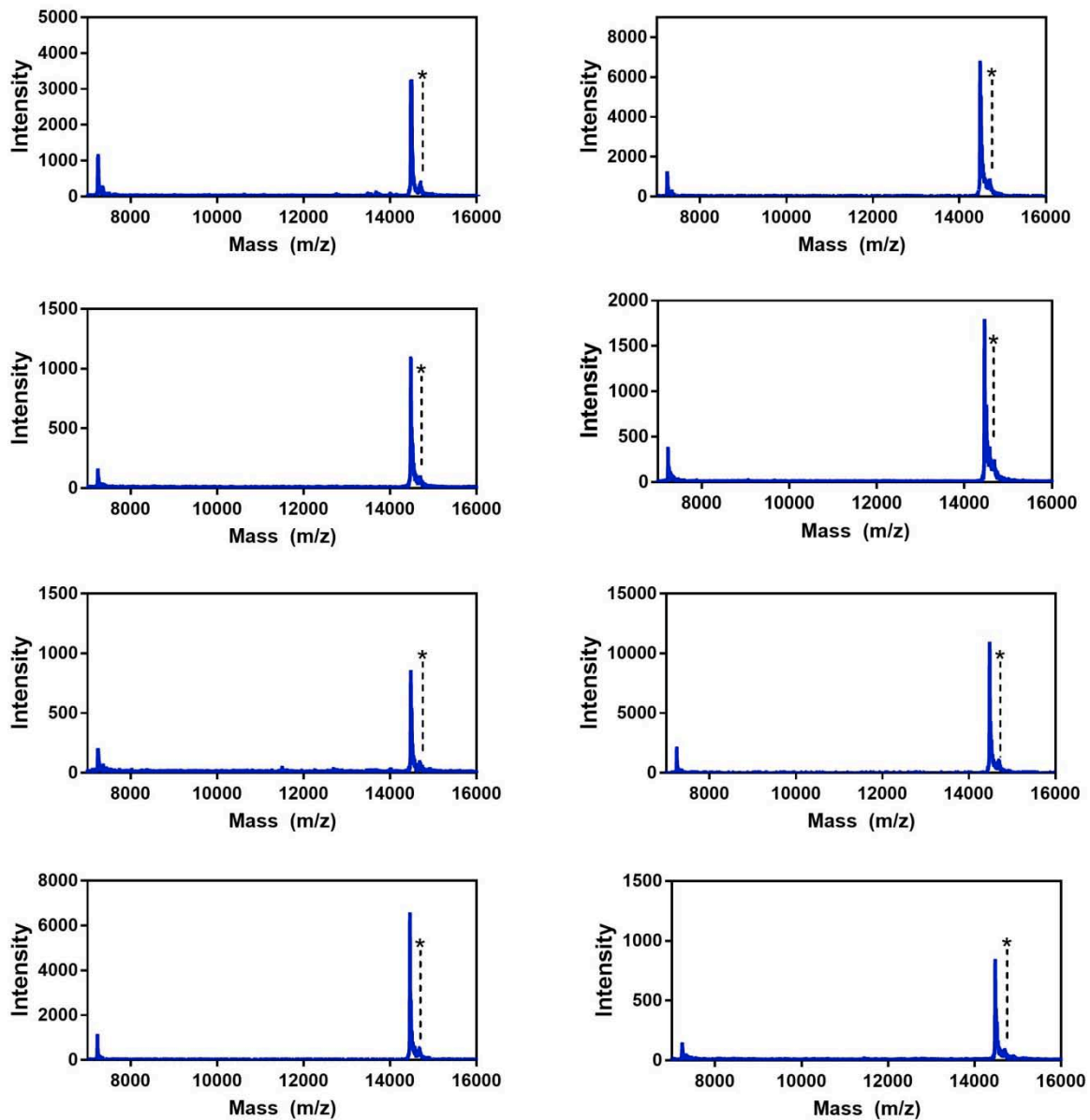


Figure S3: MALDI Spectra of Full-Length Cnf-Trp Constructs. Plots show α S-Cnf⁹⁴-Trp⁶² (top left), α S-Cnf⁹⁴-Trp⁷⁹ (top right), α S-Cnf¹²⁵-Trp²⁴ (upper middle left), α S-Cnf¹²⁵-Trp³⁹ (upper middle right), α S-Cnf¹²⁵-Trp⁶² (lower middle left), α S-Cnf¹²⁵-Trp⁷⁹ (lower middle right), α S-Cnf¹²⁵-Trp⁹⁴ (bottom left) and α S-Cnf¹³⁶-Trp²⁴ (bottom right). On each plot the matrix adduct is noted with *.

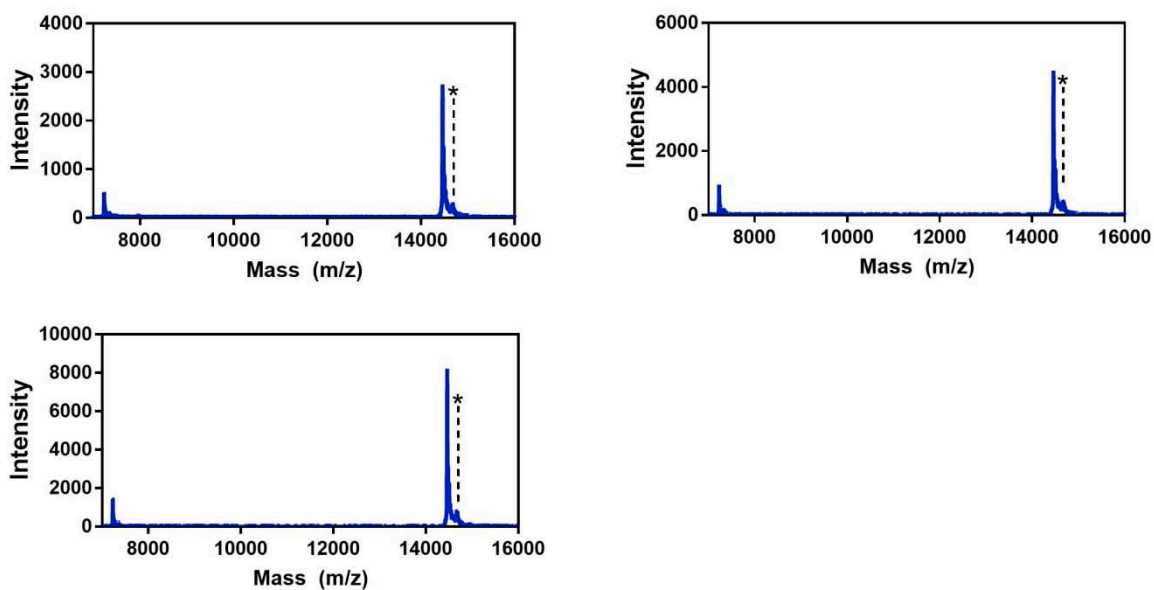


Figure S4: MALDI Spectra of Full-Length Cnf-Trp Constructs. Plots show α S-Cnf¹³⁶-Trp³⁹ (top left), α S-Cnf¹³⁶-Trp⁹⁴ (top right), α S-Cnf¹³⁶-Trp¹²⁵ (bottom). On each plot the matrix adduct is noted with *.

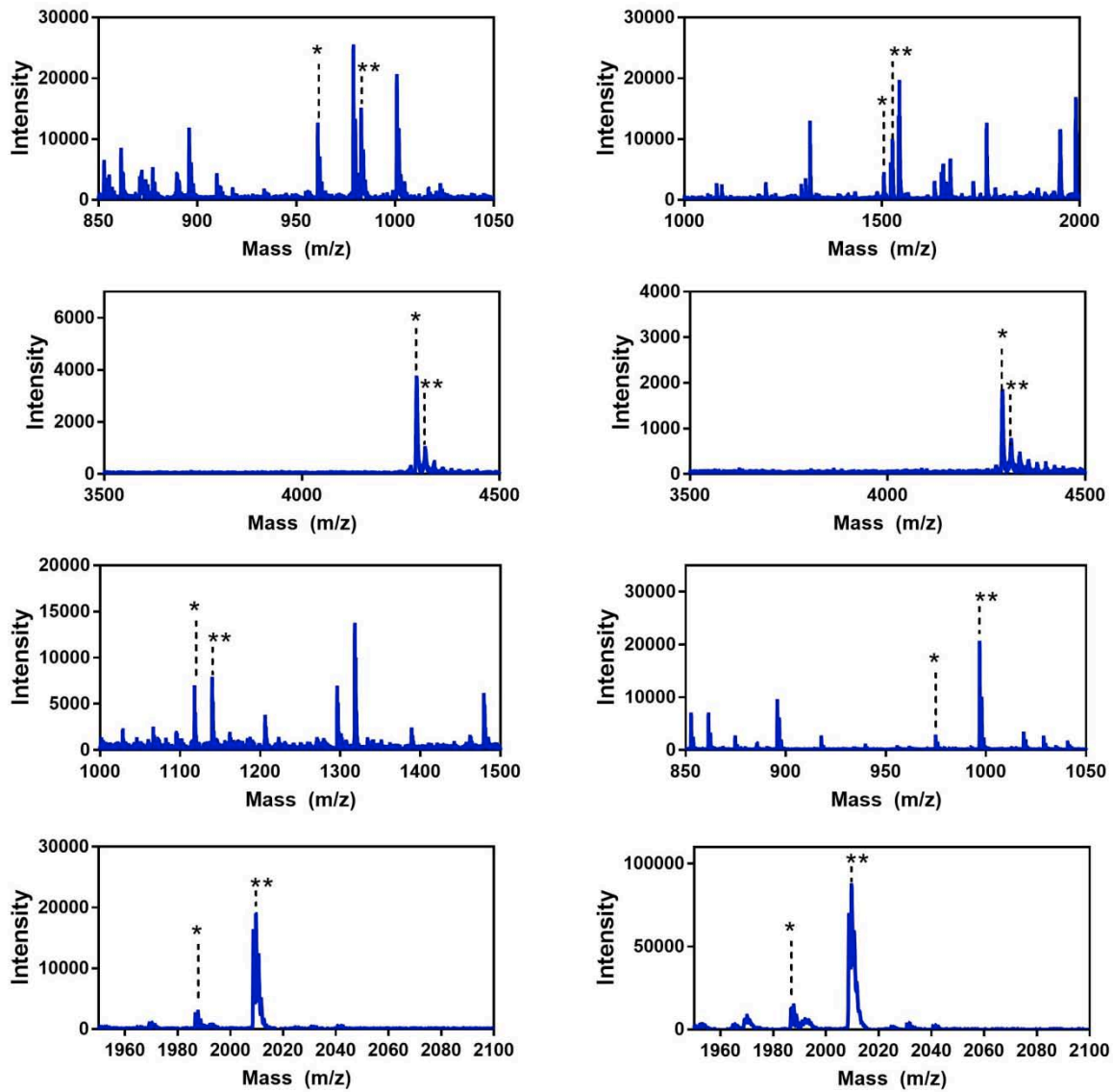


Figure S5: MALDI Spectra of Trypsin Digests of Cnf-Trp Constructs. Plots show Cnf containing fragments of α S-Cnf³⁹ (top left), α S-Cnf⁹⁴ (top right), α S-Cnf¹²⁵ (upper middle left), α S-Cnf¹³⁶ (upper middle right) and Trp containing fragments of α S-Trp²⁴ (lower middle left), α S-Trp³⁹ (lower middle right), α S-Trp⁶² (bottom left) and α S-Trp⁷⁹ (bottom right). On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

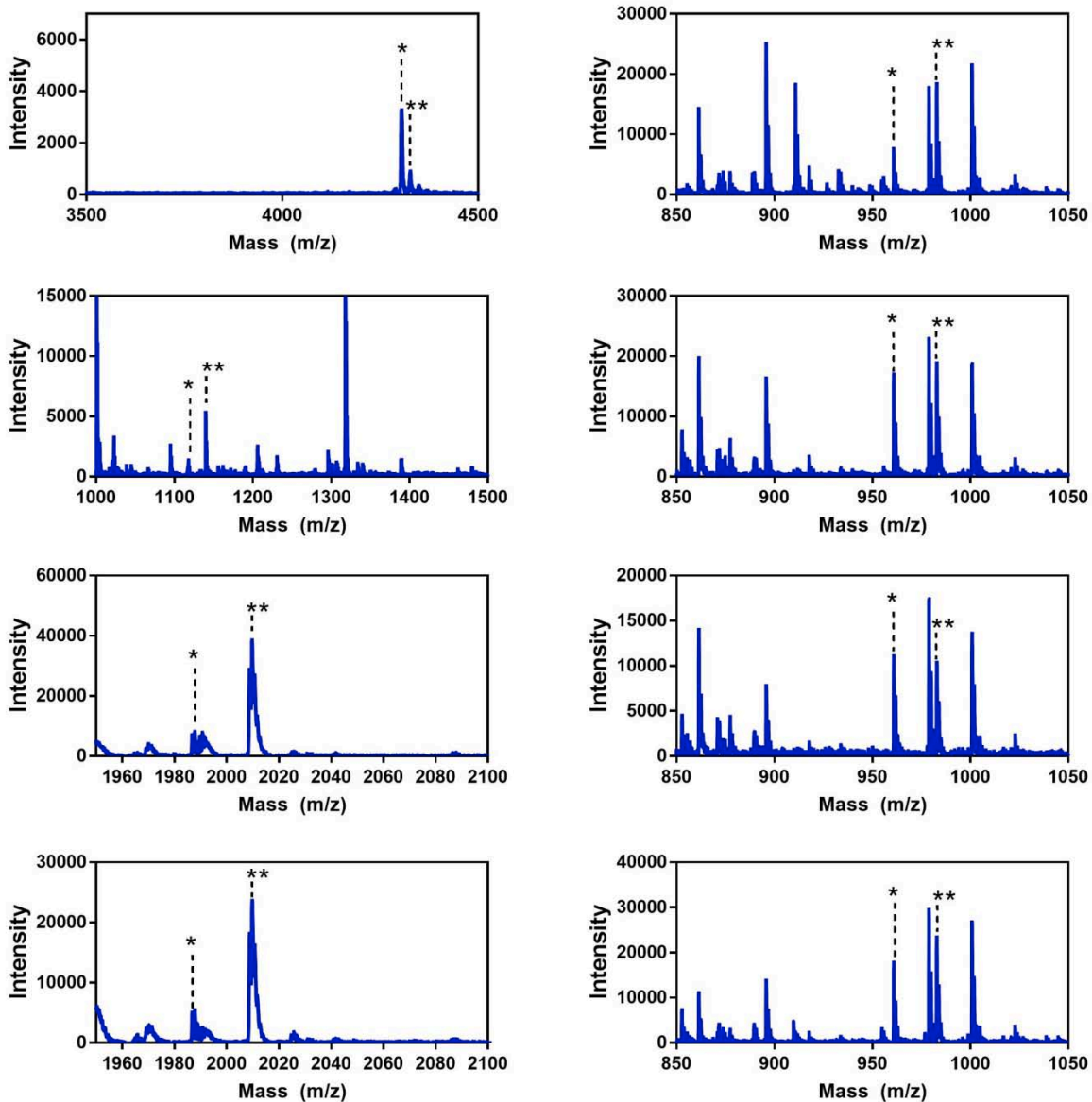


Figure S6: MALDI Spectra of Trypsin Digests of Cnf-Trp Constructs. Plots show Trp containing fragment of α S-Trp¹²⁵ (top left), Cnf (top right) and Trp (upper middle left) containing fragments of α S-Cnf³⁹-Trp²⁴, Cnf (upper middle right) and Trp (lower middle left) containing fragments of α S-Cnf³⁹-Trp⁶², Cnf (lower middle right) and Trp (bottom left) containing fragments of α S-Cnf³⁹-Trp⁷⁹ and Cnf containing fragment of α S-Cnf³⁹-Trp⁹⁴ (bottom right). On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

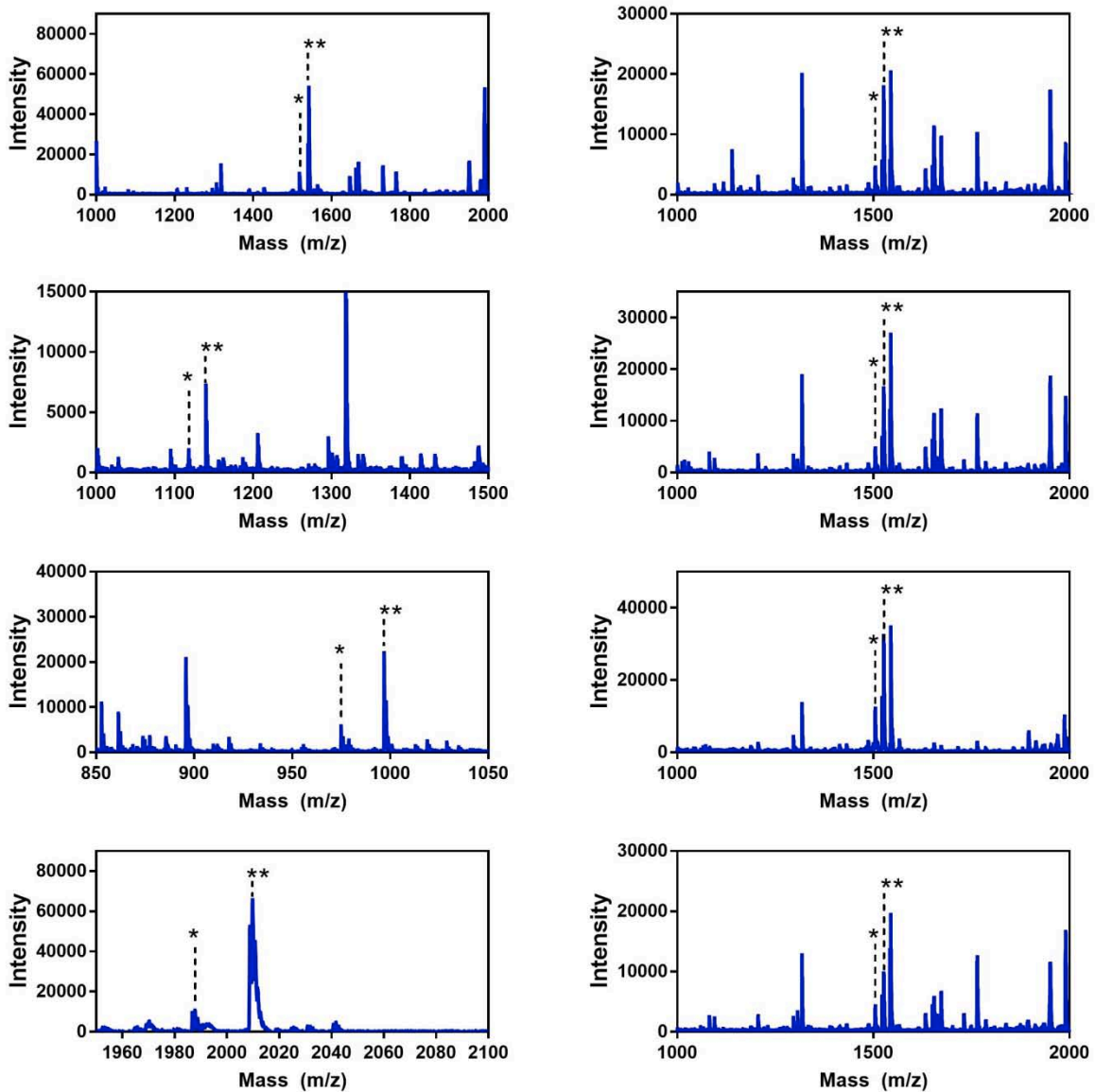


Figure S7: MALDI Spectra of Trypsin Digests of Cnf-Trp Constructs. Plots show Trp containing fragment of α S-Cnf³⁹-Trp⁹⁴ (top left), Cnf (top right) and Trp (upper middle left) containing fragments of α S-Cnf⁹⁴-Trp²⁴, Cnf (upper middle right) and Trp (lower middle left) containing fragments of α S-Cnf⁹⁴-Trp³⁹, Cnf (lower middle right) and Trp (bottom left) containing fragments of α S-Cnf⁹⁴-Trp⁶² and Cnf containing fragment of α S-Cnf⁹⁴-Trp⁷⁹ (bottom right). On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

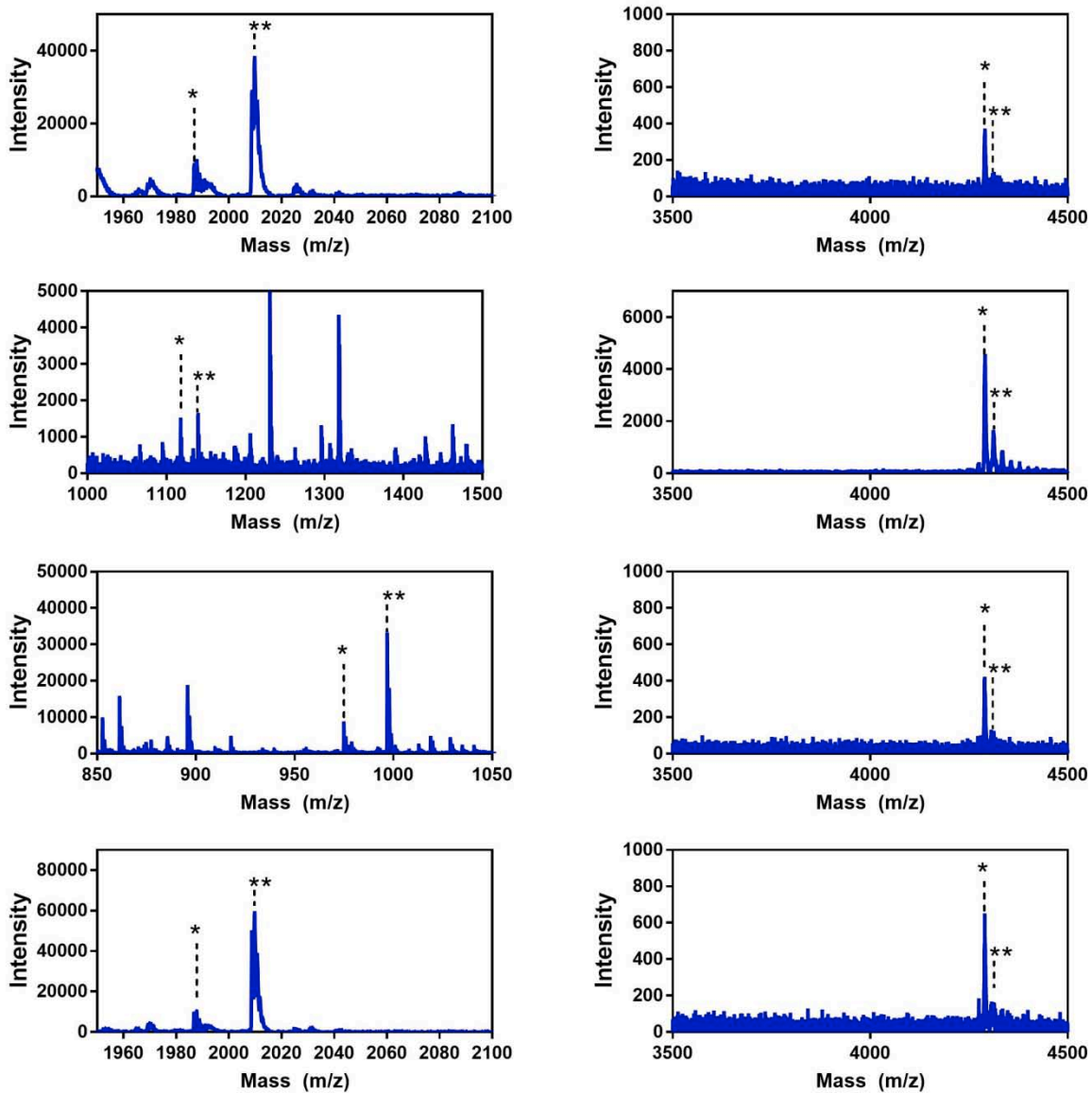


Figure S8: MALDI Spectra of Trypsin Digests of Cnf-Trp Constructs. Plots show Trp containing fragment of α S-Cnf⁹⁴-Trp⁷⁹ (top left), Cnf (top right) and Trp (upper middle left) containing fragments of α S-Cnf¹²⁵-Trp²⁴, Cnf (upper middle right) and Trp (lower middle left) containing fragments of α S-Cnf¹²⁵-Trp³⁹, Cnf (lower middle right) and Trp (bottom left) containing fragments of α S-Cnf¹²⁵-Trp⁶² and Cnf containing fragment of α S-Cnf¹²⁵-Trp⁷⁹ (bottom right). On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

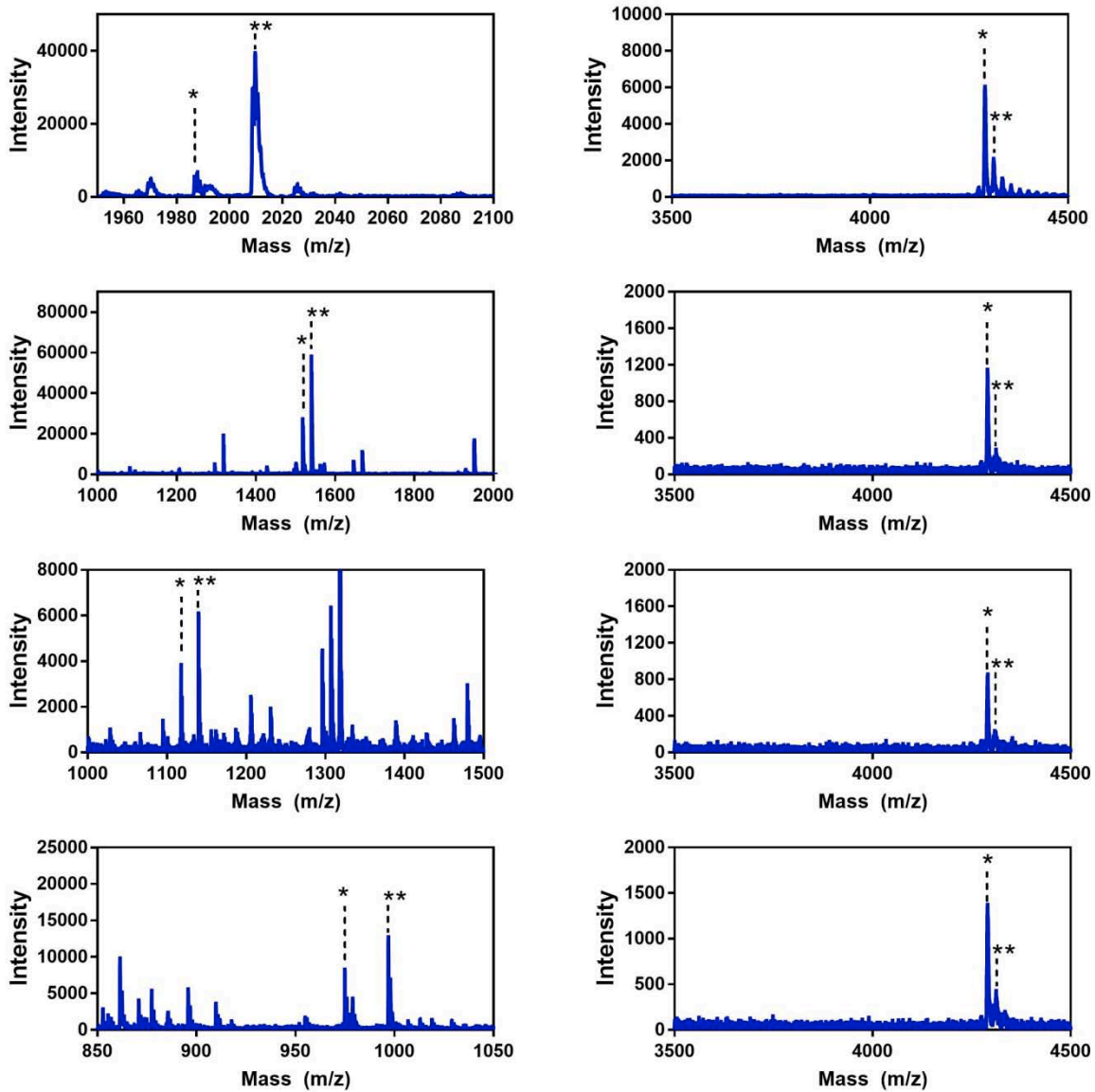


Figure S9: MALDI Spectra of Trypsin Digests of Cnf-Trp Constructs. Plots show Trp containing fragment of α S-Cnf¹²⁵-Trp⁷⁹ (top left), Cnf (top right) and Trp (upper middle left) containing fragments of α S-Cnf¹²⁵-Trp⁹⁴, Cnf (upper middle right) and Trp (lower middle left) containing fragments of α S-Cnf¹³⁶-Trp²⁴, Cnf (lower middle right) and Trp (bottom left) containing fragments of α S-Cnf¹³⁶-Trp³⁹ and Cnf containing fragment of α S-Cnf¹³⁶-Trp⁹⁴ (bottom right). On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

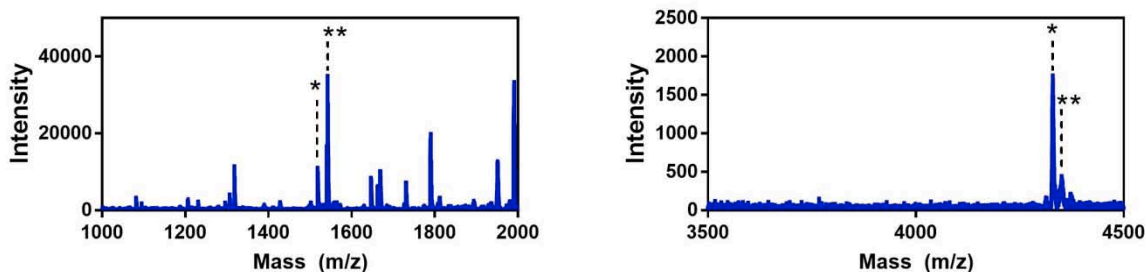


Figure S10: MALDI Spectra of Trypsin Digests of Cnf-Trp Constructs. Plots show Trp containing fragment of α S-Cnf¹³⁶-Trp⁹⁴ (left) and Cnf and Trp containing fragment of α S-Cnf¹²⁵-Trp¹³⁶ (top right). On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

Table S2. MALDI Masses from Fam-Raz Library

Construct	Full Length		Trypsin Digest				
	Exp. [M+H] ⁺	Obs. [M+H] ⁺	Fragment	Exp. [M+H] ⁺	Obs. [M+H] ⁺	Exp. [M+Na] ⁺	Obs. [M+Na] ⁺
α S-Raz ⁹⁴	15027	15027.8	81-96	2044.0	2046.6	2066.0	2068.6
α S-Raz ¹³⁶	15011	15013.3	103-140	4839.0	4837.4	4861.0	4859.4
α S-Fam ⁹ -Raz ¹³⁶	15474	15475.1	7-10	865.3	865.9	887.3	887.9
			103-140	4839.0	4837.1	4861.0	4859.1
α S-Fam ⁹ -Raz ⁹⁴	15488	15488.1	7-10	865.3	865.6	887.3	887.6
α S-Fam ²⁴ -Raz ⁹⁴	15447	15450.7	81-96	2044.0	2044.6	2066.0	2066.6
			24-32	1250.5	1250.3	1272.4	1272.3
α S-Fam ⁴² -Raz ⁹⁴	15488	15490.1	81-96	2044.0	2044.8	2066.0	2066.8
			35-43	1412.6	1413.7	1434.6	1435.7
α S-Fam ⁶² -Raz ⁹⁴	15447	15448.5	81-96	2044.0	2045.3	2066.0	2067.3
			61-80	2348.1	-	2370.1	2371.7
α S-Fam ⁸⁷ -Raz ⁹⁴	15488	15488.4	81-96	2505.1	2506.6	2527.0	2528.6
α S-Fam ¹¹⁴ -Raz ⁹⁴	15446	15446.5	103-140	4704.8	-	4726.8	4728.8
			81-96	2044.0	2046.4	2066.0	2068.4
α S-Fam ¹²³ -Raz ⁹⁴	15446	15446.1	103-140	4704.8	-	4726.8	4728.9
			81-96	2044.0	2046.4	2066.0	2068.4
α S-Fam ¹³⁶ -Raz ⁹⁴	15410	15413.8	103-140	4668.8	-	4690.7	4694.8
			81-96	2044.0	2046.4	2066.0	2068.4

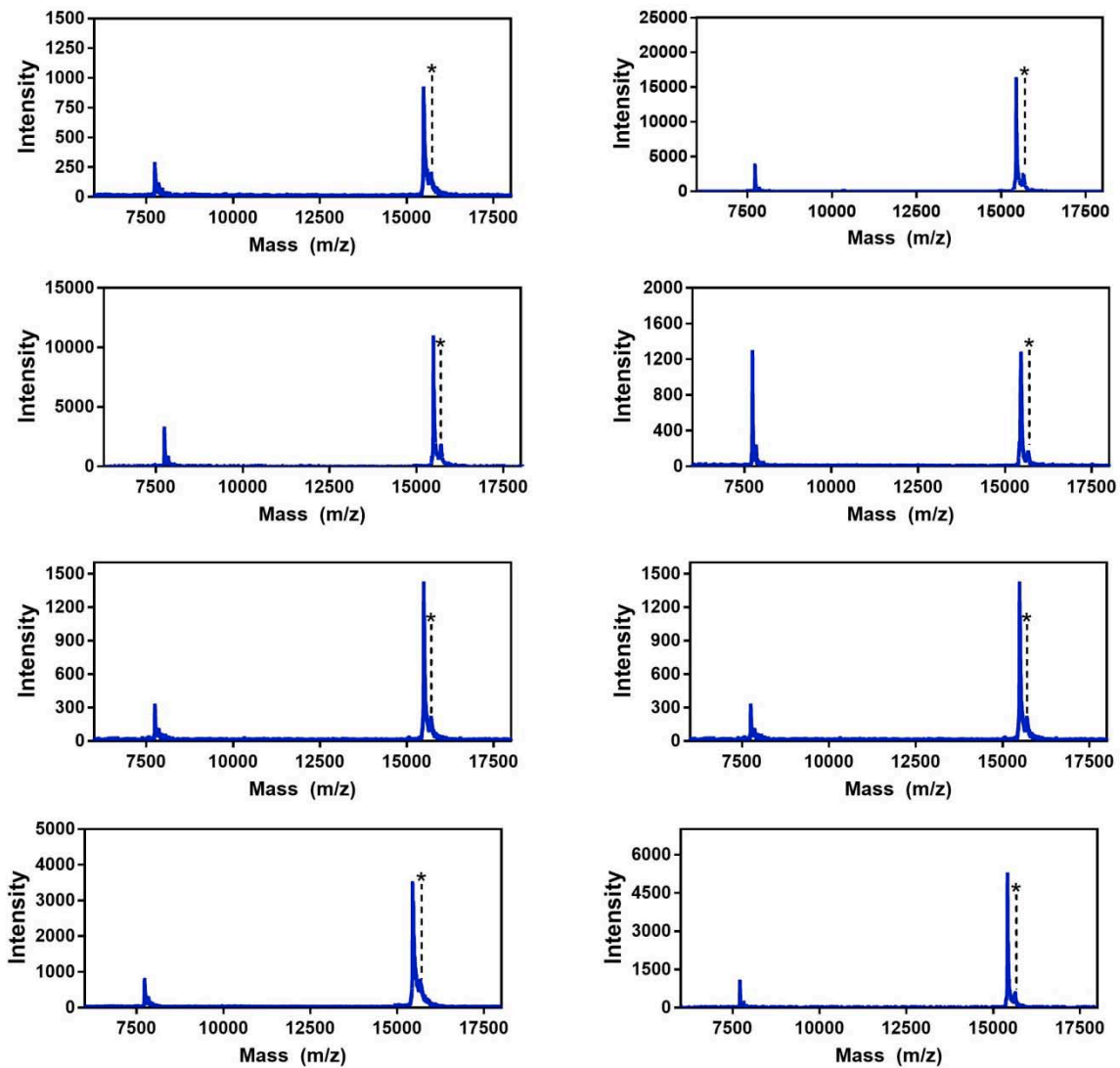


Figure S11: MALDI Spectra of Full-Length Fam-Raz Constructs. Plots show α S-Fam⁹-Raz⁹⁴ (top left), α S-Fam²⁴-Raz⁹⁴ (top right), α S-Fam⁴²-Raz⁹⁴ (upper middle left), α S-Fam⁶²-Raz⁹⁴ (upper middle right), α S-Fam⁸⁷-Raz⁹⁴ (lower middle left), α S-Fam¹¹⁴-Raz⁹⁴ (lower middle right), α S-Fam¹²³-Raz⁹⁴ (bottom left) and α S-Fam¹³⁶-Raz⁹⁴ (bottom right). On each plot the matrix adduct is noted with *.

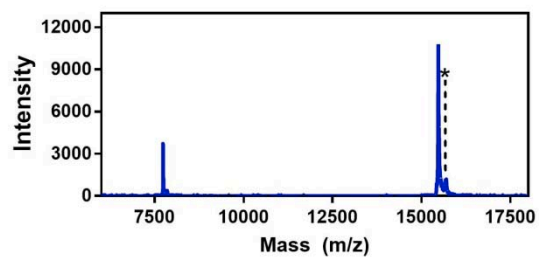


Figure S12: MALDI Spectra of Full-Length Fam-Raz Constructs. Plot shows α S-Fam⁹-Raz¹³⁶ with the matrix adduct is noted with *.

All single Fam and Raz labeled constructs have been previously reported (3, 4).

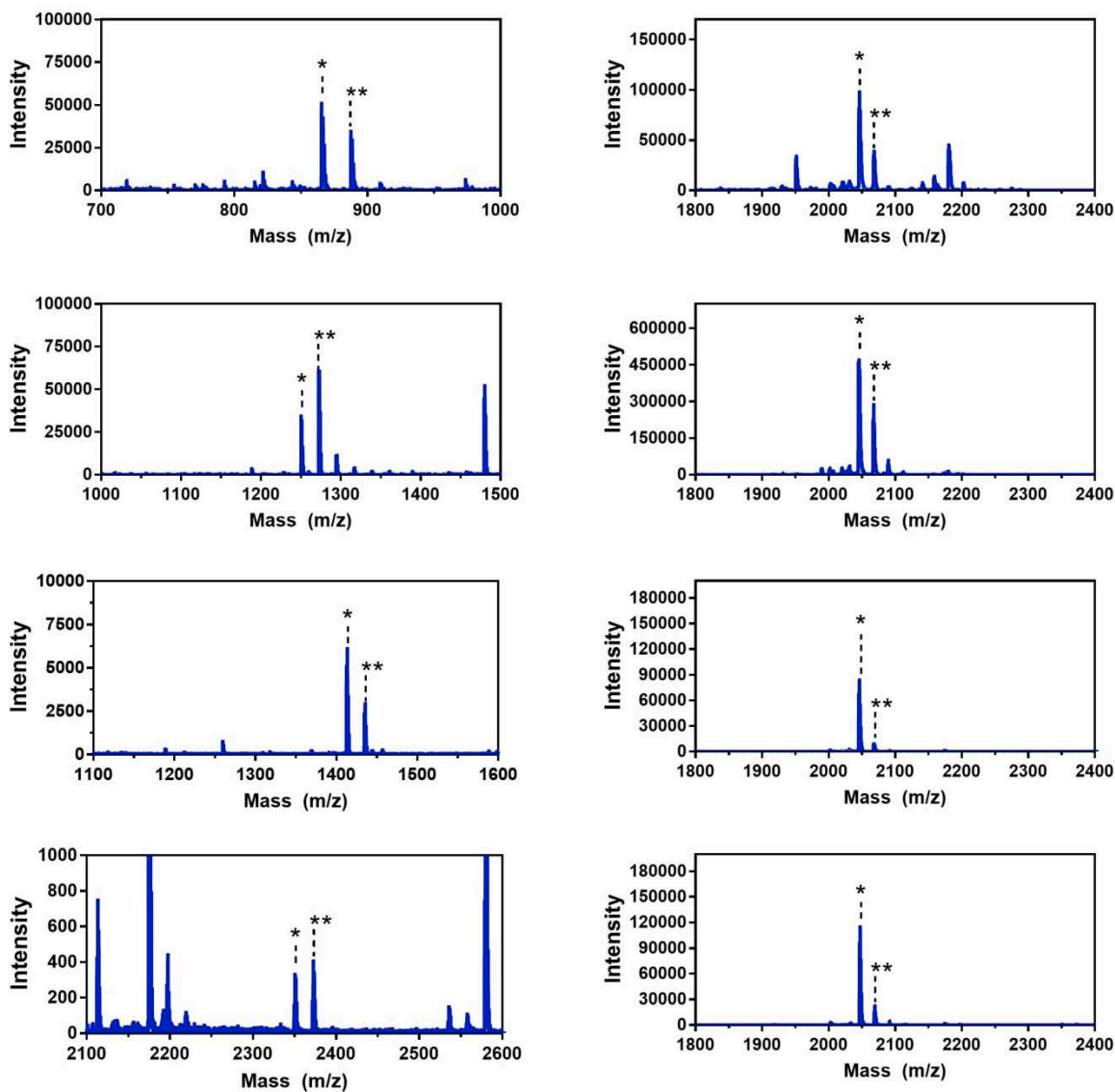


Figure S13: MALDI Spectra of Trypsin Digests of Fam-Raz Constructs. Plots show Fam (top left) and Raz (top right) containing fragments of α S-Fam⁹-Raz⁹⁴, Fam (upper middle left) and Raz (upper middle right) containing fragments of α S-Fam²⁴-Raz⁹⁴, Fam (lower middle left) and Raz (lower middle right) containing fragments of α S-Fam⁴²-Raz⁹⁴ and Fam (bottom left) and Raz (bottom right) containing fragments of α S-Fam⁶²-Raz⁹⁴. On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

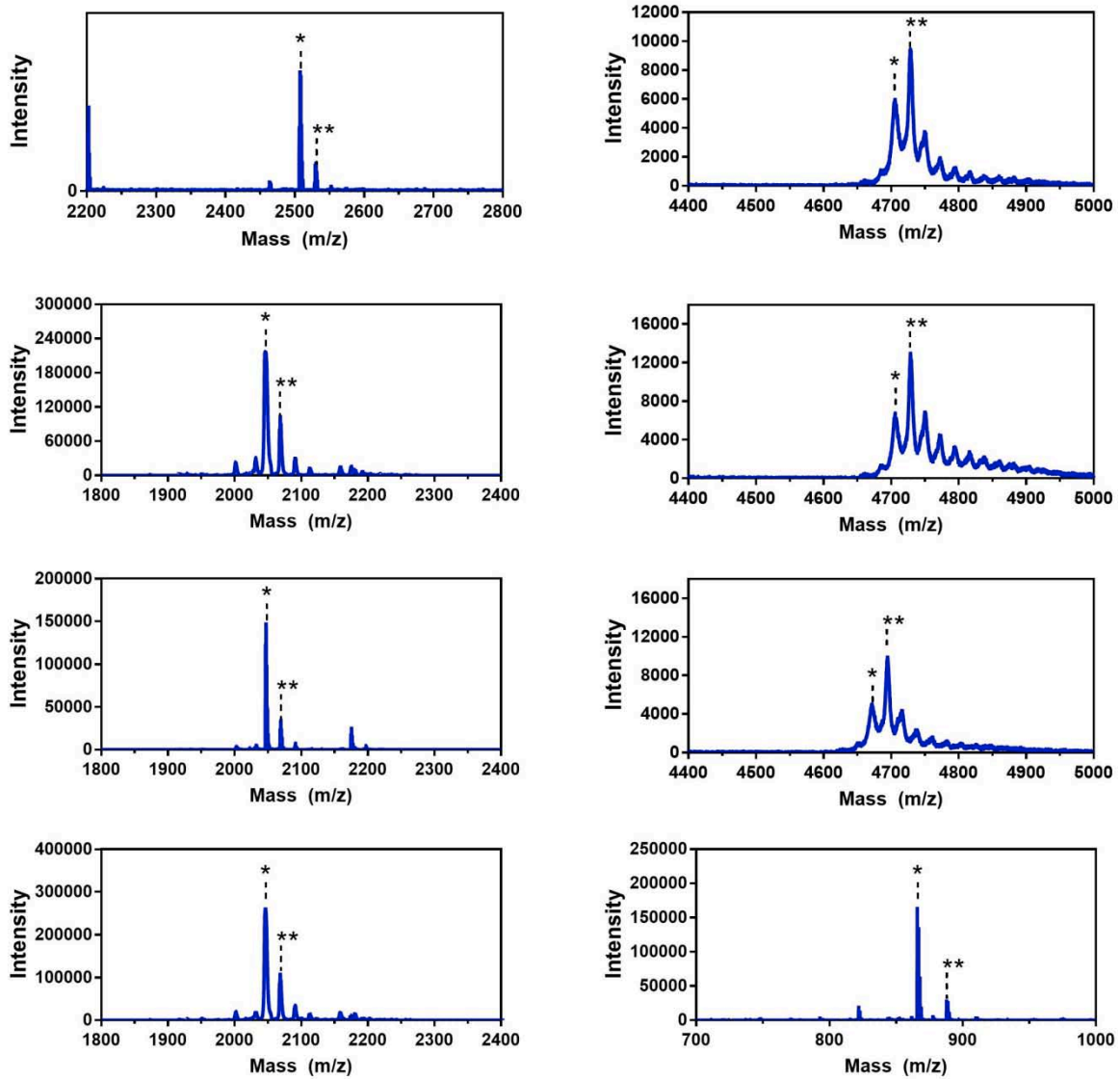


Figure S14: MALDI Spectra of Trypsin Digests of Fam-Raz Constructs. Plots show Fam and Raz containing fragments of α S-Fam⁸⁷-Raz⁹⁴ (top left), Fam (top right) and Raz (upper middle left) containing fragments of α S-Fam¹¹⁴-Raz⁹⁴, Fam (upper middle right) and Raz (lower middle left) containing fragments of α S-Fam¹²³-Raz⁹⁴, Fam (lower middle right) and Raz (bottom left) containing fragments of α S-Fam¹³⁶-Raz⁹⁴ and Fam containing fragment of α S-Fam⁹-Raz¹³⁶ (bottom right). On each plot the M+H mass of the fragment of interest is indicated with * while M+Na masses are indicated with **.

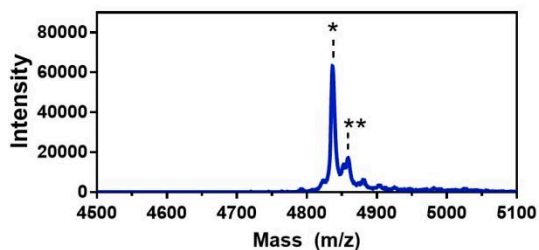


Figure S15: MALDI Spectra of Trypsin Digest of Fam-Raz Construct. Plots show Raz containing fragment of α S-Fam⁹-Raz¹³⁶. The M+H mass of the fragment of interest is indicated with * while M+Na mass is indicated with **.

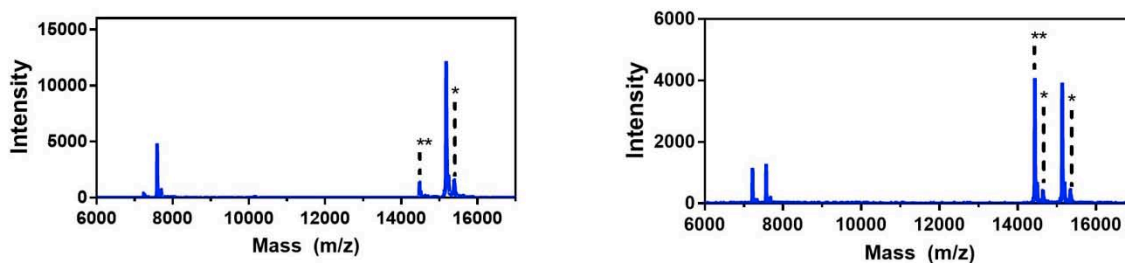


Figure S16: MALDI Spectra of Full-Length AF488 Constructs. Plots show α S-AF488⁹ (left) and α S-AF488¹¹⁴ (right). On each plot the matrix adduct is indicated with * while unlabeled Cys protein is indicated with **.

The α S-AF488¹³⁰ construct has been previously reported (6).

FRET Data Collection and Analysis

TMAO Assay: All labeled α S variants were dialyzed into 20 mM tris, 100 mM NaCl, pH 7.5. Buffers containing TMAO were also prepared with 20 mM tris, 100 mM NaCl, and the pH was adjusted to 7.5 following the addition of TMAO. Concentrations for the Cnf-Trp library was determined using the Sigma-Aldrich FluoroProfile Quantification Kit, while concentrations for Fam-Raz constructs were determined via UV-Vis absorbance. Steady-state measurements for the Cnf-Trp library and time correlated single photon counting (TCSPC) measurements of the Fam-Raz library were performed at 1 μ M, while steady-state measurements for the Fam-Raz library were performed at 100 nM. Prior to each measurement, labeled protein was mixed with TMAO containing buffer and briefly vortexed. Measurements were taken in triplicate at final concentrations of 0, 2 and 4 M TMAO at 20 °C. Steady-state measurements for the Cnf-Trp library were collected with an excitation of 240 nm over an emission range of 275 - 410 nm with excitation and emission slits set to 5 nm and 1 nm step size collecting for 0.75 seconds per step, exciting primary the Cnf fluorophore. Spectra were collected for direct excitation of Trp with an excitation of 280 nm over an emission range of 310 - 410 nm with excitation and emission slits set to 5 nm and 1 nm step size collecting for 0.75 seconds per step. Measurements of the Fam-Raz library were collected with an excitation of 486 nm over an emission range of 495 - 700 nm with excitation and emission slits set to 5 nm and 1 nm step size collecting for 0.25 seconds per step. Direct excitation of Raz was performed by exciting at 555 and measuring over an emission range of 565 - 700 nm with a 1 nm step size and a collection time of 0.25 seconds per step.

All TCSPC measurements of fluorescence lifetime decays were collected using a pulsed LED with a maximum emission at 486 nm. Fluorescence was collected at 515 nm with the slit widths adjusted for each measurement to keep the ACD value between 1 - 3 % of the SYNC value. The instrument response function (IRF) was collected for each slit width used for collection. For these experiments labeled α S was mixed with TMAO containing buffer to final protein concentrations of 2 μ M and 0, 2 or 4 M TMAO. Additionally, formation of aggregates in buffer or TMAO were assayed by mixing single-labeled α S containing FAM and single-labeled α S containing Raz in equimolar concentrations with a final protein concentration of 2 μ M in 0, 2 and 4 M TMAO.

Fitting Steady-State Data: Following data collection, the single labeled spectra were used to quantify the degree of energy transfer. First, 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. Fitting was performed by minimizing the total least squared difference using the Excel Solver feature to adjust the constants A and B :

$$\sum_{\lambda} (I(\lambda)_{DA} - AI(\lambda)_D - BI(\lambda)_A)^2 \rightarrow \min \quad (\text{Eq. 1})$$

$$I(\lambda)_{DA} = AI(\lambda)_D + BI(\lambda)_A \quad (\text{Eq. S1})$$

Here, $I(\lambda)_{DA}$, $I(\lambda)_D$ and $I(\lambda)_A$ are the wavelength dependent fluorescence intensities of the double-labeled, single labeled protein containing the donor, and single-labeled protein containing the acceptor fluorophore, respectively. Solutions to Eq. S1 were obtained by utilizing the Excel Solver functionality. This procedure was performed at each TMAO concentration, thus accounting for any changes in quantum yield or spectral shifting. 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 Eq. S2 and S3 and combined in a weighted average via Eq. S4:

$$E_D = (1 - A) \quad (\text{Eq. S2})$$

$$E_A = (B - 1) \frac{\epsilon_A}{\epsilon_D} \quad (\text{Eq. S3})$$

$$E_{FRET} = \left(\frac{1}{S_D} + \frac{1}{S_A} \right)^{-1} \left(\frac{E_D}{S_D} + \frac{E_A}{S_A} \right) \quad (\text{Eq. S4})$$

In Eqs. S2-S4, E_D and E_A are E_{FRET} values calculated from the donor and acceptor weights respectively. Additionally, Eq. S3 requires ratio of the extinction coefficient for the acceptor, ϵ_A , to the donor, ϵ_D , which are detailed in Tables S3a and S3b and have been determined from the absorption spectrum of each fluorophores scaled using published extinction coefficients (Cnf $\epsilon_{240}=13,921 \text{ M}^{-1}\text{cm}^{-1}$ (7), Trp $\epsilon_{278}=5700 \text{ M}^{-1}\text{cm}^{-1}$ (7), FAM $\epsilon_{494}=68,000 \text{ M}^{-1}\text{cm}^{-1}$ (8) and Raz $\epsilon_{555}=87,000 \text{ M}^{-1}\text{cm}^{-1}$ as indicated by the manufacturer) . 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 donor and acceptor propagated error respectively.

$$S_A = A \times \sqrt{(S_{I_A} \times B / I_D)^2 + (S_{I_{DL}} / I_D)^2 + (S_{I_D} \times (I_{DL} - B \times I_A) / I_D^2)^2} \quad (\text{Eq. S5})$$

$$S_B = B \times \sqrt{(S_{I_D} \times S / I_A)^2 + (S_{I_{DL}} / I_A)^2 + (S_{I_A} \times (I_{DL} - A \times I_D) / I_A^2)^2} \quad (\text{Eq. S6})$$

$$S_{EFRET} = \sqrt{2} / \left(\frac{1}{S_A} + \frac{1}{S_B} \right) \quad (\text{Eq. S7})$$

Lastly, 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:

$$R_0^6 = (9 \ln(10) \kappa^2 \Phi_D J) / (128 \pi^2 N_A n^4) \quad (\text{Eq. S8})$$

Here, N_A is Avogadro's number, κ^2 is the dipole orientation factor, approximated at 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 of the medium. 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 free fluorophores in buffer through application of the integral:

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

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:

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

where $F_D(\lambda)$ is the fluorescence emission spectrum of the donor dye. Following determination of R_0 average distance values from the polymer scaled Förster equation (Eq. 2), using either Eq 3 or

Eq 4 to determine $P_n(r,x)$, along with the associated error and constraint weights for each constraining function, were determined in Wolfram Mathematica.

$$E_{FRET} = \sum_r P_n(r, x) / (1 + (r/R_0)^6) \quad (\text{Eq. 2})$$

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

$$P_2(r, x) = \frac{1}{\sqrt{2\pi\sigma^2}} \exp\left(-\frac{1}{2} \frac{(r-x)^2}{\sigma^2} \right) \quad (\text{Eq. 4})$$

Fluorescence measurements of labeled proteins have shown that the quantum yield is sensitive to the local environment, as well as TMAO concentration. Therefore, changes in quantum yield must also be taken into account to effectively determine R_0 via Eq. S8. The quantum yield was calculated by fitting the emission spectrum of the free fluorophore in buffer, without TMAO, to the emission spectrum of each labeled analog at each concentration of TMAO, again using a linear least squared difference approach.

$$\sum_{\lambda} (I_D - C \times I_{Dye})^2 \quad (\text{Eq. S11})$$

The sum in Eq. S11 was minimized using the Excel solver function by adjusting the constant, C . Here, I_D and I_{Dye} represent the sum of the fluorescence intensity over all wavelengths of the labeled protein and the free fluorophore, respectively. The donor quantum yield is then defined as:

$$\Phi_D = C \times \Phi_0 \quad (\text{Eq. S12})$$

where Φ_0 is the quantum yield of the free fluorophore. By using this empirically adjusted quantum yield in the calculation of R_0 , we effectively reduce inaccuracies in the determined interchromophore distance arising from changes in quantum yield. Lastly, the distance of interest, R , is determined from the above variables as described in the main text.

Finally, error was propagated through the calculation of the interprobe distance. This was performed by determining the inverse function of $P_n(r,x)$, here represented as $F(E_{FRET})$, using Wolfram Mathematica as shown in Eq. S13.

$$F(E_{FRET}) = P_n^{-1}(r, x) \quad (\text{Eq. S13})$$

The error is then simply propagated following the determination of the partial derivative of $P_n^{-1}(E_{FRET})$ with respect to the E_{FRET} variable as shown in Eq. S14.

$$S_{Distance} = S_{EFRET} \times \frac{\delta F(E_{FRET})}{\delta E_{FRET}} \quad (\text{Eq. S14})$$

Fitting Lifetime Data: Lifetime data were fit using PowerFit10 distributed by PTI. 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 $1 - (\tau_{DA}/\tau_D)$ where τ_{DA} and τ_D are the lifetimes for double-labeled and donor-only constructs respectively. For biexponential decays both the amplitude average and the intensity average lifetimes were used to calculate E_{FRET} values for comparison to the E_{FRET} values extracted from steady-state measurement.

Steady-state Fluorescence Spectra and Fitting

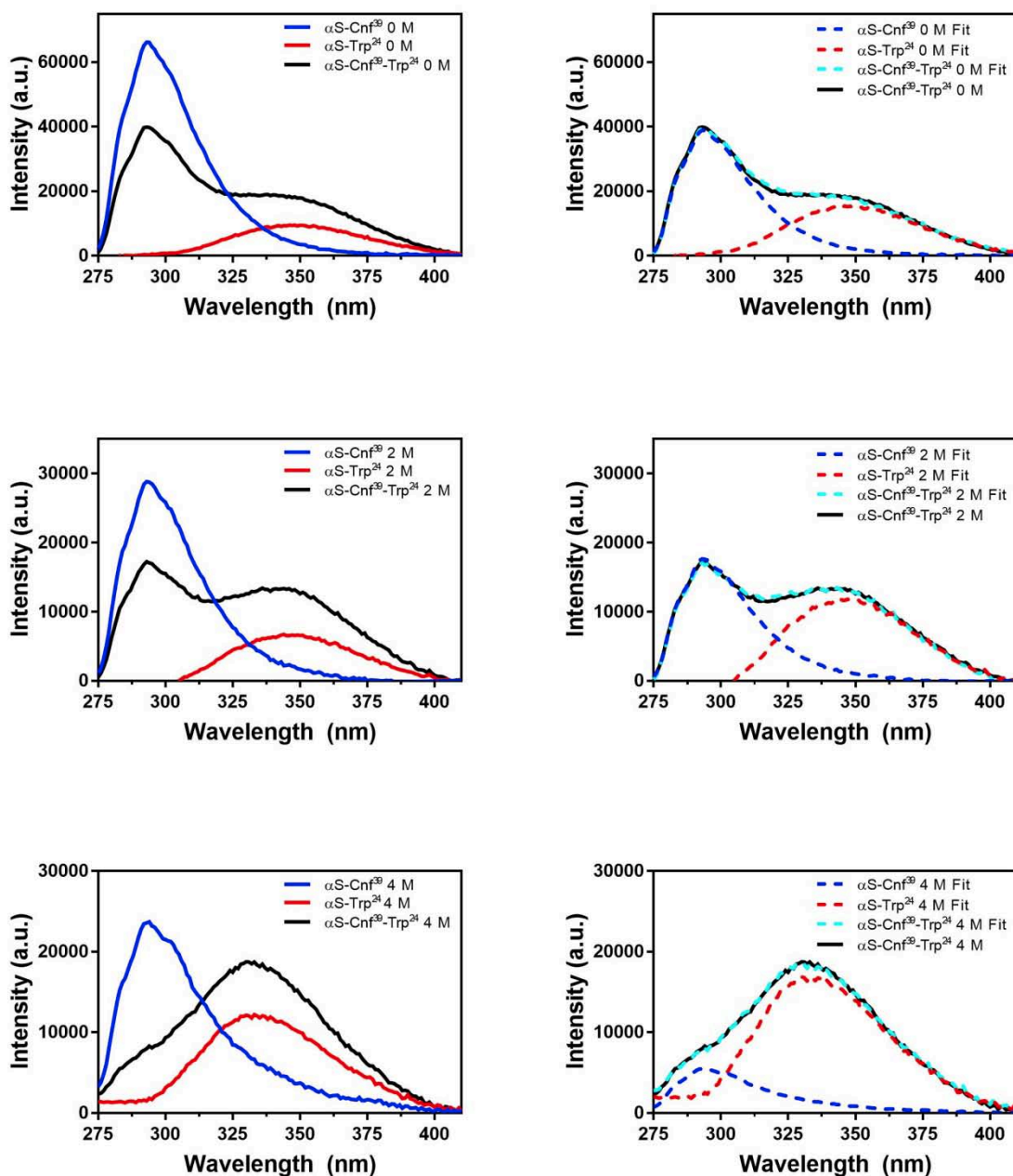


Figure S17: Steady-state Fluorescence Spectra of α S-Cnf³⁹-Trp²⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

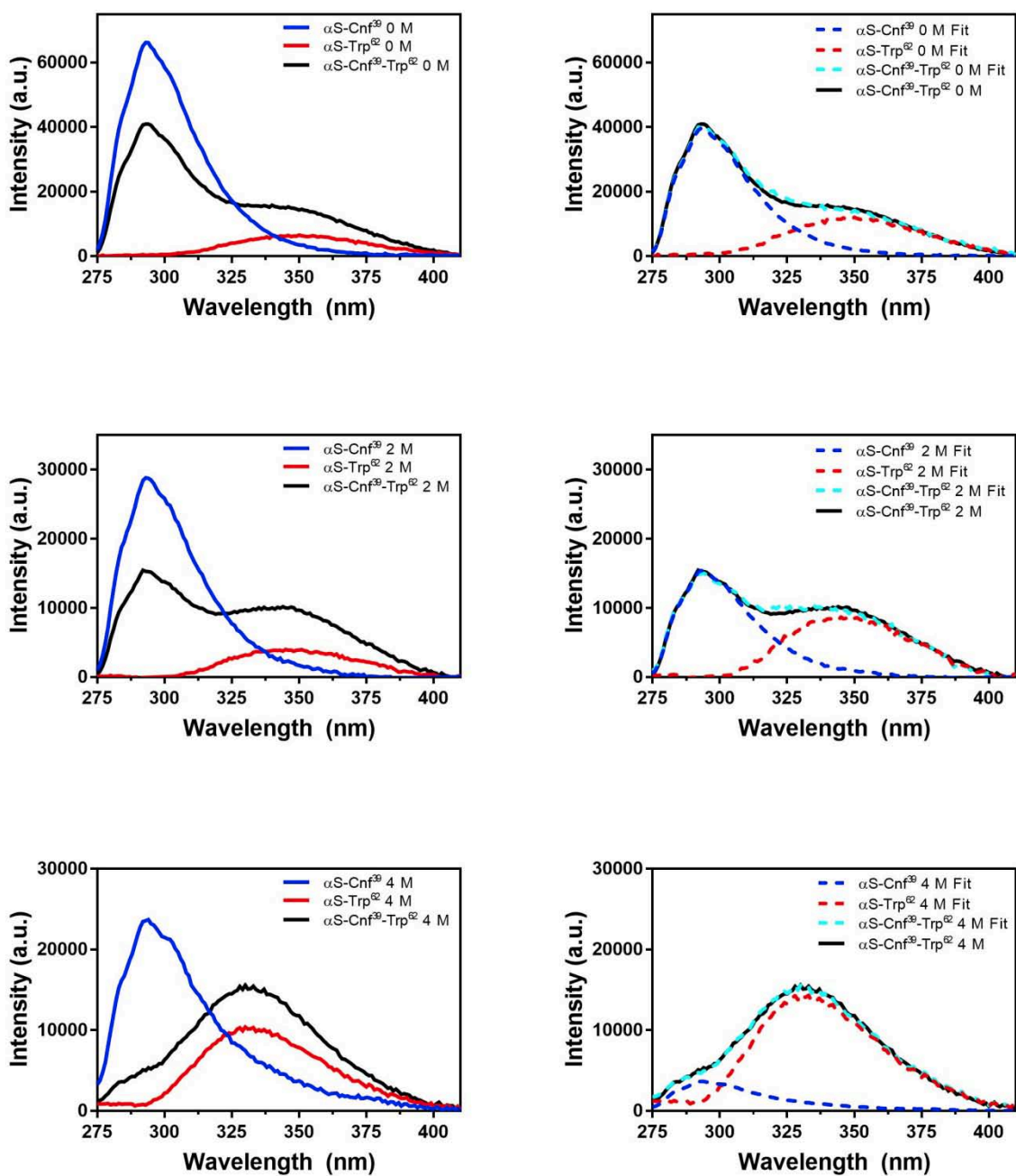


Figure S18: Steady-state Fluorescence Spectra of α S-Cnf³⁹-Trp⁶². Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

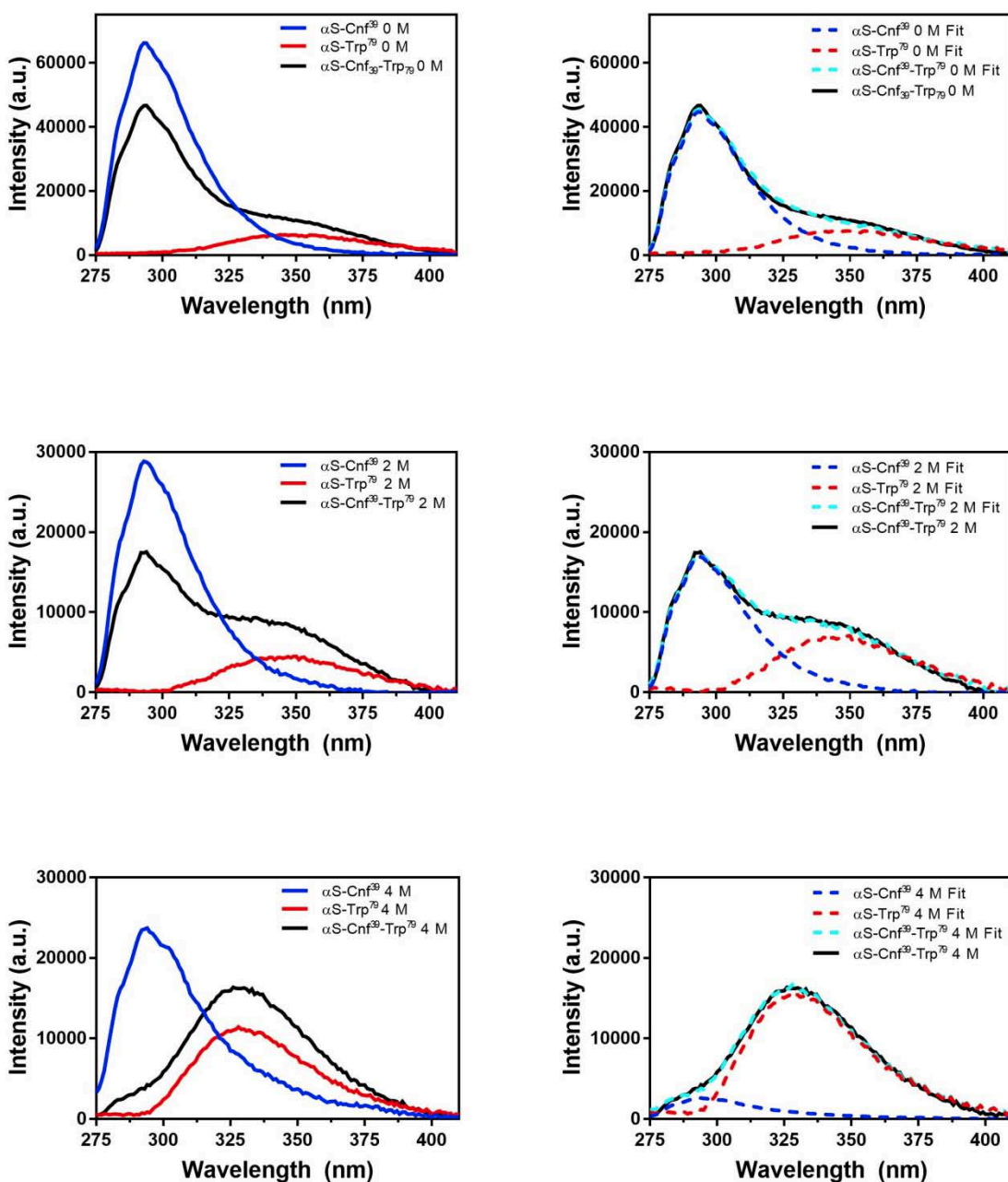


Figure S19: Steady-state Fluorescence Spectra of α S-Cnf³⁹-Trp⁷⁹. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

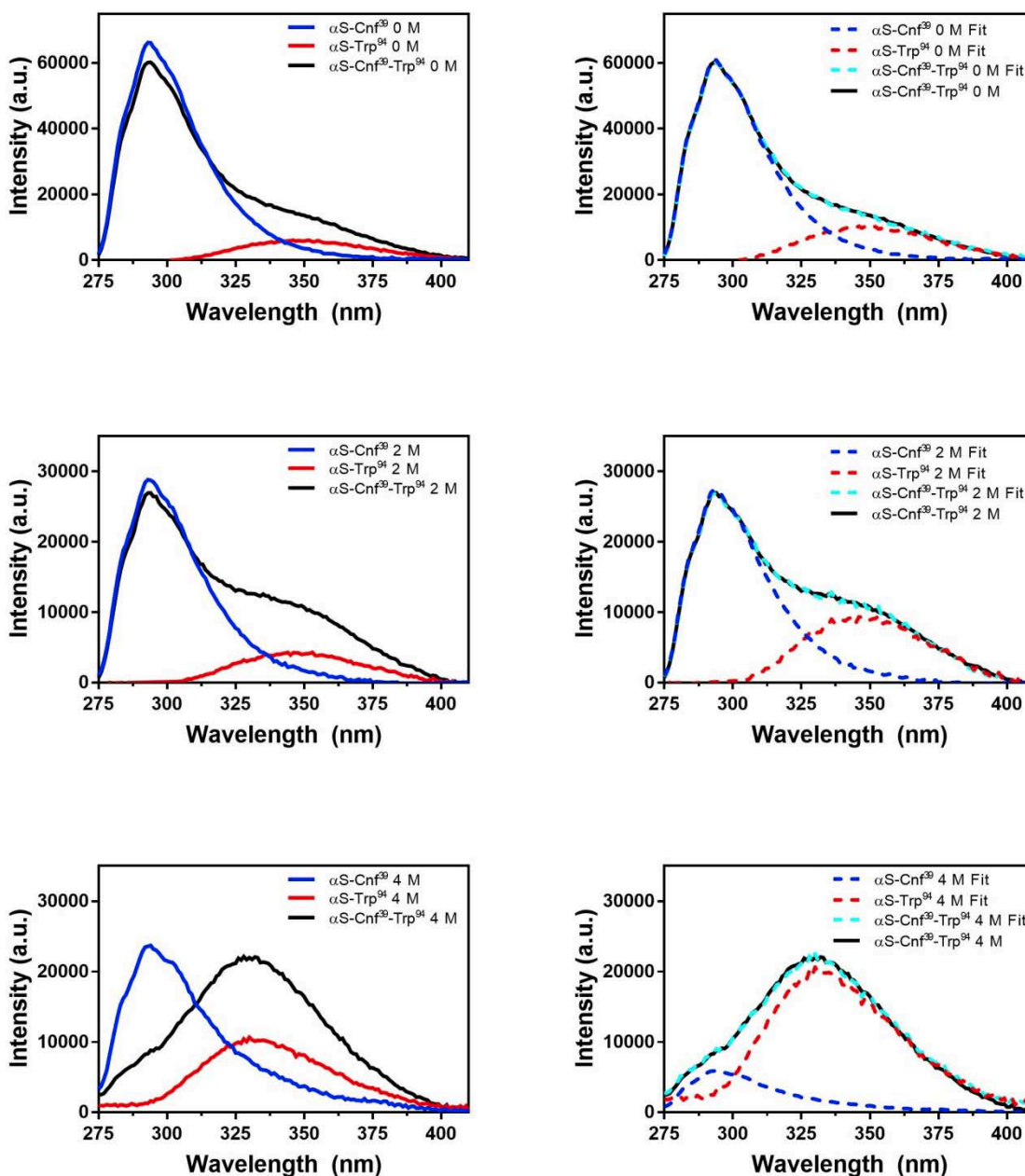


Figure S20: Steady-state Fluorescence Spectra of $\alpha\text{S-Cnf}^{39}\text{-Trp}^{94}$. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled αS along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

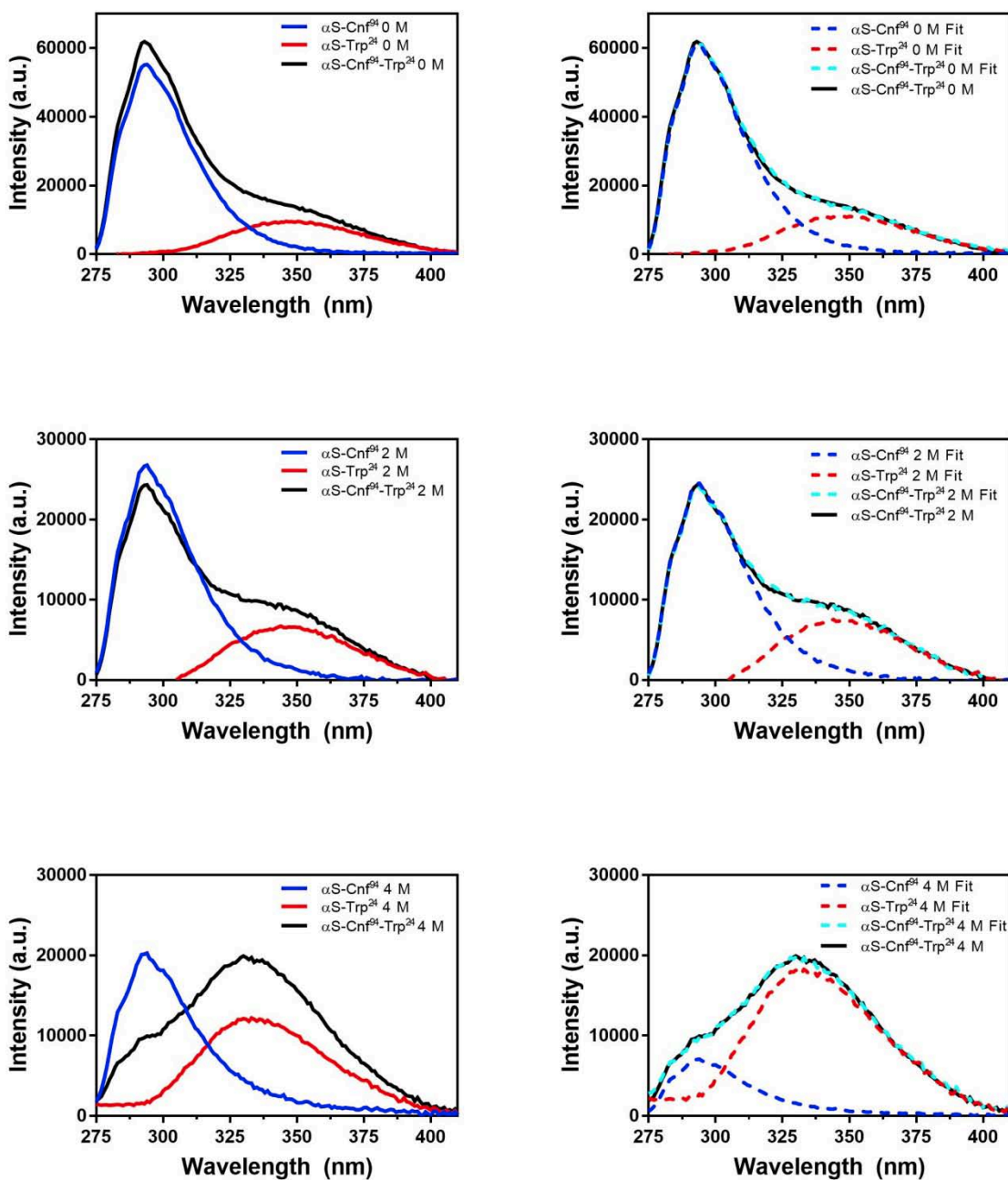


Figure S21: Steady-state Fluorescence Spectra of $\alpha\text{S-Cnf}^{94}\text{-Trp}^{24}$. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled αS along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

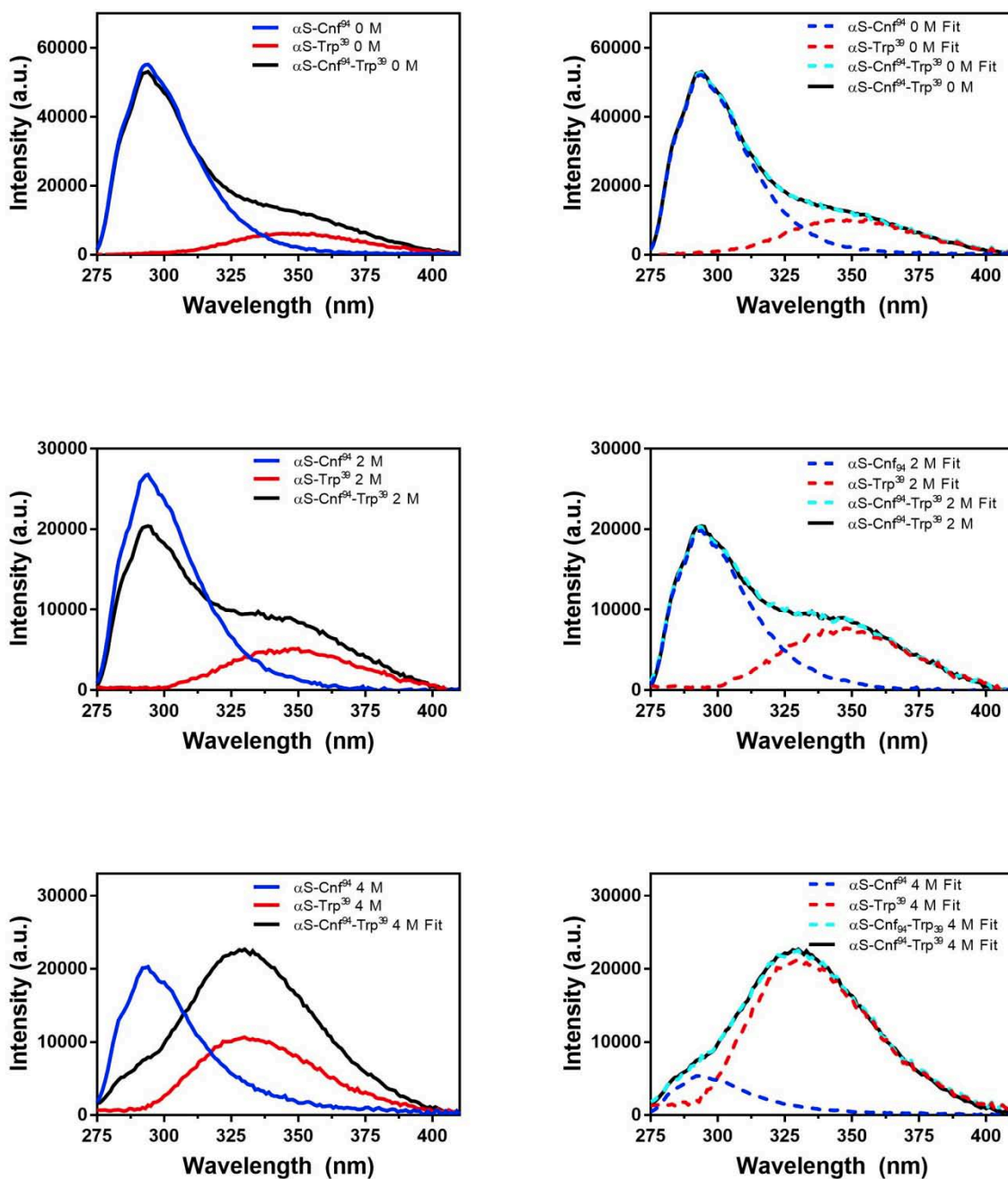


Figure S22: Steady-state Fluorescence Spectra of $\alpha\text{S-Cnf}^{94}\text{-Trp}^{39}$. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled αS along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

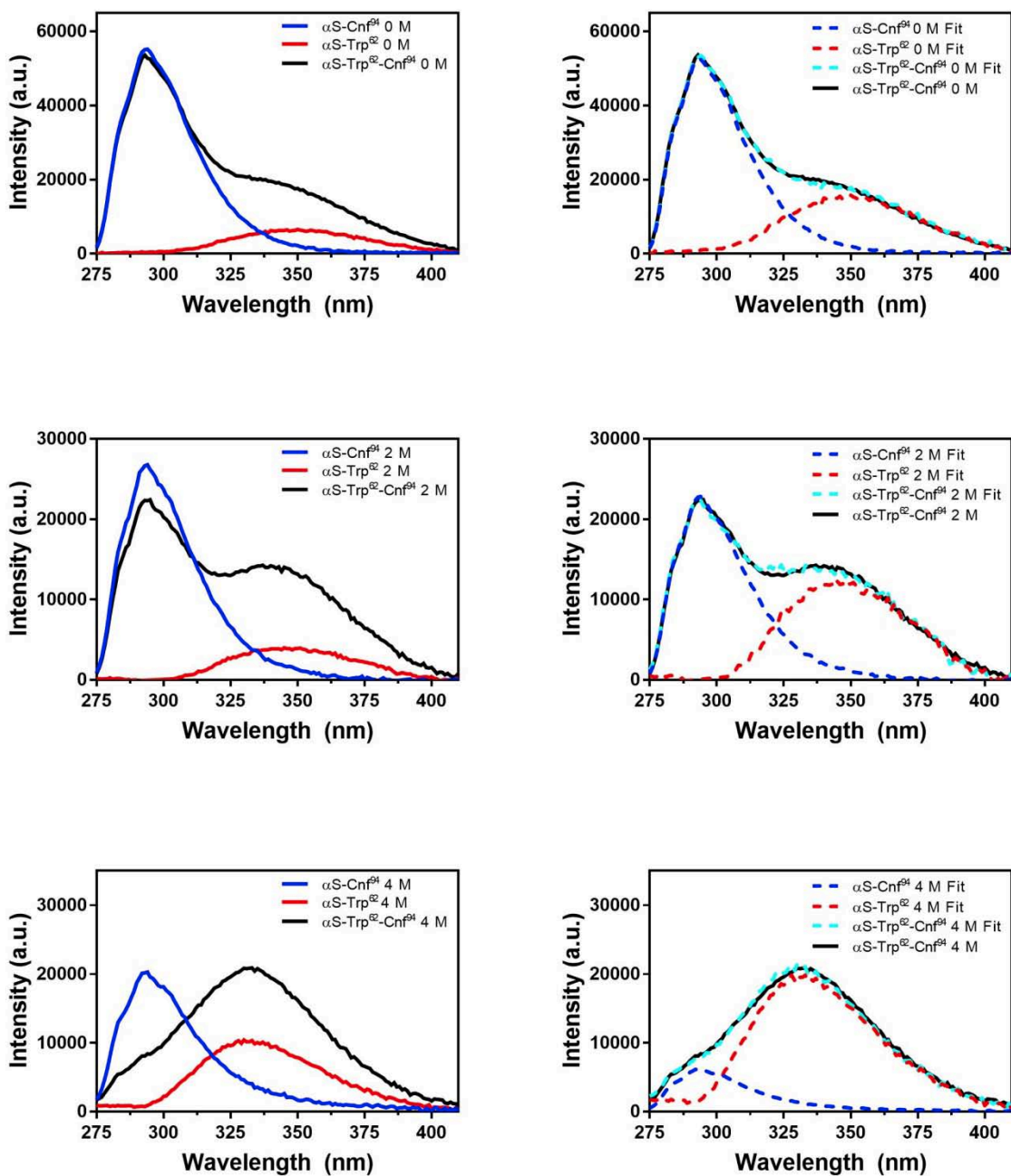


Figure S23: Steady-state Fluorescence Spectra of α S-Cnf⁹⁴-Trp⁶². Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

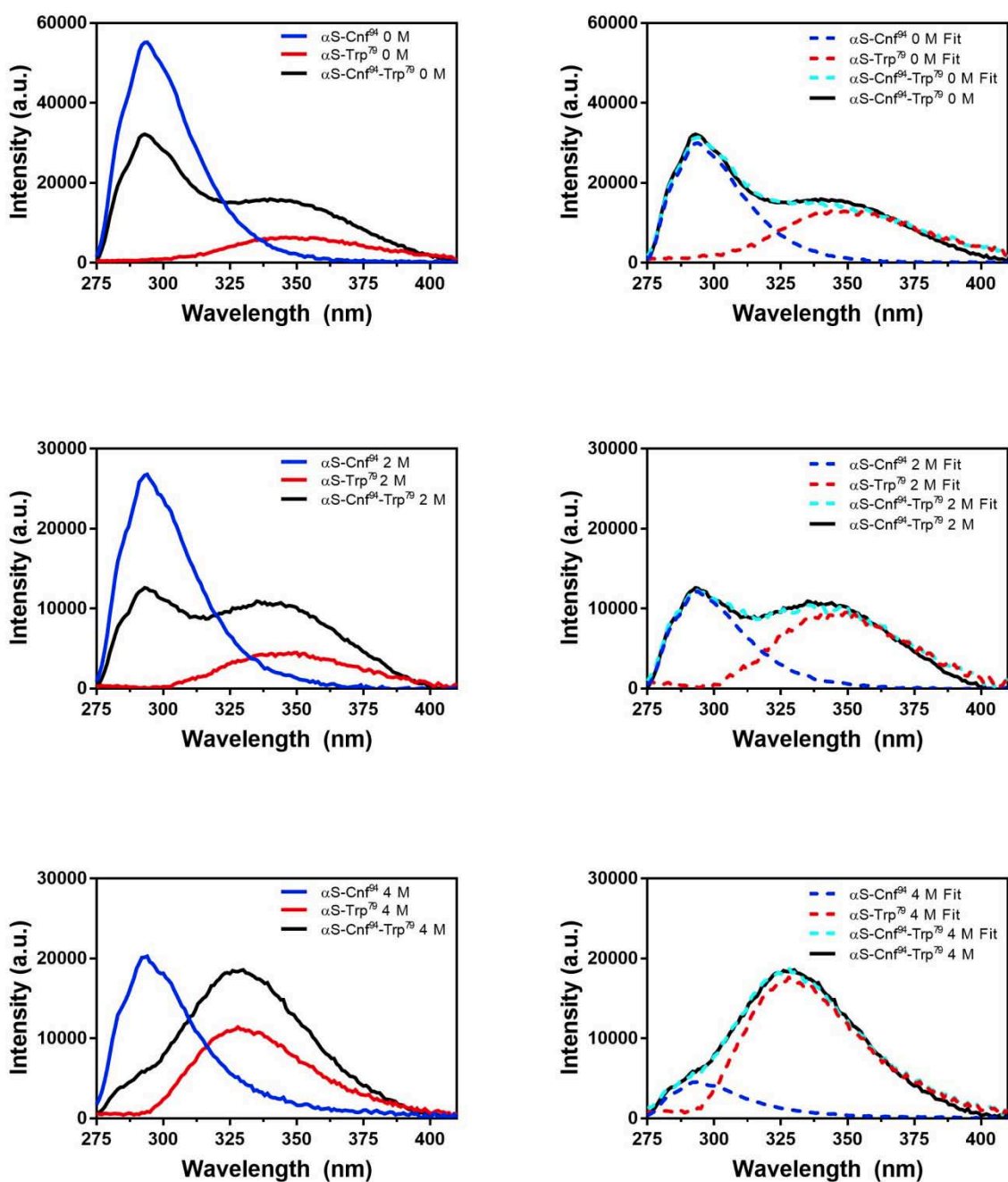


Figure S24: Steady-state Fluorescence Spectra of $\alpha\text{S-Cnf}^{94}\text{-Trp}^{79}$. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled αS along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

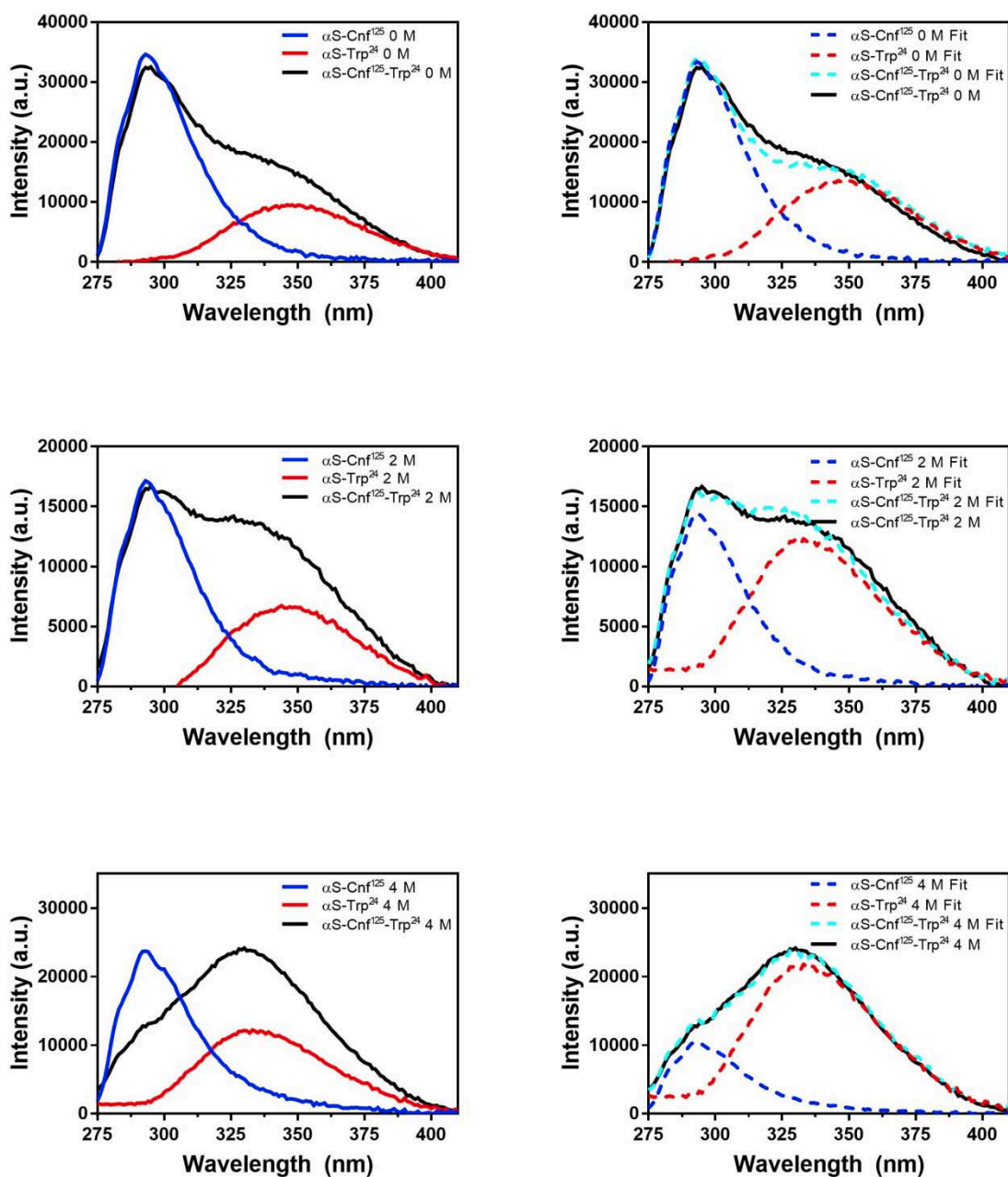


Figure S25: Steady-state Fluorescence Spectra of α S-Cnf¹²⁵-Trp²⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

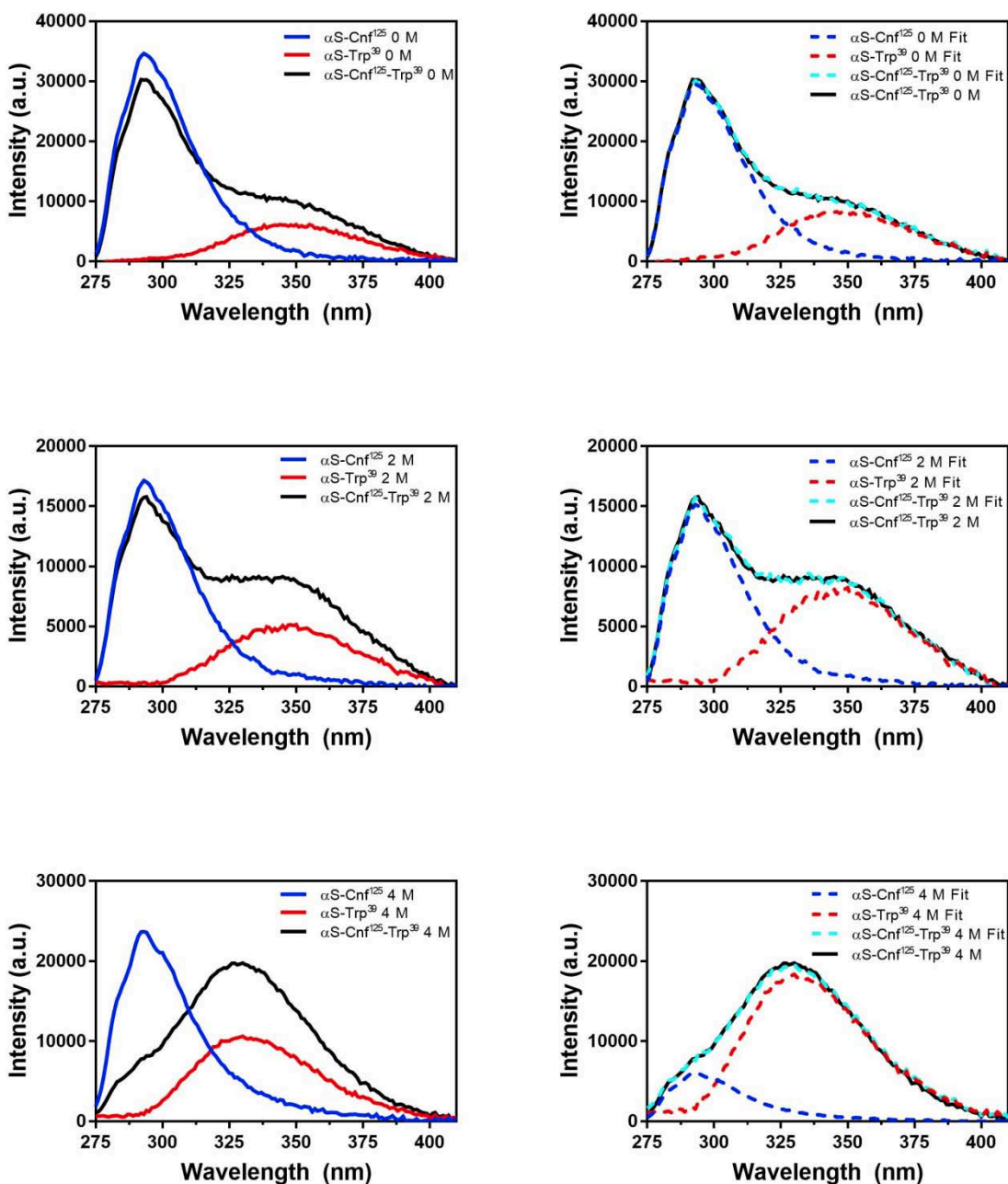


Figure S26: Steady-state Fluorescence Spectra of $\alpha\text{S-Cnf}^{125}\text{-Trp}^{39}$. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled αS along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

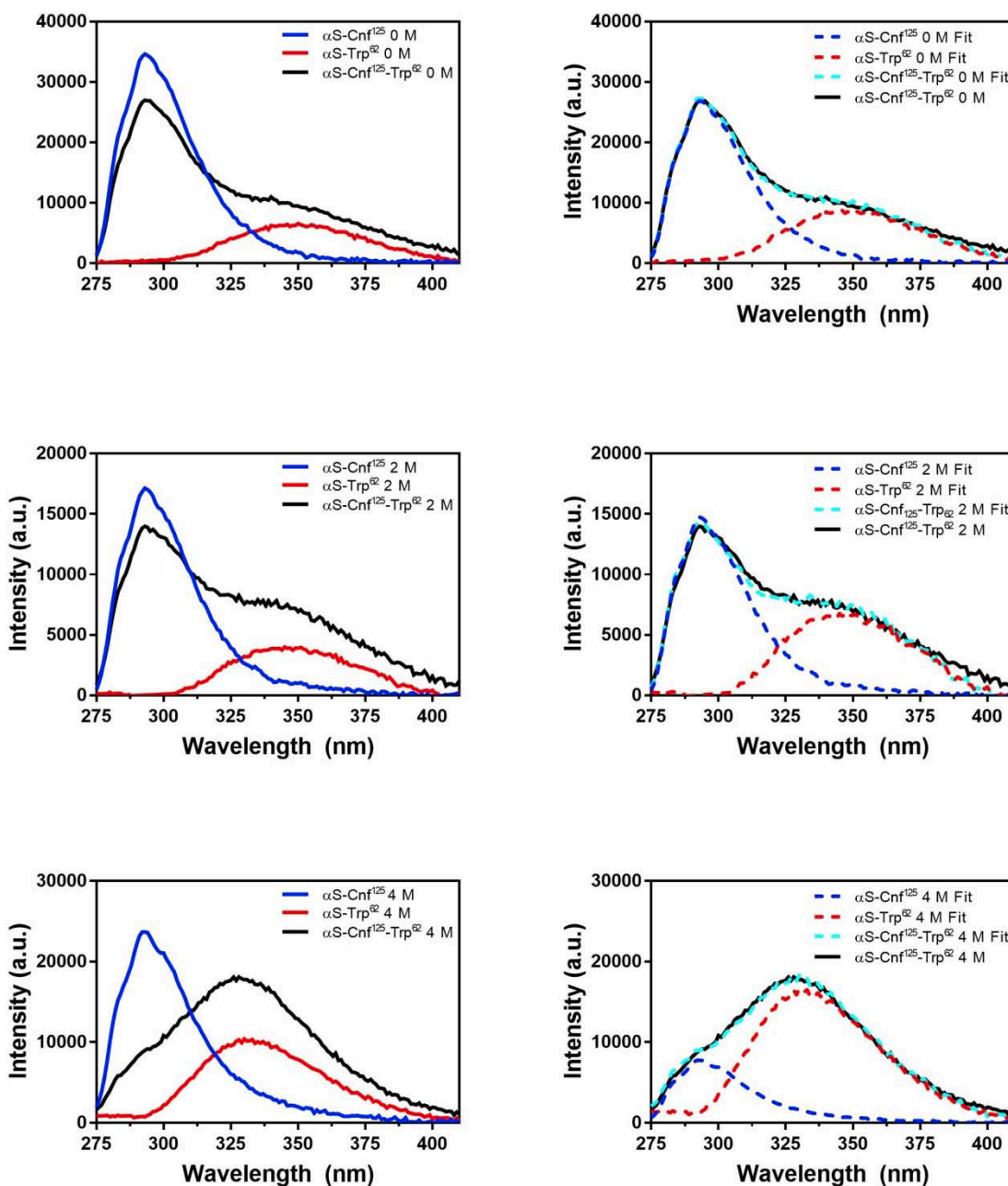


Figure S27: Steady-state Fluorescence of $\alpha\text{S-Cnf}^{125}\text{-Trp}^{62}$. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled αS along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

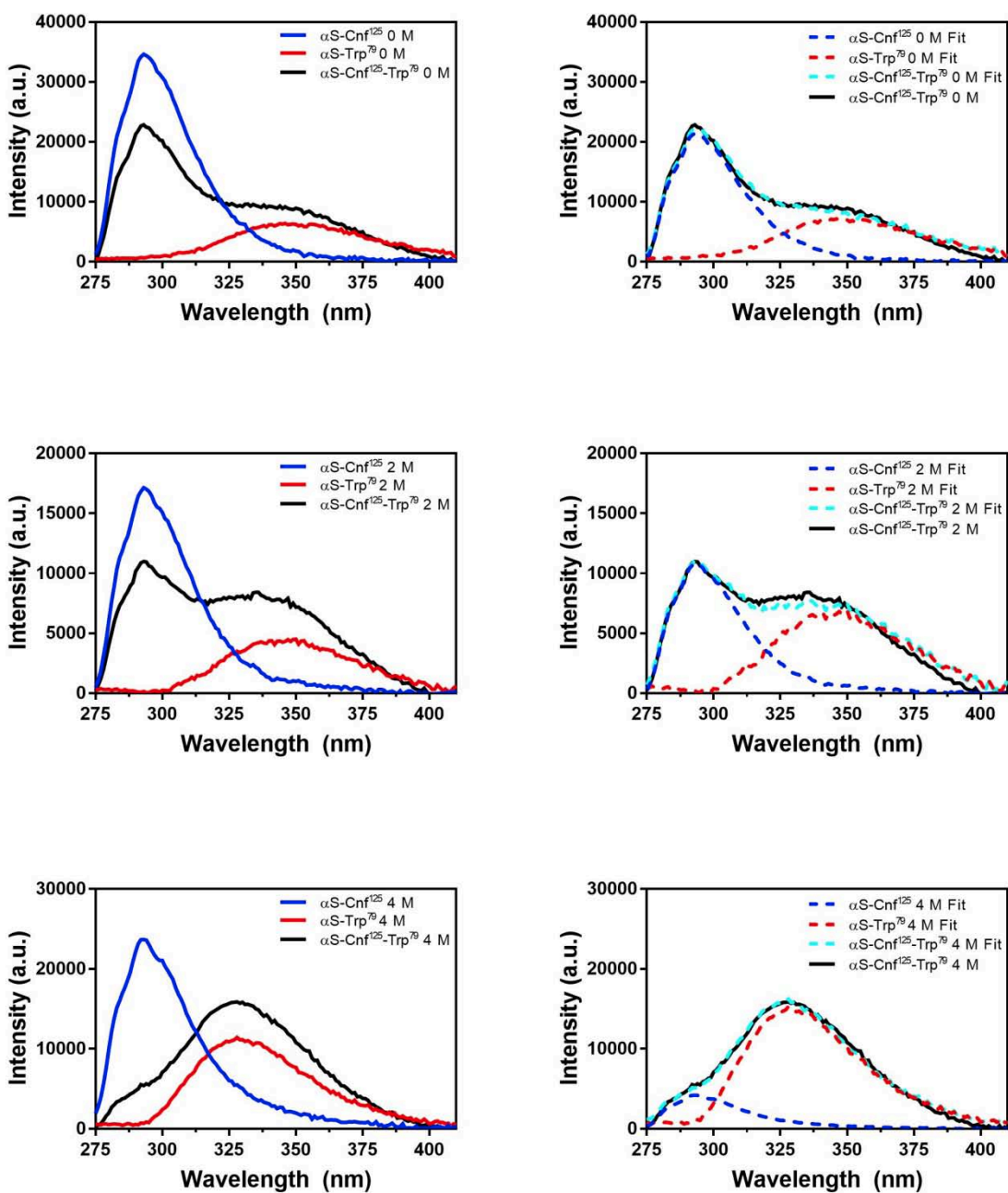


Figure S28: Steady-state Fluorescence Spectra of α S-Cnf¹²⁵-Trp⁷⁹. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

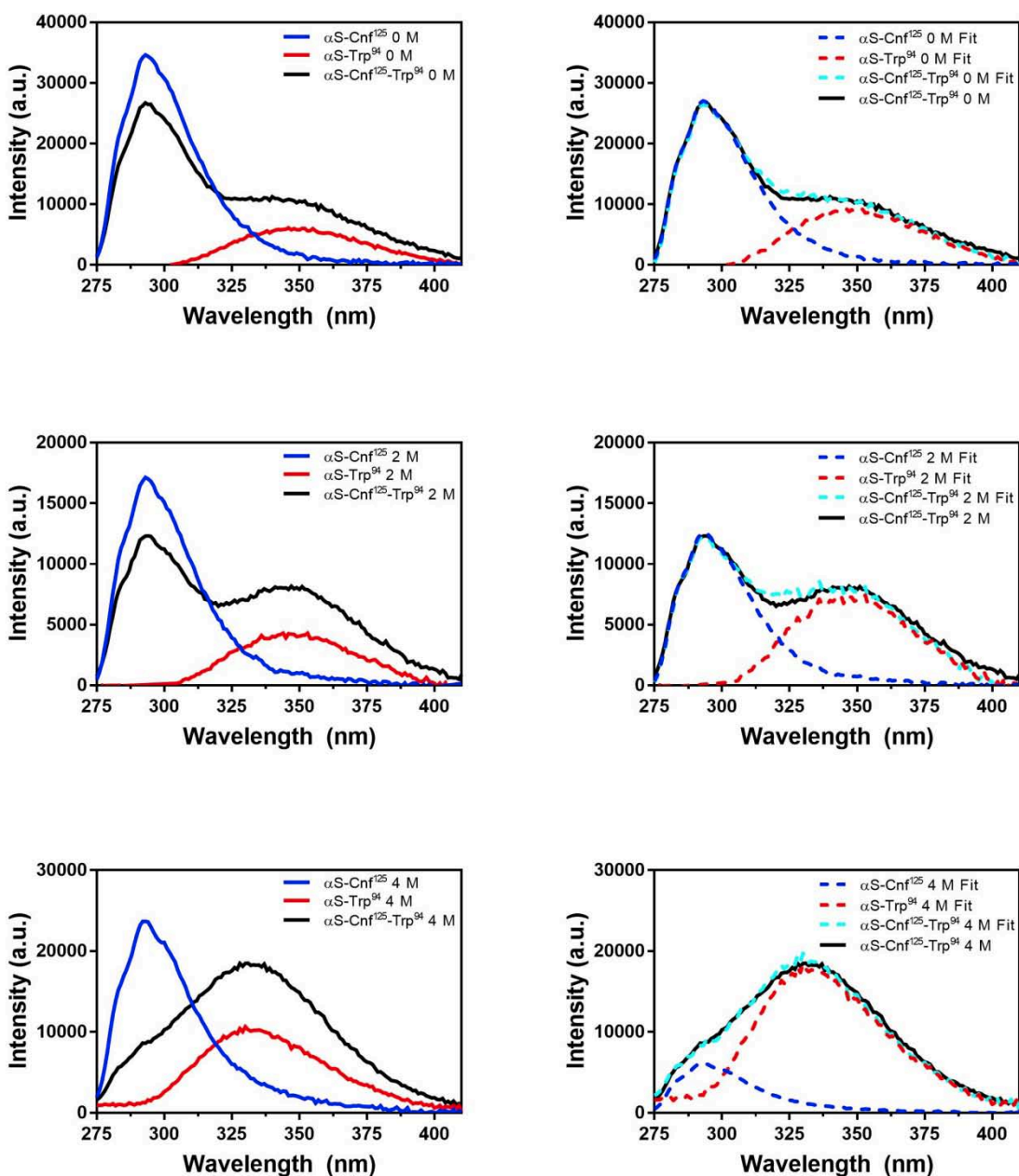


Figure S29: Steady-state Fluorescence Spectra of α S-Cnf¹²⁵-Trp⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

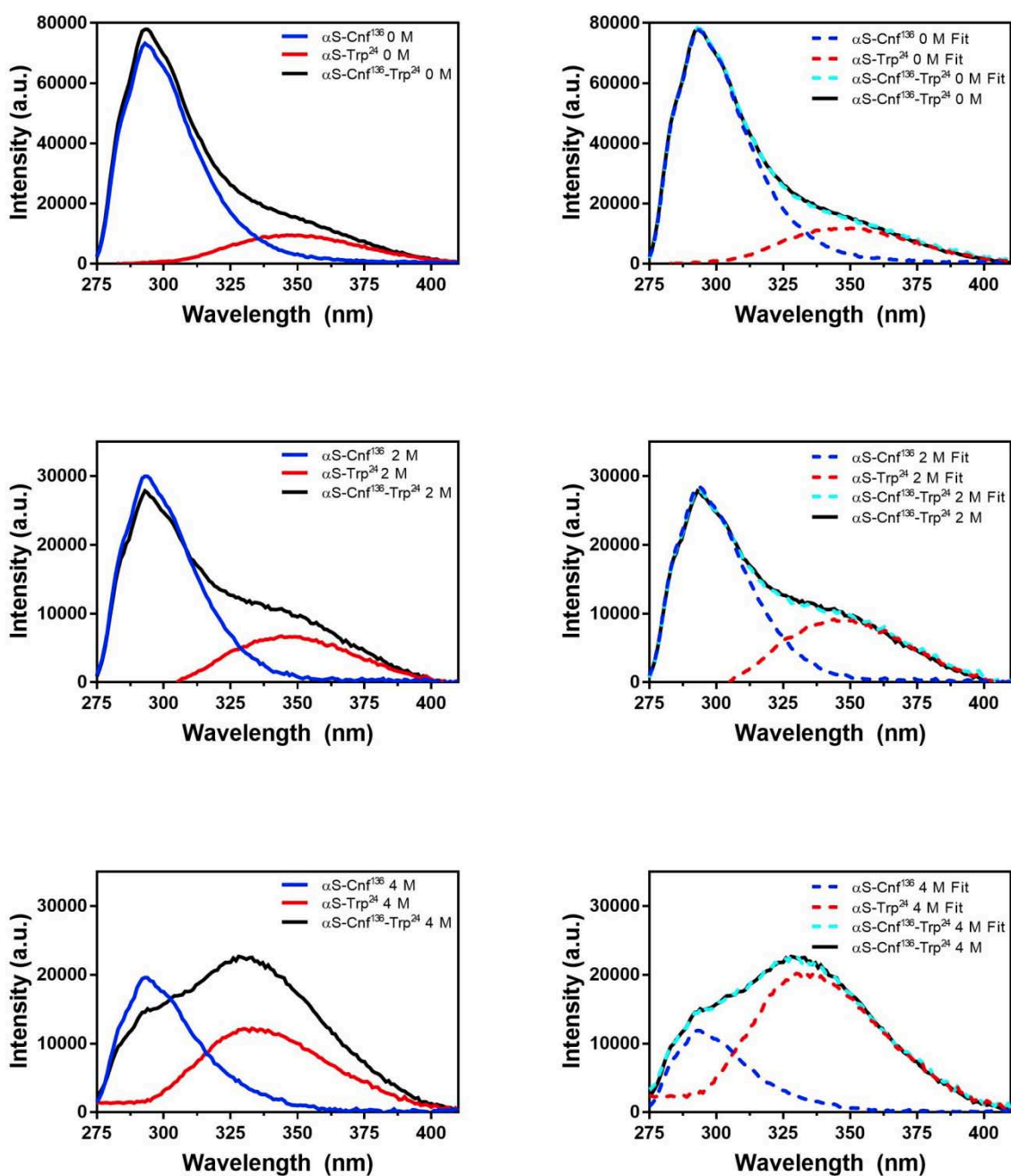


Figure S30: Steady-state Fluorescence Spectra of α S-Cnf¹³⁶-Trp²⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

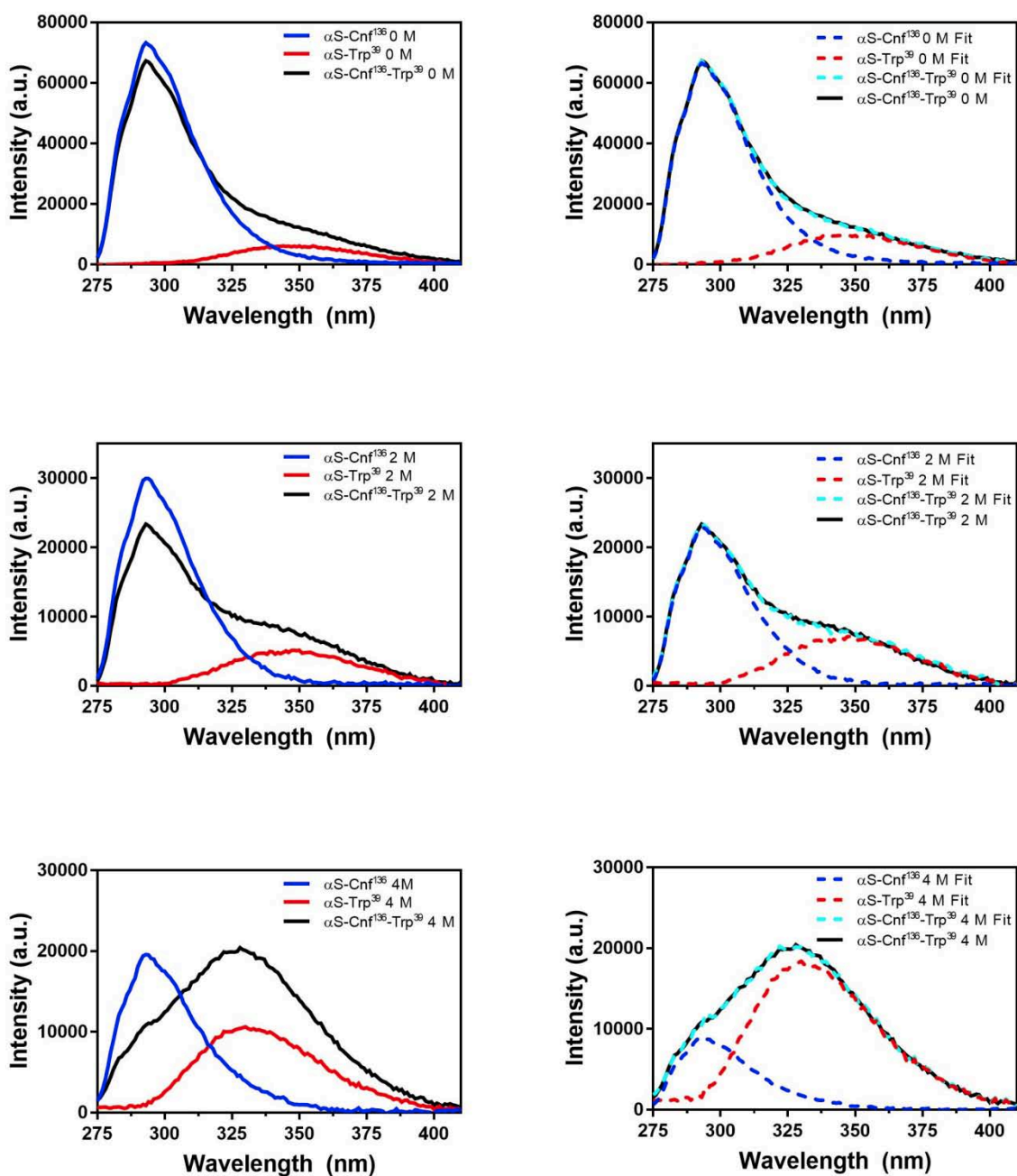


Figure S31: Steady-state Fluorescence Spectra of α S-Cnf¹³⁶-Trp³⁹. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

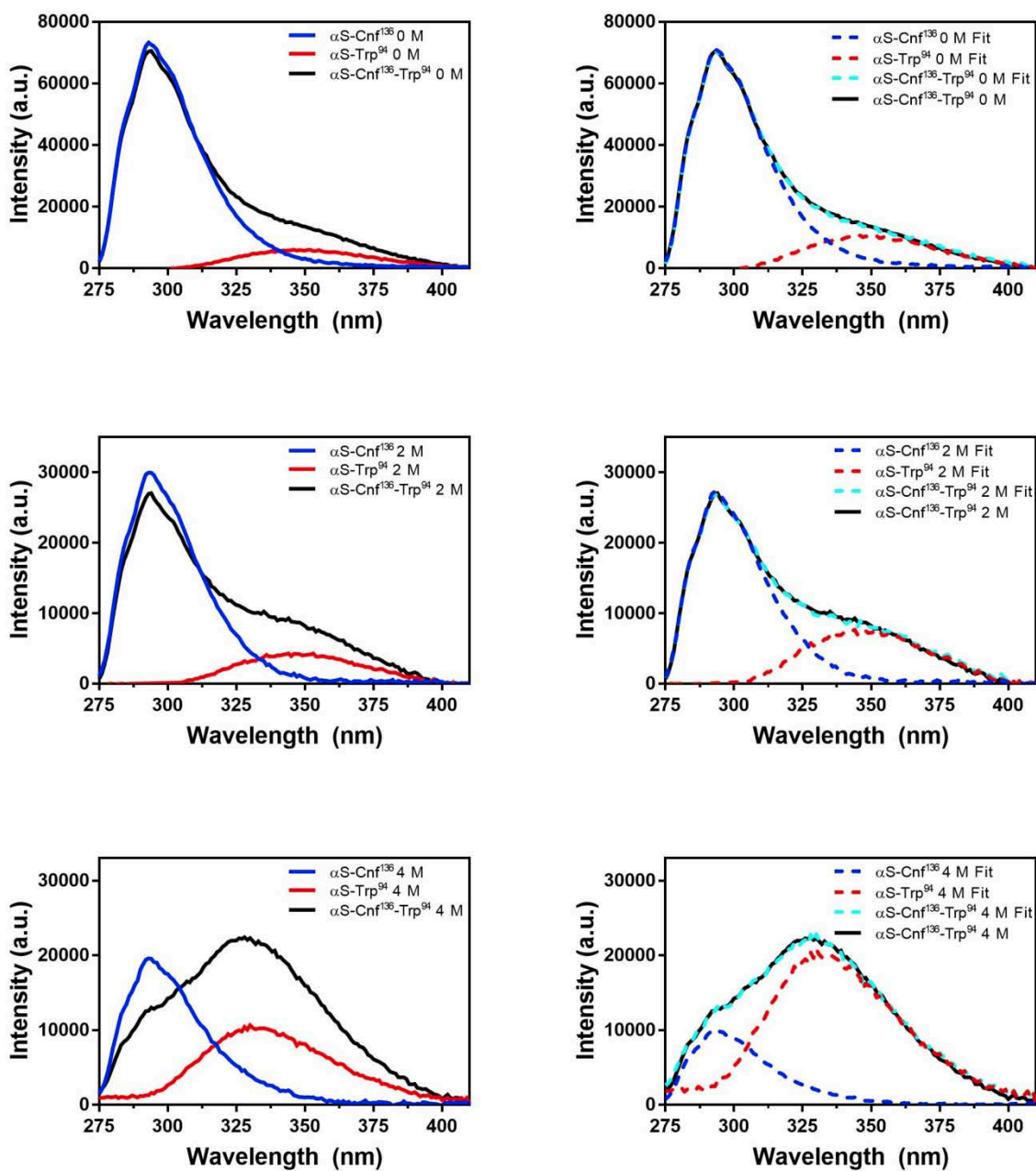


Figure S32: Steady-state Fluorescence Spectra of α S-Cnf¹³⁶-Trp⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

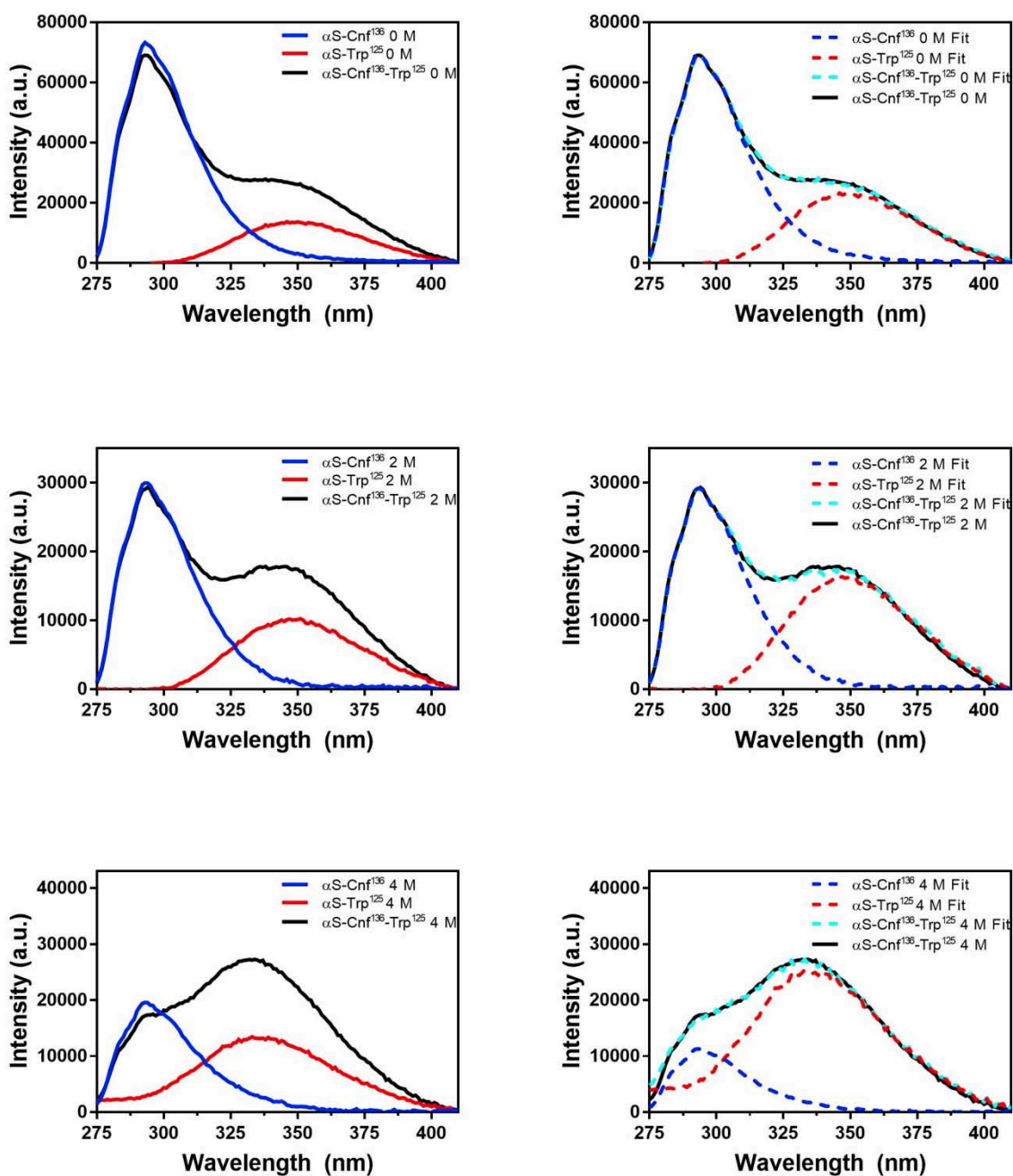


Figure S33: Steady-state Fluorescence Spectra of α S-Cnf¹³⁶-Trp¹²⁵. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Cnf (blue) and Trp (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Cnf (red dashed) and Trp (blue dashed) to the fit.

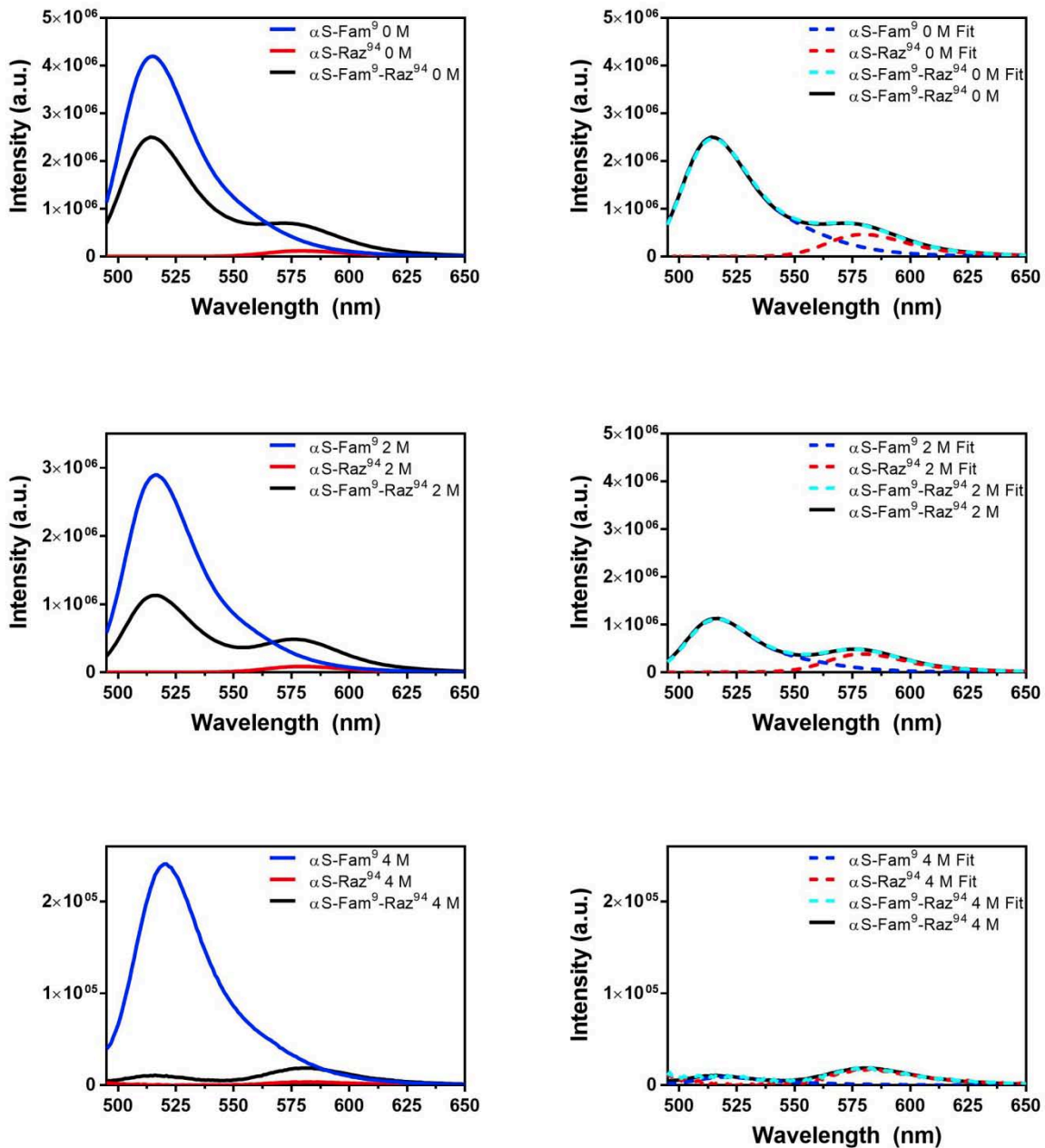


Figure S34: Steady-state Fluorescence Spectra of α S-Fam⁹-Raz⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

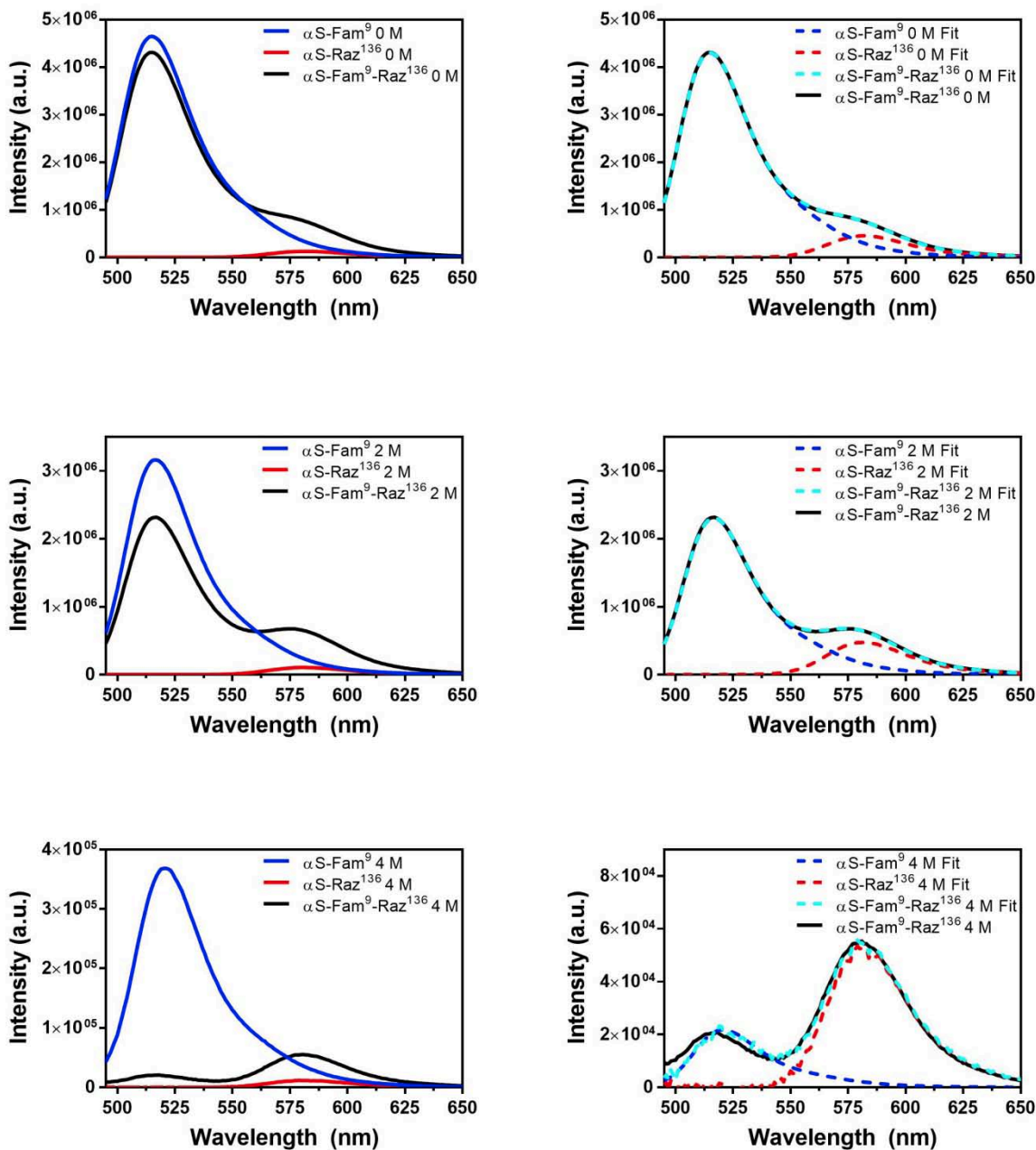


Figure S35: Steady-state Fluorescence Spectra of α S-Fam⁹-Raz¹³⁶. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

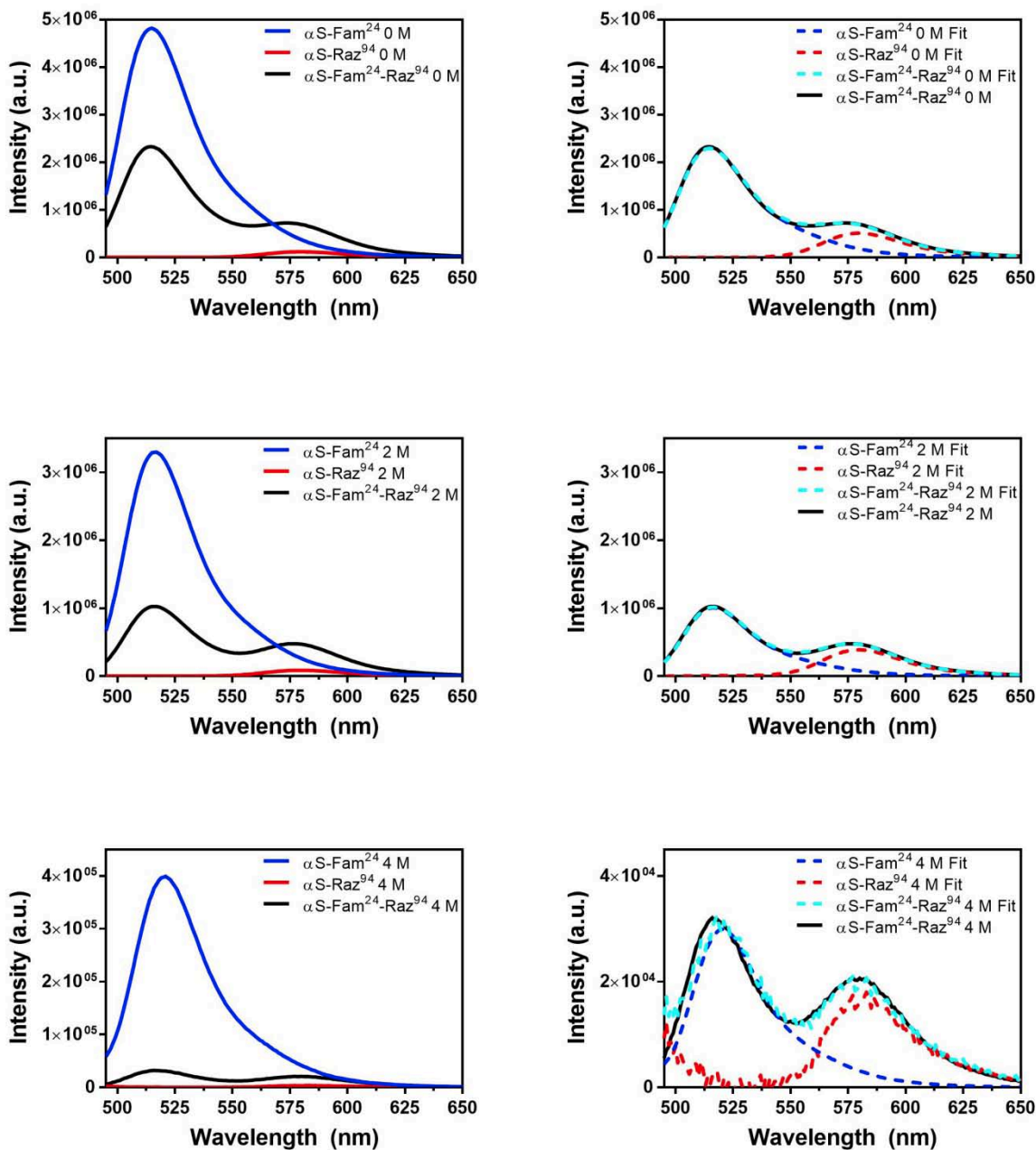


Figure S36: Steady-state Fluorescence Spectra of α S-Fam²⁴-Raz⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

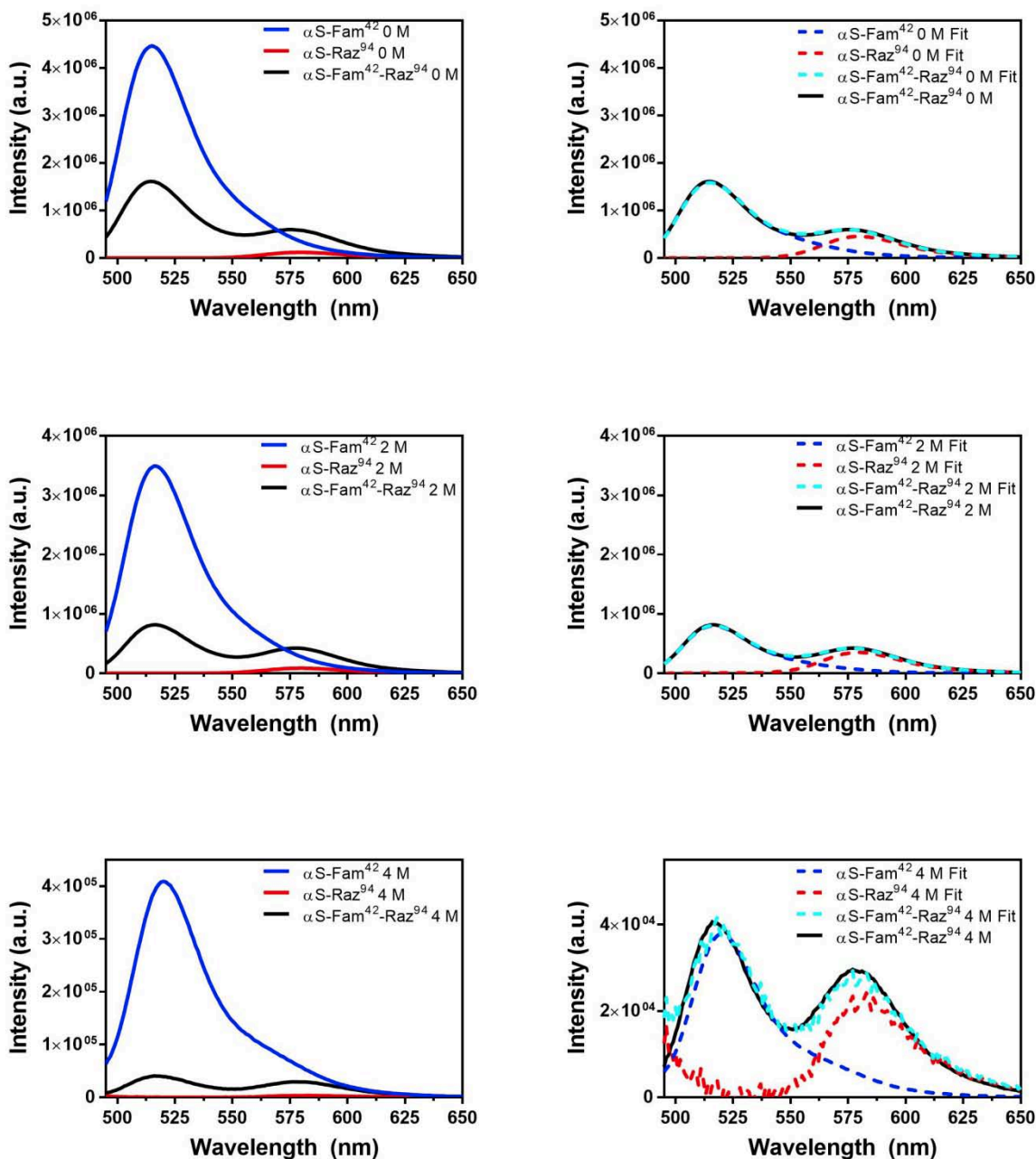


Figure S37: Steady-state Fluorescence Spectra of α S-Fam⁴²-Raz⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

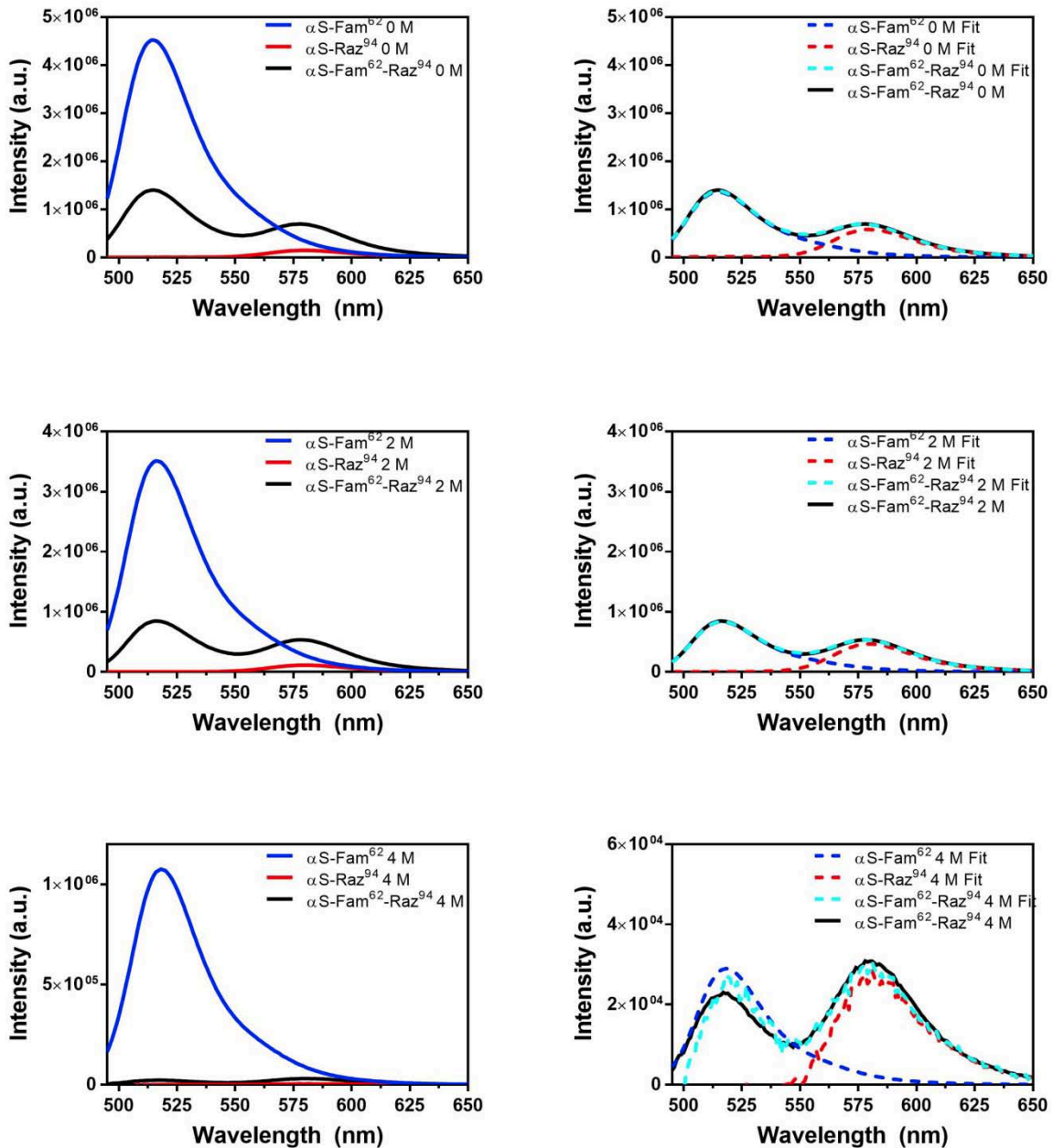


Figure S38: Steady-state Fluorescence Spectra of α S-Fam⁶²-Raz⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

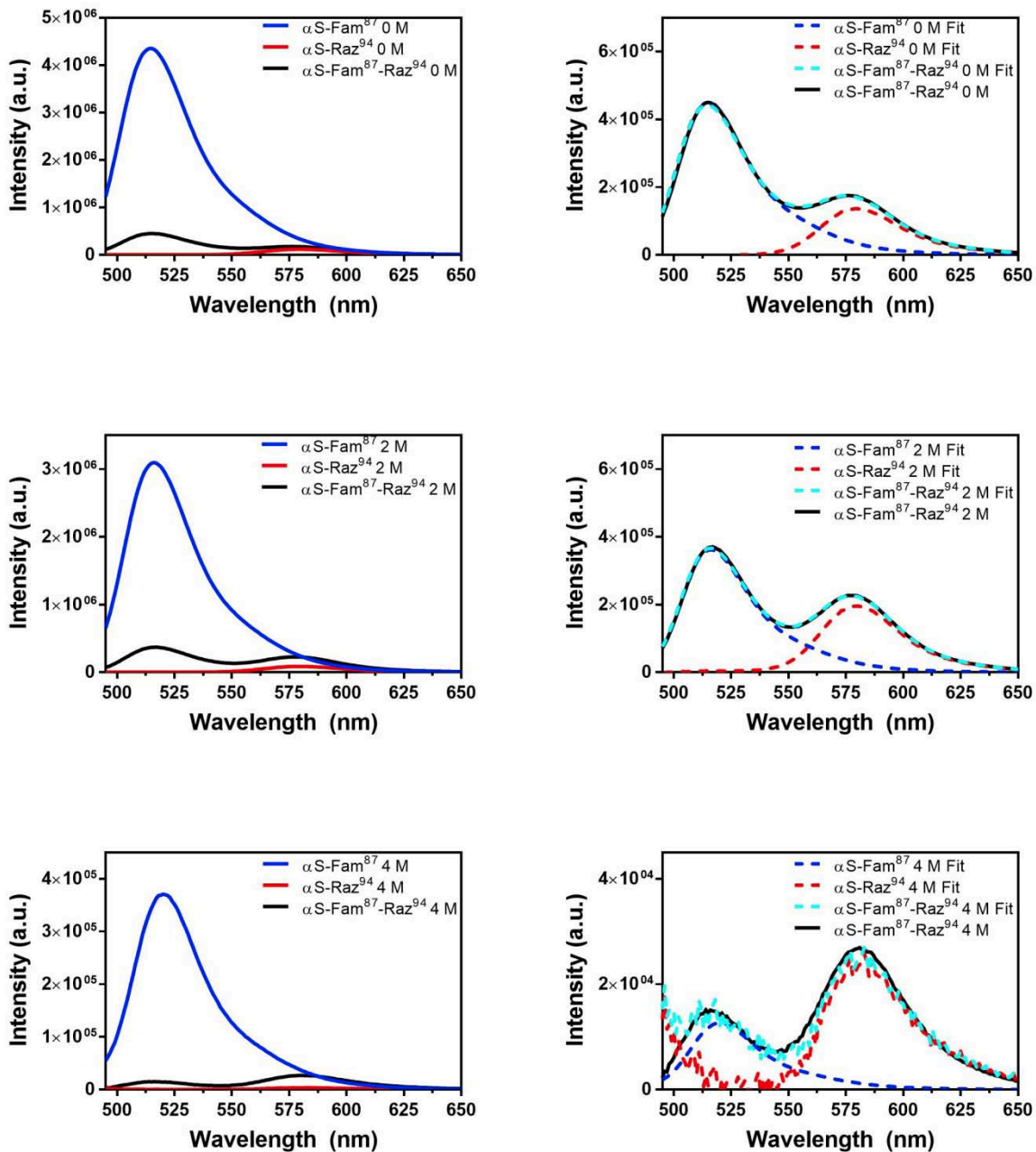


Figure S39: Steady-state Fluorescence Spectra of α S-Fam⁸⁷-Raz⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

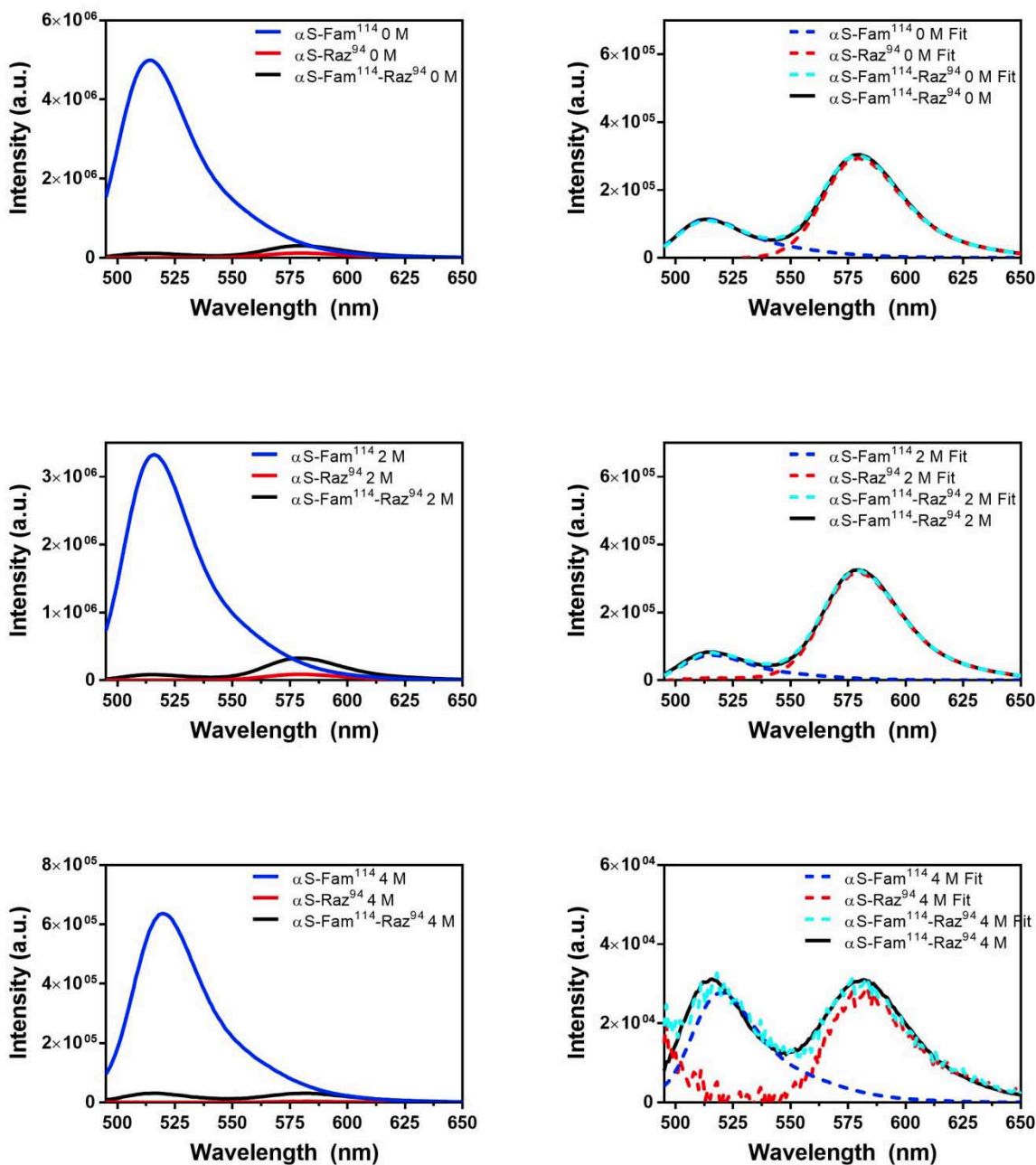


Figure S40: Steady-state Fluorescence Spectra of α S-Fam¹¹⁴-Raz⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

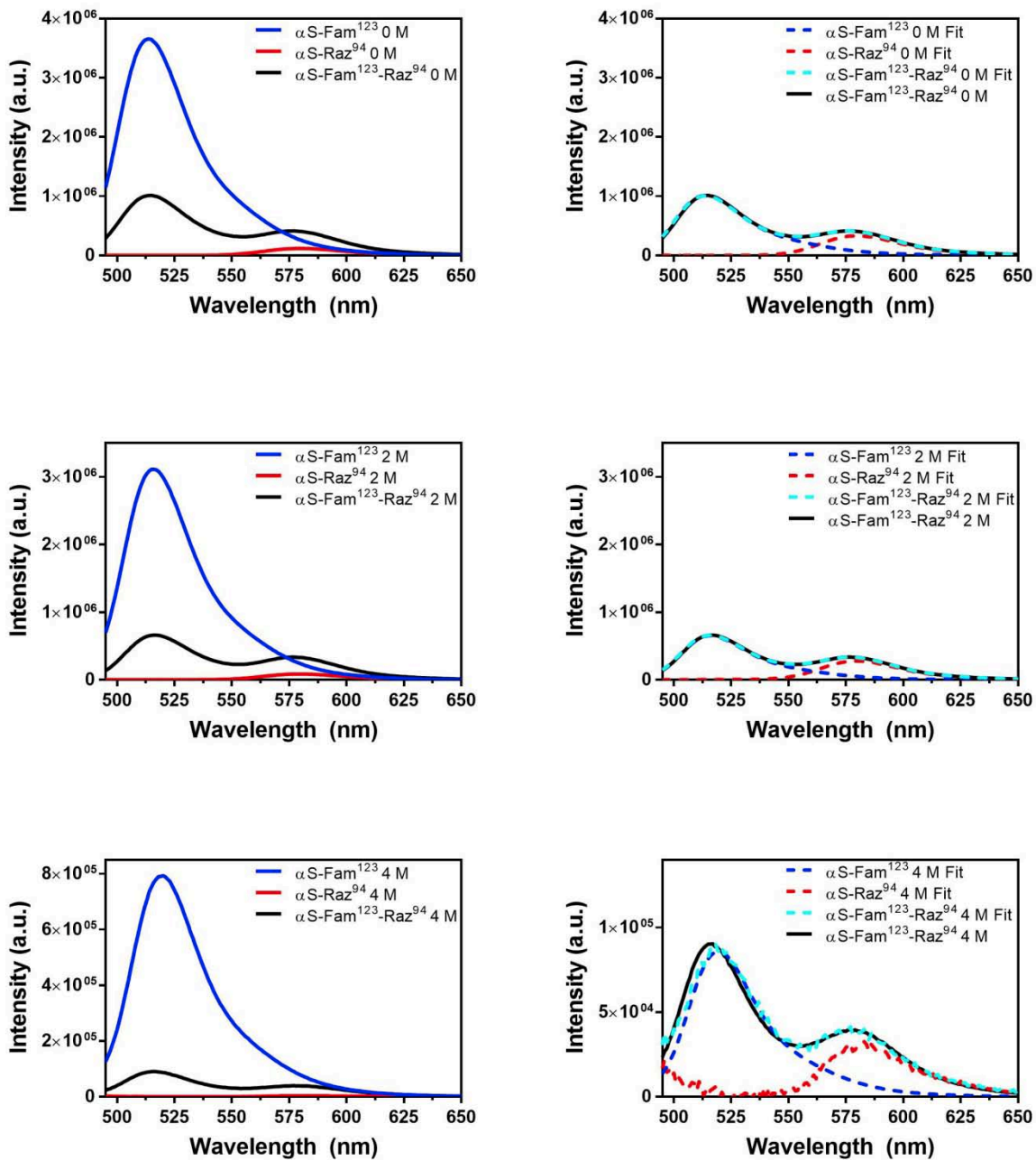


Figure S41: Steady-state Fluorescence Spectra of $\alpha\text{S-Fam}^{123}\text{-Raz}^{94}$. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain cooresponding single-labeled Fam (blue) and Raz (red) labeled αS along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

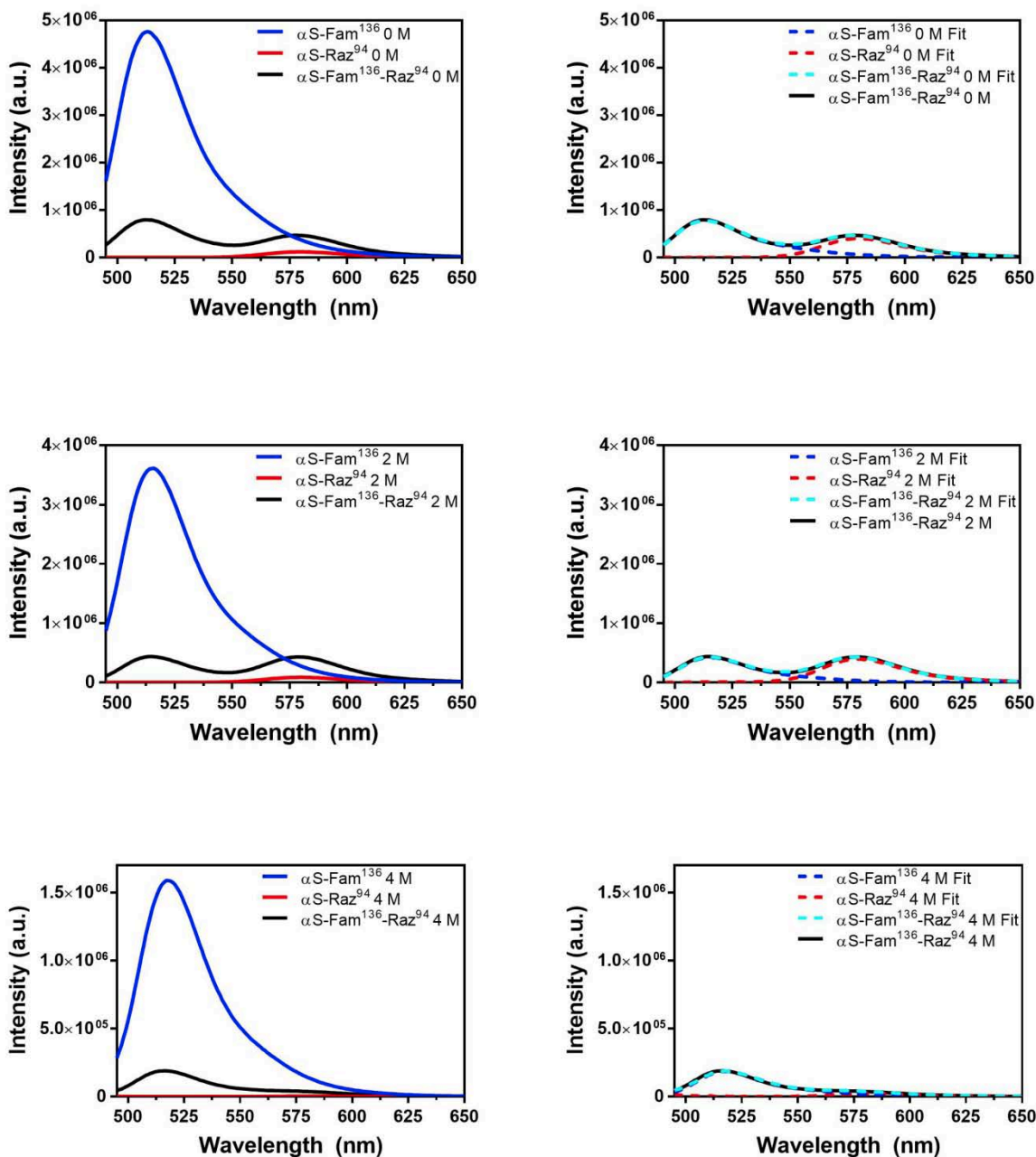


Figure S42: Steady-state Fluorescence Spectra of α S-Fam¹³⁶-Raz⁹⁴. Plots show background subtracted data (Left) and fit data (Right) for 0, 2 and 4 M TMAO (Top-Bottom). Background subtracted plots contain corresponding single-labeled Fam (blue) and Raz (red) labeled α S along with the double-labeled protein (black). Plots showing the fits contain the background subtracted double-labeled protein (black) spectrum along with the fit (cyan dashed) and contributions of Fam (red dashed) and Raz (blue dashed) to the fit.

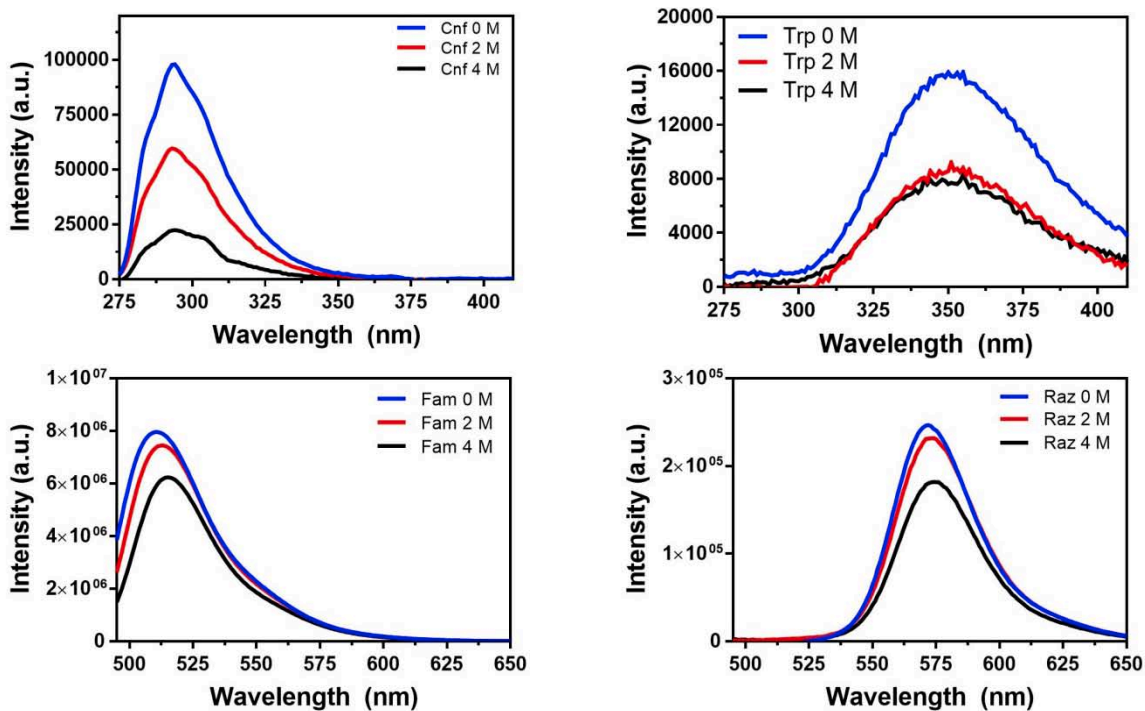


Figure S43: Steady-state Fluorescence Spectra of Free Fluorophores. Plots show background subtracted spectra of Cnf (top left), Trp (top right), Fam (bottom left) and Raz (bottom right) for 0 (blue), 2 (red) and 4 M TMAO (black).

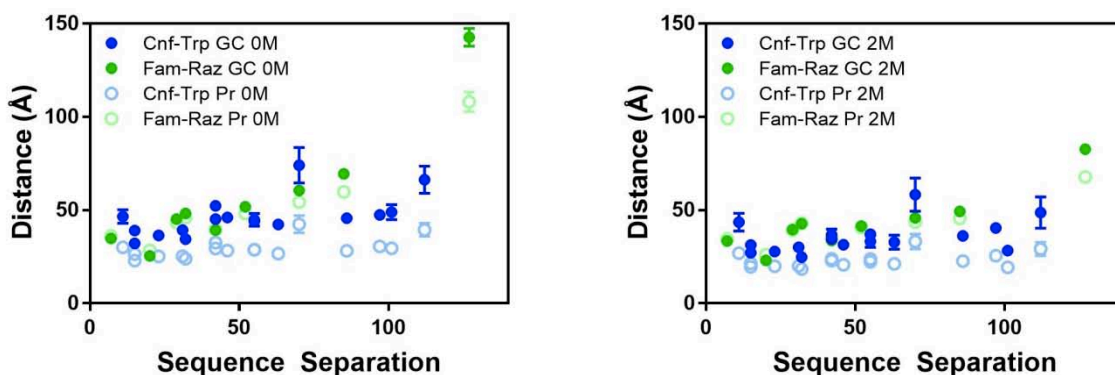


Figure S44: Distance from Polymer-Scaled Förster Equation. Plot show distances obtained from steady-state derived E_{FRET} values through the Gaussian chain (GC) or semi-empirical (Pr) forms of the polymer-scaled Förster equation. Plots are shown for data obtained in 0 M (left) and 2 M (right) TMAO.

Table S3a. Steady-State FRET Fitting and Distance Determination

Pair	PSS	TMAO	Q _D	R ₀	ε _A /ε _D	A	E _D	S _A	B	E _A	S _B	E _{FRET}	S _{FRET}	Gaussian Chain		Semi-Empirical	
														r (Å)	S _{Dist}	r (Å)	S _{Dist}
Cnf ⁶⁹ -Trp ²⁴	15	0M	0.10	16.7	0.13	0.587	0.413	0.094	1.619	0.080	0.005	0.096	0.006	39.1	0.8	26.4	0.4
	15	2M	0.04	14.6	0.13	0.613	0.387	0.140	1.782	0.102	0.008	0.116	0.010	31.3	0.9	21.4	0.4
	15	4M	0.04	14.2	0.13	0.233	0.767	0.146	1.390	0.051	0.010	0.094	0.013	30.0	1.0	20.7	0.5
Cnf ⁶⁹ -Trp ⁶²	23	0M	0.10	16.7	0.13	0.599	0.401	0.138	1.841	0.109	0.006	0.121	0.008	36.5	0.7	25.2	0.3
	23	2M	0.04	14.6	0.13	0.535	0.465	0.218	2.173	0.153	0.007	0.163	0.010	27.8	0.7	19.9	0.3
	23	4M	0.04	14.2	0.13	0.155	0.845	0.209	1.386	0.050	0.010	0.085	0.013	31.7	1.3	21.4	0.6
Cnf ⁶⁹ -Trp ⁷⁹	42	0M	0.10	16.7	0.13	0.675	0.325	0.078	1.201	0.026	0.005	0.044	0.007	52.3	2.2	32.6	1.0
	42	2M	0.04	14.6	0.13	0.589	0.411	0.198	1.564	0.073	0.011	0.090	0.014	34.6	1.5	22.9	0.7
	42	4M	0.04	14.2	0.13	0.111	0.889	0.177	1.382	0.050	0.009	0.089	0.012	31.0	0.5	21.1	0.2
Cnf ⁶⁹ -Trp ⁹⁴	55	0M	0.10	16.7	0.13	0.920	0.080	0.097	1.697	0.091	0.010	0.090	0.012	44.1	2.3	28.8	1.1
	55	2M	0.04	14.6	0.13	0.950	0.050	0.206	2.194	0.155	0.025	0.144	0.031	33.2	3.1	22.3	1.4
	55	4M	0.04	14.2	0.13	0.250	0.750	0.168	1.938	0.122	0.006	0.145	0.009	25.5	0.5	18.6	0.2
Cnf ⁶⁴ -Trp ²⁴	70	0M	0.08	16.2	0.13	1.104	-0.104	0.033	1.156	0.020	0.007	0.020	0.007	74.1	9.5	42.5	4.7
	70	2M	0.04	14.4	0.13	0.916	0.084	0.059	1.122	0.016	0.010	0.026	0.012	58.4	8.9	33.2	4.0
	70	4M	0.03	13.8	0.13	0.350	0.650	0.090	1.496	0.064	0.009	0.123	0.011	27.4	1.0	19.2	0.4
Cnf ⁶⁴ -Trp ³⁹	55	0M	0.08	16.2	0.13	0.945	0.055	0.097	1.655	0.085	0.013	0.082	0.016	44.8	3.3	28.7	1.5
	55	2M	0.04	14.4	0.13	0.741	0.259	0.088	1.496	0.064	0.008	0.081	0.010	37.0	1.7	23.9	0.7
	55	4M	0.03	13.8	0.13	0.265	0.735	0.094	2.002	0.130	0.006	0.166	0.008	23.3	0.5	17.4	0.2
Cnf ⁶⁴ -Trp ⁶²	32	0M	0.08	16.2	0.13	0.950	0.050	0.080	2.401	0.182	0.009	0.169	0.011	34.4	1.1	23.9	0.5
	32	2M	0.04	14.4	0.13	0.853	0.147	0.135	3.059	0.268	0.012	0.258	0.015	24.8	0.7	18.4	0.3
	32	4M	0.03	13.8	0.13	0.305	0.695	0.092	1.920	0.120	0.006	0.153	0.008	24.2	0.5	17.8	0.2
Cnf ⁶⁴ -Trp ⁷⁹	15	0M	0.08	16.2	0.13	0.543	0.457	0.134	2.045	0.136	0.006	0.149	0.008	32.2	0.5	22.9	0.2
	15	2M	0.04	14.4	0.13	0.453	0.547	0.182	2.114	0.145	0.008	0.162	0.011	27.2	0.6	19.5	0.3
	15	4M	0.03	13.8	0.13	0.226	0.774	0.103	1.543	0.071	0.008	0.119	0.010	26.4	0.8	18.8	0.4
Cnf ¹²⁵ -Trp ²⁴	101	0M	0.05	15.0	0.13	0.965	0.035	0.057	1.423	0.055	0.009	0.052	0.011	48.9	4.0	29.6	1.7
	101	2M	0.02	13.3	0.13	0.842	0.158	0.105	2.116	0.145	0.024	0.148	0.028	28.3	2.0	19.3	0.8
	101	4M	0.03	14.1	0.13	0.440	0.560	0.121	1.785	0.102	0.013	0.146	0.017	27.3	0.8	19.4	0.4
Cnf ¹²⁵ -Trp ³⁹	86	0M	0.05	15.0	0.13	0.853	0.147	0.035	1.361	0.047	0.003	0.055	0.004	45.7	1.4	28.2	0.6
	86	2M	0.02	13.3	0.13	0.884	0.116	0.032	1.598	0.078	0.005	0.083	0.006	36.2	0.9	22.7	0.4
	86	4M	0.03	14.1	0.13	0.254	0.746	0.037	1.729	0.095	0.003	0.141	0.004	27.8	0.5	19.6	0.2
Cnf ¹²⁵ -Trp ⁶²	63	0M	0.05	15.0	0.13	0.777	0.223	0.080	1.358	0.047	0.009	0.064	0.011	42.3	2.5	26.7	1.1
	63	2M	0.02	13.3	0.13	0.860	0.140	0.163	1.691	0.090	0.029	0.097	0.034	32.8	3.8	21.2	1.6
	63	4M	0.03	14.1	0.13	0.328	0.672	0.146	1.598	0.078	0.008	0.110	0.011	28.4	0.8	19.9	0.4
Cnf ¹²⁵ -Trp ⁷⁹	46	0M	0.05	15.0	0.13	0.619	0.381	0.009	1.116	0.015	0.001	0.045	0.001	46.0	0.3	28.3	0.1
	46	2M	0.02	13.3	0.13	0.633	0.367	0.015	1.527	0.068	0.002	0.096	0.002	31.4	0.2	20.7	0.1
	46	4M	0.03	14.1	0.13	0.177	0.823	0.015	1.336	0.044	0.001	0.088	0.001	30.5	0.1	20.8	0.0
Cnf ¹²⁵ -Trp ⁹⁴	31	0M	0.05	15.0	0.13	0.782	0.218	0.098	1.523	0.068	0.010	0.082	0.013	39.3	2.3	25.3	1.0
	31	2M	0.02	13.3	0.13	0.737	0.263	0.166	1.728	0.095	0.021	0.113	0.026	30.1	2.4	20.1	1.0
	31	4M	0.03	14.1	0.13	0.258	0.742	0.140	1.729	0.095	0.008	0.128	0.010	26.3	0.7	18.9	0.3

Table S3b. Steady-State FRET Fitting and Distance Determination

Pair	PSS	TMAO	Q ₀	R ₀	ε _A /ε _D	A	E _D	S _A	B	E _A	S _B	E _{FRET}	S _{EFFRET}	Gaussian Chain		Semi-Empirical	
														r (Å)	S _{DIST}	r (Å)	S _{DIST}
Cnf ¹³⁶ -Trp ²⁴	112	0M	0.10	17.0	0.13	1.065	-0.065	0.050	1.243	0.032	0.009	0.032	0.009	66.3	7.3	39.5	3.5
	112	2M	0.04	14.6	0.13	0.947	0.053	0.114	1.362	0.047	0.020	0.048	0.024	48.7	8.5	29.2	3.7
	112	4M	0.03	13.7	0.13	0.607	0.393	0.160	1.663	0.086	0.014	0.111	0.018	27.7	1.6	19.3	0.7
Cnf ¹³⁶ -Trp ³⁹	97	0M	0.10	17.0	0.13	0.909	0.091	0.072	1.571	0.074	0.007	0.076	0.009	47.5	2.0	30.5	0.9
	97	2M	0.04	14.6	0.13	0.761	0.239	0.129	1.391	0.051	0.011	0.065	0.014	40.4	2.8	25.5	1.3
	97	4M	0.03	13.7	0.13	0.458	0.542	0.201	1.730	0.095	0.011	0.118	0.015	26.2	1.1	18.6	0.5
Cnf ¹³⁶ -Trp ⁹⁴	42	0M	0.10	17.0	0.13	0.973	0.027	0.077	1.752	0.098	0.008	0.091	0.010	45.2	2.0	29.5	0.9
	42	2M	0.04	14.6	0.13	0.909	0.091	0.163	1.786	0.102	0.017	0.101	0.022	36.8	3.1	23.9	1.4
	42	4M	0.03	13.7	0.13	0.511	0.489	0.199	1.954	0.124	0.009	0.140	0.012	24.9	1.0	18.1	0.5
Cnf ¹³⁶ -Trp ¹²⁵	11	0M	0.10	17.0	0.13	0.946	0.054	0.085	1.693	0.090	0.014	0.085	0.017	46.6	3.6	30.1	1.7
	11	2M	0.04	14.6	0.13	0.973	0.027	0.089	1.607	0.079	0.018	0.070	0.022	43.5	4.8	26.9	2.1
	11	4M	0.03	13.7	0.13	0.577	0.423	0.147	1.887	0.115	0.013	0.140	0.017	25.2	1.0	18.2	0.4
Fam ⁹ -Raz ⁹⁴	85	0M	0.49	54.3	0.24	0.590	0.410	0.009	3.932	0.717	0.075	0.444	0.012	69.5	1.2	59.8	0.8
	85	2M	0.33	50.9	0.24	0.384	0.616	0.005	4.439	0.841	0.033	0.643	0.006	49.3	0.4	45.6	0.3
	85	4M	0.03	33.9	0.24	0.037	0.963	0.010	4.961	0.968	0.123	0.964	0.013	16.4	1.2	18.5	1.0
Fam ²⁴ -Raz ⁹⁴	70	0M	0.57	55.6	0.24	0.477	0.523	0.023	4.325	0.813	0.219	0.551	0.030	60.5	2.5	54.3	1.7
	70	2M	0.38	52.1	0.24	0.305	0.695	0.005	4.454	0.844	0.043	0.711	0.007	45.8	0.5	43.5	0.3
	70	4M	0.05	36.8	0.24	0.075	0.925	0.007	4.938	0.963	0.138	0.927	0.009	20.9	0.6	22.8	0.5
Fam ⁴² -Raz ⁹⁴	52	0M	0.52	54.9	0.24	0.357	0.643	0.008	3.864	0.700	0.068	0.649	0.010	51.8	0.7	48.2	0.5
	52	2M	0.40	52.6	0.24	0.229	0.771	0.004	4.051	0.746	0.038	0.769	0.005	41.4	0.4	40.7	0.3
	52	4M	0.05	37.1	0.24	0.093	0.907	0.008	6.634	1.378	0.174	0.907	0.011	22.3	0.7	23.9	0.5
Fam ⁶² -Raz ⁹⁴	32	0M	0.54	55.2	0.24	0.301	0.699	0.003	3.955	0.722	0.019	0.702	0.004	48.2	0.2	45.9	0.2
	32	2M	0.45	53.5	0.24	0.238	0.762	0.003	4.116	0.762	0.021	0.762	0.004	42.7	0.3	43.0	0.2
	32	4M	0.16	45.3	0.24	0.027	0.973	0.003	6.541	1.355	0.220	0.973	0.003	20.6	0.5	23.3	0.4
Fam ⁸⁷ -Raz ⁹⁴	7	0M	0.51	54.7	0.24	0.102	0.898	0.001	1.154	0.038	0.010	0.807	0.002	34.8	0.1	36.1	0.1
	7	2M	0.35	51.5	0.24	0.117	0.883	0.002	2.244	0.304	0.015	0.817	0.002	33.4	0.2	34.6	0.2
	7	4M	0.04	36.3	0.24	0.034	0.966	0.009	7.093	1.490	0.176	0.966	0.009	17.3	1.0	19.6	0.8
Fam ¹¹⁴ -Raz ⁹⁴	20	0M	0.59	56.0	0.24	0.023	0.977	0.001	2.486	0.363	0.012	0.920	0.002	25.4	0.3	28.4	0.3
	20	2M	0.38	52.1	0.24	0.022	0.978	0.001	3.635	0.644	0.013	0.942	0.002	23.1	0.4	26.0	0.4
	20	4M	0.08	39.7	0.24	0.043	0.957	0.006	8.040	1.721	0.199	0.957	0.006	20.0	0.6	22.2	0.6
Fam ¹²³ -Raz ⁹⁴	29	0M	0.42	53.0	0.24	0.276	0.724	0.006	2.821	0.445	0.040	0.689	0.007	45.2	0.5	43.4	0.4
	29	2M	0.36	51.5	0.24	0.210	0.790	0.003	3.205	0.539	0.023	0.762	0.004	39.6	0.3	39.2	0.2
	29	4M	0.09	41.2	0.24	0.108	0.892	0.005	8.941	1.941	0.220	0.892	0.005	25.8	0.3	27.2	0.3
Fam ¹³⁶ -Raz ⁹⁴	42	0M	0.56	55.5	0.24	0.164	0.836	0.002	3.393	0.585	0.015	0.811	0.002	39.4	0.2	39.7	0.1
	42	2M	0.42	52.9	0.24	0.116	0.884	0.004	4.536	0.864	0.032	0.882	0.004	33.7	0.4	35.1	0.3
	42	4M	0.19	46.2	0.24	0.116	0.884	0.002	6.291	1.293	0.195	0.884	0.002	29.5	0.2	30.8	0.1
Fam ⁹ -Raz ¹³⁶	127	0M	0.55	55.5	0.24	0.925	0.075	0.006	3.558	0.625	0.048	0.134	0.007	142.7	4.8	108.1	5.3
	127	2M	0.40	52.6	0.24	0.729	0.271	0.013	4.500	0.856	0.090	0.348	0.017	82.7	2.7	67.9	1.7
	127	4M	0.06	37.9	0.24	0.059	0.941	0.005	4.517	0.860	0.036	0.931	0.006	20.4	0.6	22.5	0.5

Fluorescence Lifetime Data and Fitting

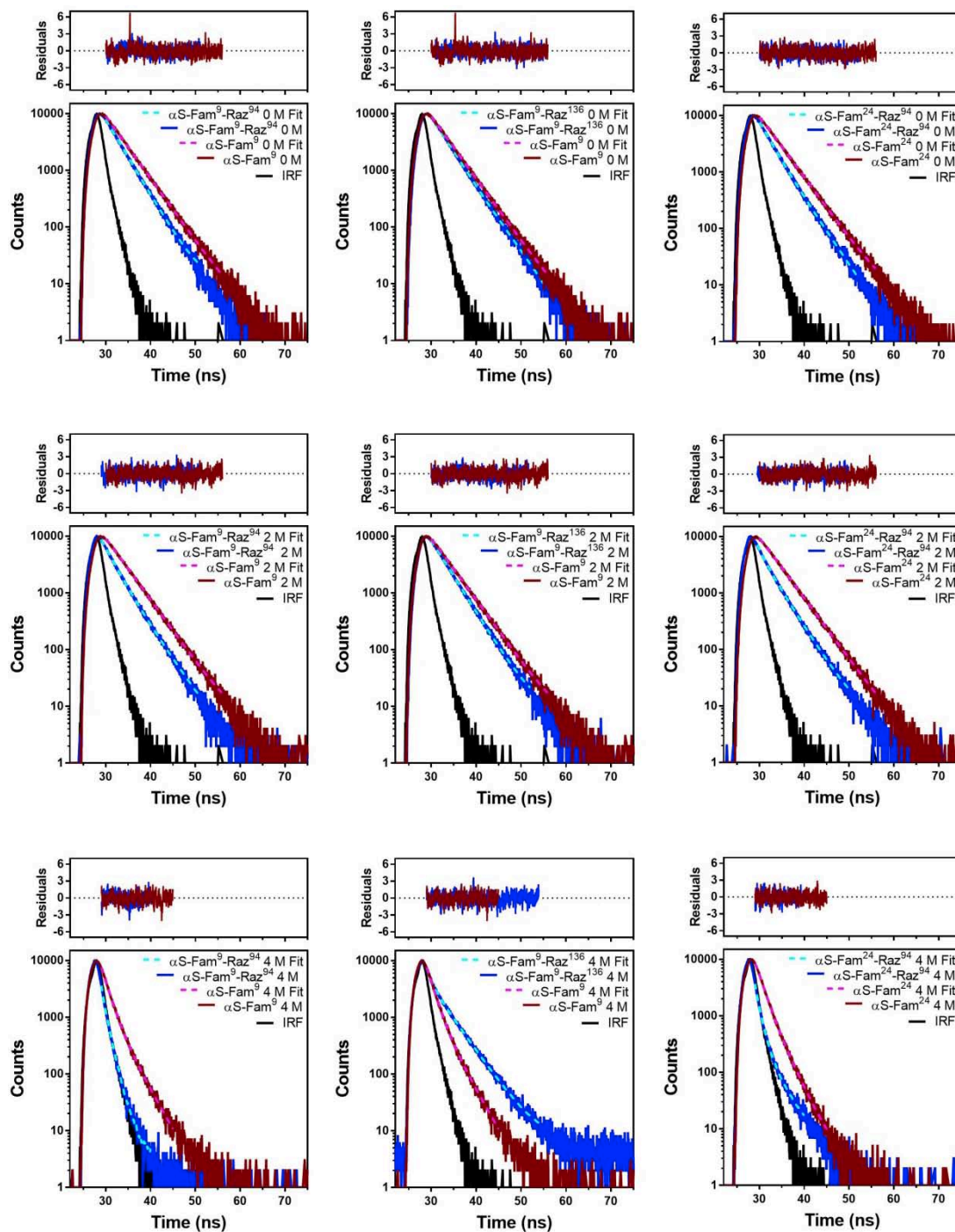


Figure S45: Intramolecular FRET Lifetime Decays and Fits. Plots show lifetime decays and fits of (Left) α S-Fam⁹-Raz⁹⁴, (Middle) α S-Fam⁹-Raz¹³⁶ and (Right) α S-Fam²⁴-Raz⁹⁴ in (Top-Bottom) 0, 2 and 4 M TMAO. Double-labeled construct decays are shown in solid blue with associated single-labeled Fam constructs shown in red with fits shown as dashed lines.

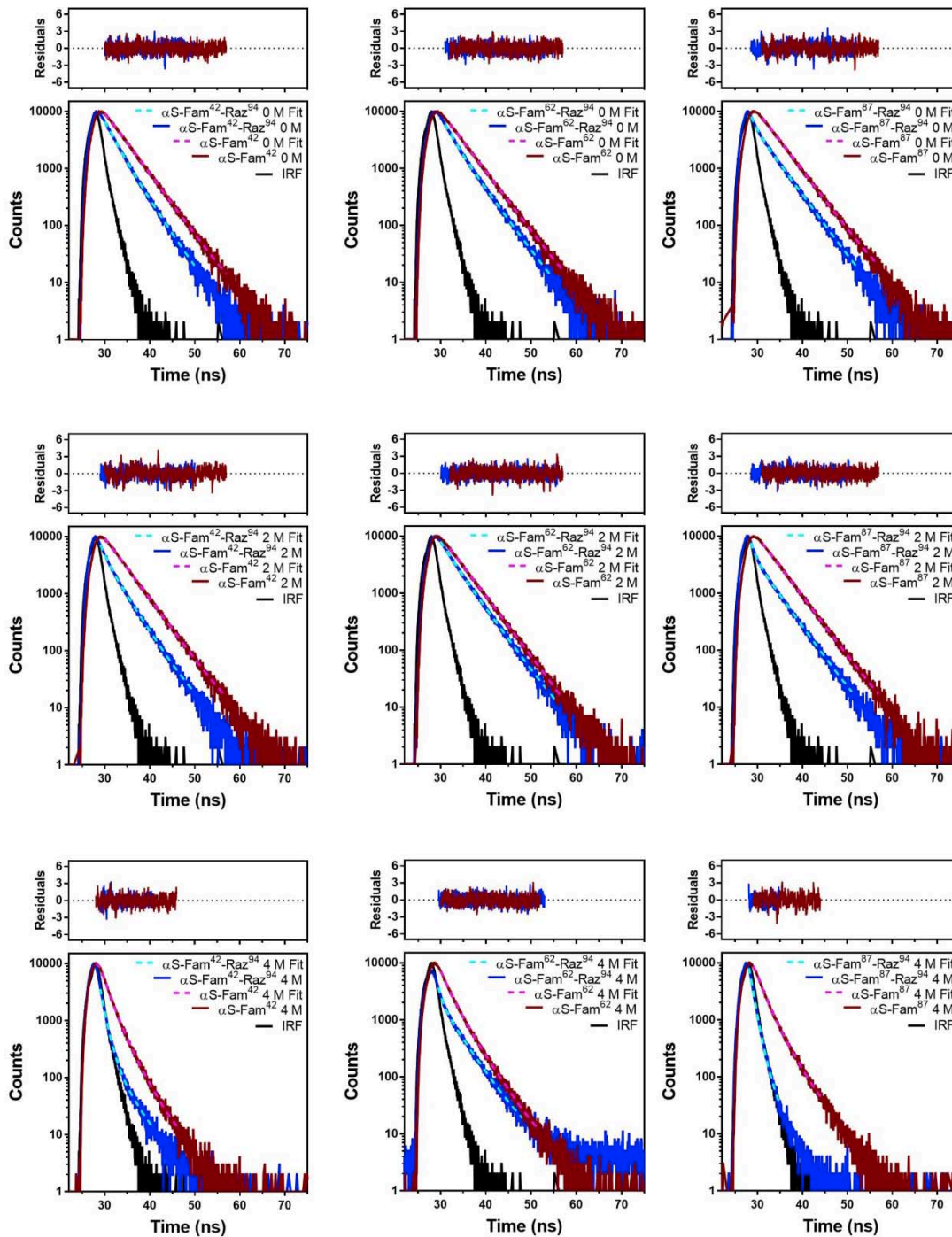


Figure S46: Intramolecular FRET Lifetime Decays and Fits. Plots show lifetime decays and fits of (Left) α S-Fam⁴²-Raz⁹⁴, (Middle) α S-Fam⁶²-Raz⁹⁴ and (Right) α S-Fam⁸⁷-Raz⁹⁴ in (Top-Bottom) 0, 2 and 4 M TMAO. Double-labeled construct decays are shown in solid blue with associated single-labeled Fam constructs shown in red with fits shown as dashed lines.

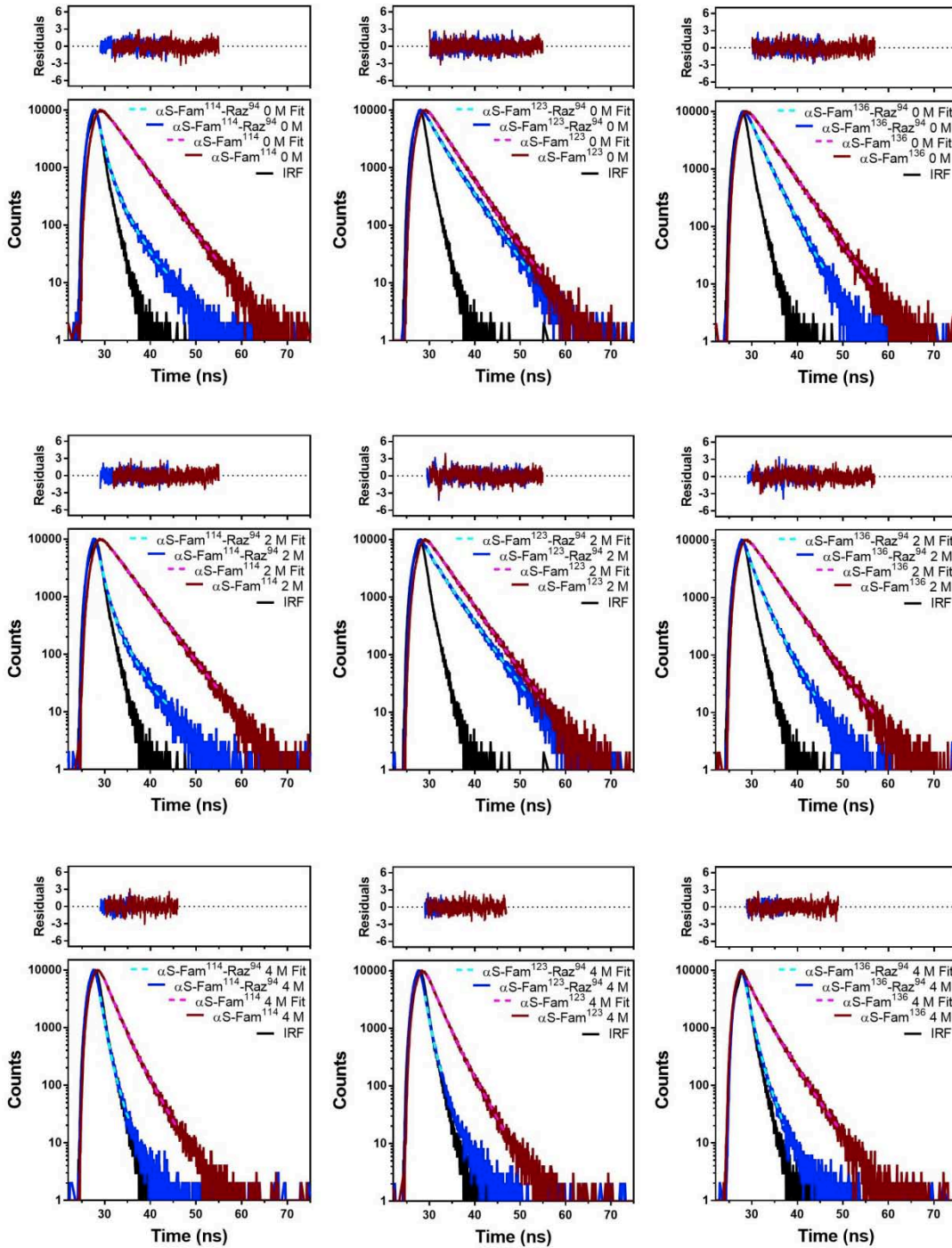


Figure S47: Intramolecular FRET Lifetime Decays and Fits. Plots show lifetime decays and fits of (Left) α S-Fam¹¹⁴-Raz⁹⁴, (Middle) α S-Fam¹²³-Raz¹³⁶ and (Right) α S-Fam¹³⁶-Raz⁹⁴ in (Top-Bottom) 0, 2 and 4 M TMAO. Double-labeled construct decays are shown in solid blue with associated single-labeled Fam constructs shown in red with fits shown as dashed lines.

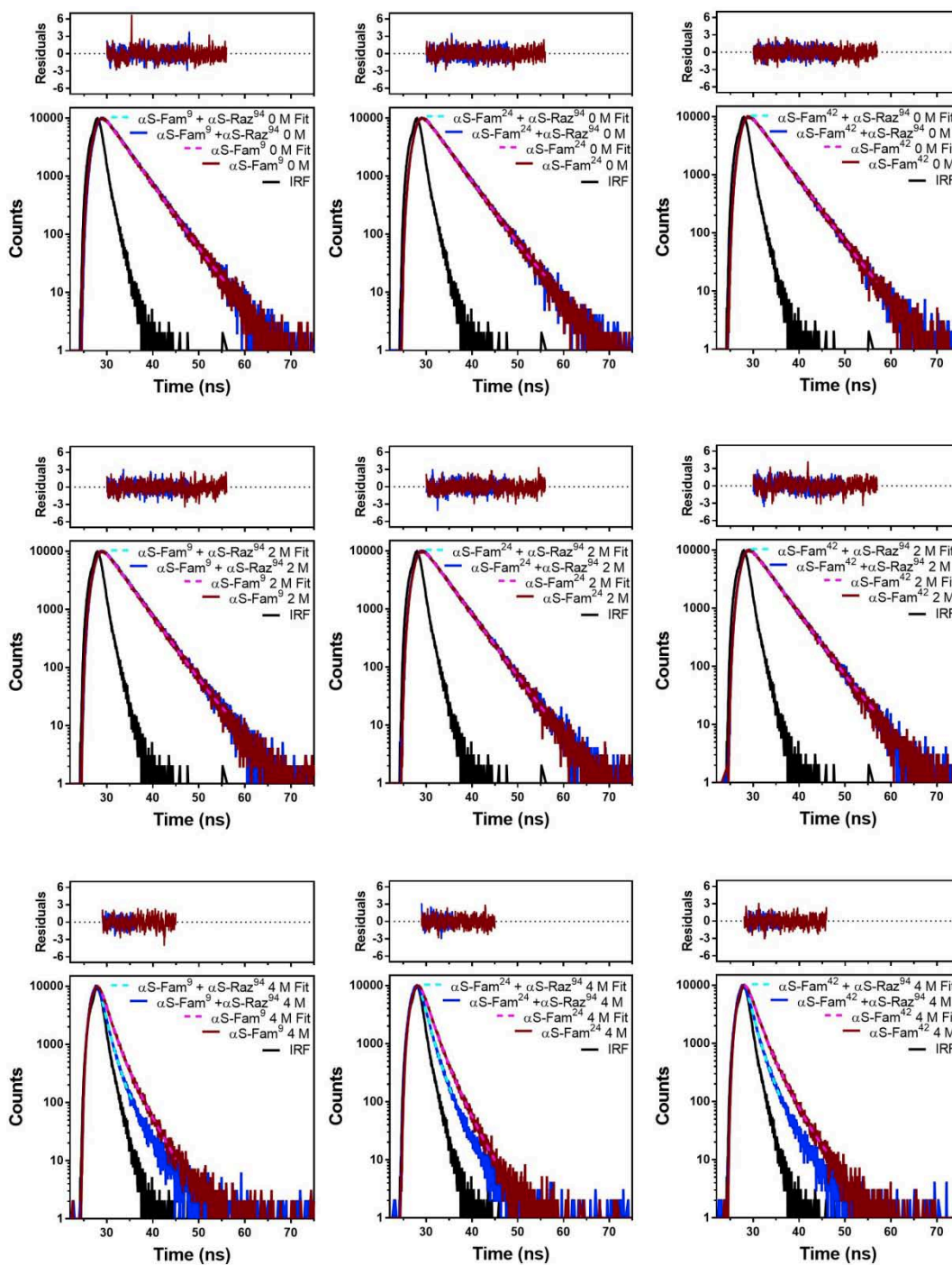


Figure S48: Intermolecular FRET Lifetime Decays and Fits. Plots show lifetime decays and fits of (Left) α S-Fam⁹ + α S-Raz⁹⁴, (Middle) α S-Fam⁹ + α S-Raz¹³⁶ and (Right) α S-Fam²⁴ + α S-Raz⁹⁴ in (Top-Bottom) 0, 2 and 4 M TMAO. Decay of single-labeled Fam construct decays are shown before (red) and after (blue) the addition of the Raz containing construct with fits shown as dashed lines.

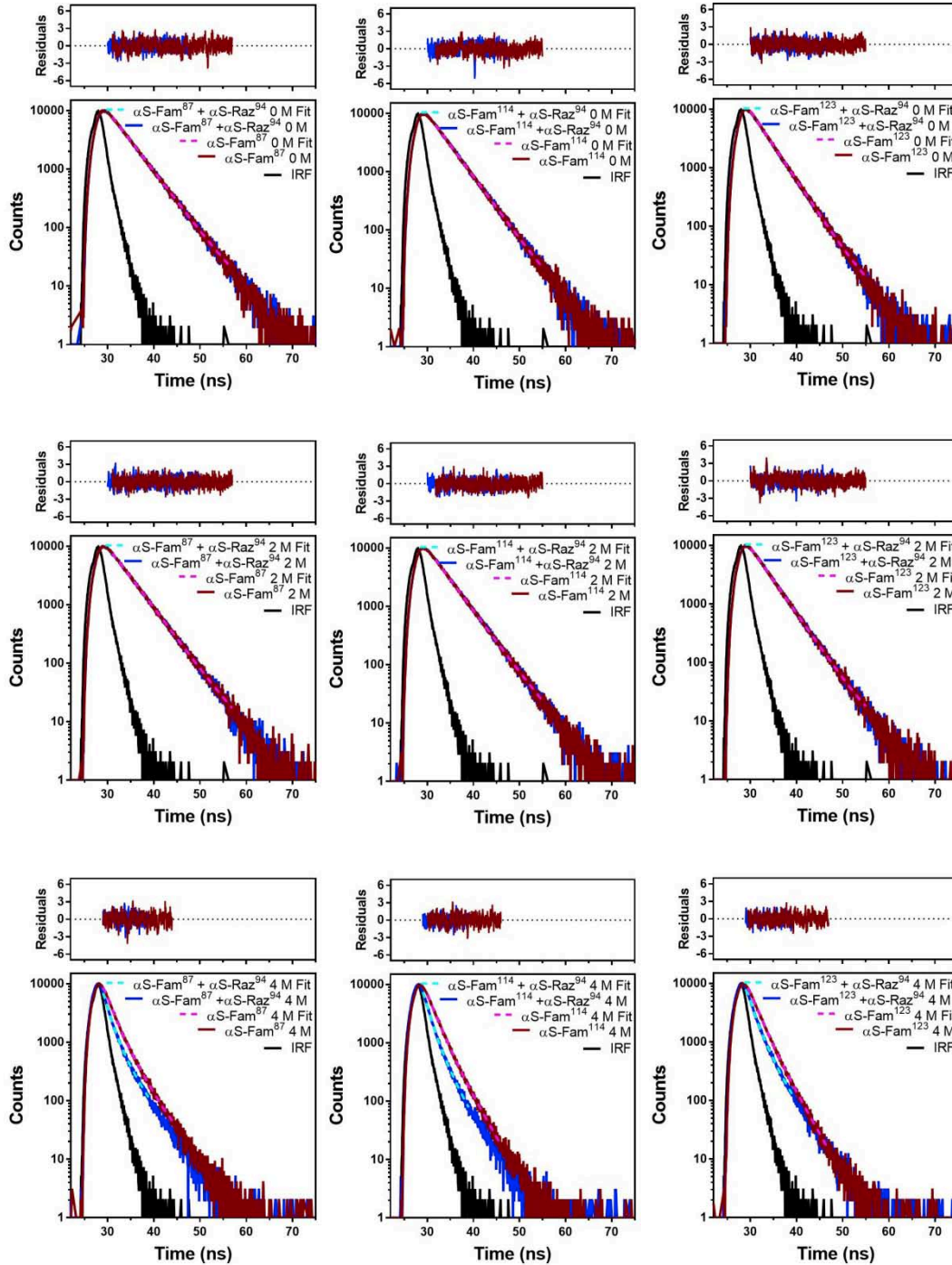


Figure S49: Intermolecular FRET Lifetime Decays and Fits. Plots show lifetime decays and fits of (Left) α S-Fam⁸⁷ + α S-Raz⁹⁴, (Middle) α S-Fam¹¹⁴ + α S-Raz⁹⁴ and (Right) α S-Fam¹²³ + α S-Raz⁹⁴ in (Top-Bottom) 0, 2 and 4 M TMAO. Decay of single-labeled Fam construct decays are shown before (red) and after (blue) the addition of the Raz containing construct with fits shown as dashed lines.

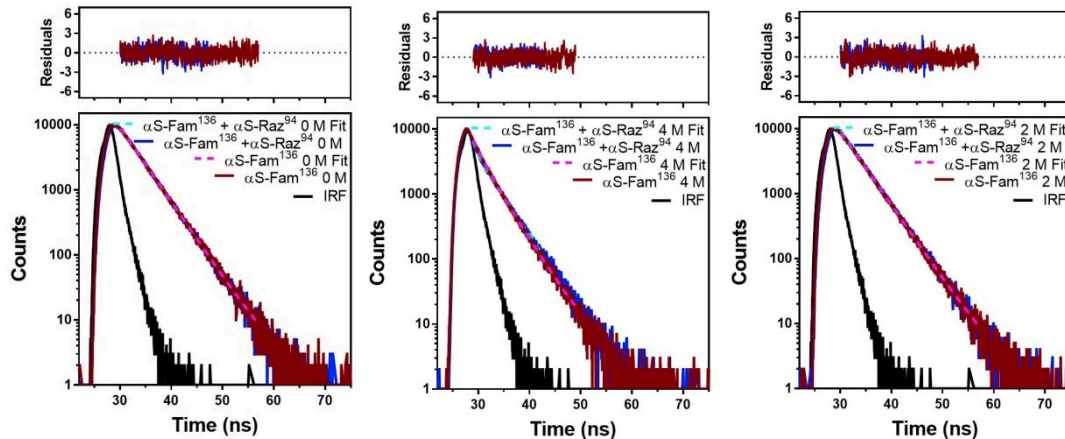


Figure S50: Intermolecular FRET Lifetime Decays and Fits. Plots show lifetime decays and fits of α S-Fam¹³⁶ + α S-Raz⁹⁴ in (Left-Right) 0, 2 and 4 M TMAO. Decay of single-labeled Fam construct decays are shown before (red) and after (blue) the addition of the Raz containing construct with fits shown as dashed lines.

Table S4a. TCSPC Data and Fitting

Construct	TMAO	T ₁	T ₂	Amplitude ₁	Amplitude ₂	Intensity ₁	Intensity ₂	T _{Amp.} -Avg.	T _{Int.} -Avg.	χ ²
αS-Fam ⁹	0 M	3.97E+00 ± 5.02E-03	-	1.15E+00 ± 8.16E-03	-	1.00E+00	-	3.97E+00	3.97E+00	9.57E-01
	2 M	3.98E+00 ± 7.48E-03	-	7.66E-01 ± 3.70E-02	-	1.00E+00	-	3.98E+00	3.98E+00	9.40E-01
	4 M	3.03E+00 ± 6.06E-02	1.27E+00 ± 5.36E-02	2.79E-01 ± 2.29E-02	1.10E+00 ± 1.05E-01	2.01E-01	7.99E-01	1.63E+00	1.94E+00	8.64E-01
	0 M	3.99202 ± 5.72E-03	-	7.93E-01 ± 1.34E-02	-	1.00E+00	-	3.99202	3.99202	9.86E-01
αS-Fam ²⁴	2 M	4.02857 ± 7.13E-03	-	7.77E-01 ± 2.35E-02	-	1.00E+00	-	4.02857	4.02857	1.02E+00
	4 M	2.68377 ± 8.74E-02	1.16144 ± 4.39E-02	1.77E-01 ± 1.88E-02	9.12E-01 ± 2.45E-02	3.10E-01	6.90E-01	1.40886	1.63276	9.64E-01
	0 M	4.03269 ± 7.48E-03	-	7.83E-01 ± 2.63E-02	-	1.00E+00	-	4.03269	4.03269	9.49E-01
	2 M	4.02E+00 ± 6.07E-03	-	8.06E-01 ± 2.58E-02	-	1.00E+00	-	4.02E+00	4.02E+00	1.1847
αS-Fam ⁶²	4 M	2.88E+00 ± 5.03E-02	1.12E+00 ± 2.36E-02	1.88E-01 ± 1.01E-02	8.98E-01 ± 5.93E-03	3.50E-01	6.50E-01	1.42E+00	1.73E+00	1.02E+00
	0 M	4.14E+00 ± 6.45E-03	-	9.65E-01 ± 6.35E-02	-	1.00E+00	-	4.14E+00	4.14E+00	9.82E-01
	2 M	4.13E+00 ± 5.64E-03	-	9.77E-01 ± 7.47E-03	-	1.00E+00	-	4.13E+00	4.13E+00	1.10E+00
	4 M	3.54E+00 ± 5.82E-03	1.46E+00 ± 5.23E-03	3.33E-01 ± 1.50E-03	1.02E+00 ± 6.77E-03	4.43E-01	5.57E-01	1.97E+00	2.38E+00	9.18E-01
αS-Fam ⁶⁷	0 M	4.16E+00 ± 8.86E-03	-	7.72E-01 ± 5.40E-02	-	1.00E+00	-	4.16E+00	4.16E+00	1.08E+00
	2 M	4.12E+00 ± 8.53E-03	-	7.21E-01 ± 3.60E-02	-	1.00E+00	-	4.12E+00	4.12E+00	8.96E-01
	4 M	3.07E+00 ± 7.69E-02	1.15E+00 ± 4.94E-02	2.36E-01 ± 1.47E-02	7.52E-01 ± 9.02E-03	4.57E-01	5.43E-01	1.61E+00	2.03E+00	1.30E+00
	0 M	4.04E+00 ± 6.09E-03	-	1.35E+00 ± 1.14E-02	-	1.00E+00	-	4.04E+00	4.04E+00	1.01E+00
αS-Fam ¹¹⁴	2 M	4.07E+00 ± 5.90E-03	-	1.52E+00 ± 3.87E-02	-	1.00E+00	-	4.07E+00	4.07E+00	9.03E-01
	4 M	3.95E+00 ± 5.31E-03	1.92E+00 ± 1.19E-03	1.21E-01 ± 4.47E-04	1.61E+00 ± 2.65E-03	1.34E-01	8.66E-01	2.06E+00	2.19E+00	1.05E+00
	0 M	3.64E+00 ± 4.64E-03	-	1.21E+00 ± 8.97E-03	-	1.00E+00	-	3.64E+00	3.64E+00	9.56E-01
	2 M	3.81E+00 ± 1.52E-03	-	8.70E-01 ± 6.82E-04	-	1.00E+00	-	3.81E+00	3.81E+00	1.02E+00
αS-Fam ¹²³	4 M	3.15E+00 ± 1.26E-01	1.56E+00 ± 9.23E-02	2.39E-01 ± 3.34E-02	7.03E-01 ± 5.11E-02	4.06E-01	5.94E-01	1.96E+00	2.20E+00	9.78E-01
	0 M	3.77E+00 ± 7.07E-03	-	9.01E-01 ± 4.82E-02	-	1.00E+00	-	3.77E+00	3.77E+00	9.87E-01
	2 M	3.82E+00 ± 6.62E-03	-	8.28E-01 ± 3.28E-02	-	1.00E+00	-	3.82E+00	3.82E+00	1.08E+00
	4 M	3.51E+00 ± 4.72E-02	1.47E+00 ± 8.81E-02	4.82E-01 ± 4.91E-02	9.22E-01 ± 1.48E-01	5.55E-01	4.45E-01	2.17E+00	2.60E+00	9.44E-01
αS-Fam ^{9-Raz} ²⁴	0 M	3.25E+00 ± 5.53E-03	-	2.62E+00 ± 2.91E-01	-	1.00E+00	-	3.25E+00	3.25E+00	9.41E-01
	2 M	3.67E+00 ± 3.10E-02	2.03E+00 ± 4.24E-02	4.60E-01 ± 1.80E-02	7.19E-01 ± 2.91E-02	5.37E-01	4.63E-01	2.67E+00	2.91E+00	1.10E+00
	4 M	3.88E-01 ± 1.79E-02	-	5.97E+00 ± 4.08E-01	-	1.00E+00	-	3.88E-01	3.88E-01	1.19E+00
	0 M	4.57E+00 ± 1.44E-02	3.01E+00 ± 7.35E-03	1.58E-01 ± 1.55E-03	6.29E-01 ± 3.18E-03	2.75E-01	7.25E-01	3.32E+00	3.44E+00	9.50E-01
αS-Fam ^{9-Raz} ¹³⁶	2 M	3.41E+00 ± 2.47E-03	-	3.40E+00 ± 9.08E-03	-	1.00E+00	-	3.41E+00	3.41E+00	1.04E+00
	4 M	3.87E+00 ± 1.23E-02	5.41E-01 ± 4.71E-02	2.93E-01 ± 5.70E-03	3.06E+00 ± 5.15E-01	4.07E-01	5.93E-01	8.32E-01	1.90E+00	1.11E+00
	0 M	3.94E+00 ± 1.63E-02	2.51E+00 ± 1.82E-02	4.34E-01 ± 7.96E-03	9.94E-01 ± 1.65E-02	4.07E-01	5.93E-01	2.94E+00	3.09E+00	9.46E-01
	2 M	3.47E+00 ± 3.68E-02	1.34E+00 ± 8.49E-02	5.65E-01 ± 5.40E-02	9.89E-01 ± 1.58E-01	5.96E-01	4.04E-01	2.12E+00	2.61E+00	8.80E-01
αS-Fam ^{24-Raz} ²⁴	4 M	3.48E+00 ± 2.52E-01	4.27E-01 ± 3.18E-02	1.89E-02 ± 2.02E-03	3.46E+00 ± 4.48E-01	4.28E-02	9.57E-01	4.43E-01	5.57E-01	1.13E+00
	0 M	3.77E+00 ± 1.48E-02	2.09E+00 ± 1.84E-02	3.89E-01 ± 5.91E-03	7.53E-01 ± 1.31E-02	4.81E-01	5.19E-01	2.66E+00	2.90E+00	1.04E+00
	2 M	3.53E+00 ± 2.01E-02	9.99E-01 ± 4.10E-02	3.49E-01 ± 2.10E-02	9.01E-01 ± 1.07E-01	5.78E-01	4.22E-01	1.70E+00	2.46E+00	1.03E+00
	4 M	3.82E+00 ± 2.90E-01	5.50E-01 ± 1.54E-02	1.51E-02 ± 1.58E-03	2.17E+00 ± 1.39E-01	4.62E-02	9.54E-01	5.73E-01	7.02E-01	9.94E-01

Table S4b. TCSPC Data and Fitting

Construct	TMAO	T ₁	T ₂	Amplitude ₁	Amplitude ₂	Intensity ₁	Intensity ₂	T _{Amp, Avg.}	T _{Int, Avg.}	χ ²
αS-Fam ⁶² -Raz ⁹⁴	0M	4.02E+00 ± 3.27E-02	1.71E+00 ± 6.02E-02	3.90E-01 ± 1.25E-02	6.57E-01 ± 3.40E-02	5.82E-01	4.18E-01	2.57E+00	3.06E+00	8.68E-01
	2M	3.98E+00 ± 2.25E-02	1.57E+00 ± 8.34E-02	5.09E-01 ± 1.33E-02	4.21E-01 ± 1.95E-02	7.54E-01	2.46E-01	2.89E+00	3.39E+00	9.24E-01
	4M	3.77E+00 ± 2.18E-02	2.35E-01 ± 3.29E-02	1.12E-01 ± 1.57E-03	3.47E+00 ± 5.95E-01	3.40E-01	6.60E-01	3.46E-01	1.44E+00	9.16E-01
αS-Fam ⁶⁷ -Raz ⁹⁴	0M	4.04E+00 ± 1.42E-02	7.74E-01 ± 2.87E-02	3.98E-01 ± 1.64E-02	1.40E+00 ± 1.90E-01	5.98E-01	4.02E-01	1.50E+00	2.73E+00	1.09E+00
	2M	3.97E+00 ± 1.57E-02	4.83E-01 ± 3.72E-02	2.83E-01 ± 6.14E-03	2.51E+00 ± 3.68E-01	4.81E-01	5.19E-01	8.37E-01	2.16E+00	1.01E+00
	4M	4.86E-01 ± 6.49E-03	-	3.04E+00 ± 5.30E-02	-	1.00E+00	-	4.86E-01	4.86E-01	1.06E+00
αS-Fam ¹¹⁴ -Raz ⁹⁴	0M	3.25E+00 ± 1.88E-02	7.56E-01 ± 3.34E-03	6.22E-02 ± 7.98E-04	1.99E+00 ± 1.81E-02	1.19E-01	8.81E-01	8.31E-01	1.05E+00	1.01E+00
	2M	3.14E+00 ± 7.13E-02	6.01E-01 ± 1.70E-02	4.98E-02 ± 2.27E-03	1.57E+00 ± 9.54E-02	1.43E-01	8.57E-01	6.79E-01	9.63E-01	9.38E-01
	4M	4.95E-01 ± 1.90E-02	-	2.63E+00 ± 2.12E-01	-	1.00E+00	-	4.95E-01	4.95E-01	8.61E-01
αS-Fam ¹²³ -Raz ⁹⁴	0M	3.86E+00 ± 5.96E-02	1.81E+00 ± 1.04E-01	5.11E-01 ± 3.72E-02	7.49E-01 ± 7.82E-02	5.93E-01	4.07E-01	2.65E+00	3.03E+00	1.00E+00
	2M	3.87E+00 ± 1.08E-02	1.32E+00 ± 3.04E-02	5.84E-01 ± 4.95E-03	8.56E-01 ± 3.90E-02	6.67E-01	3.33E-01	2.35E+00	3.02E+00	1.09E+00
	4M	5.25E-01 ± 1.06E-02	-	2.18E+00 ± 1.13E-01	-	1.00E+00	-	5.25E-01	5.25E-01	9.64E-01
αS-Fam ¹³⁶ -Raz ⁹⁴	0M	2.78E+00 ± 1.00E-02	1.39E+00 ± 1.99E-02	5.25E-01 ± 6.82E-03	7.57E-01 ± 1.93E-02	5.81E-01	4.19E-01	1.96E+00	2.20E+00	1.02E+00
	2M	2.75E+00 ± 4.57E-02	9.53E-01 ± 4.36E-02	2.63E-01 ± 1.92E-02	1.03E+00 ± 1.04E-01	4.24E-01	5.76E-01	1.32E+00	1.71E+00	1.05E+00
	4M	3.67E+00 ± 9.63E-01	5.80E-01 ± 2.70E-02	1.52E-02 ± 4.99E-03	5.65E+00 ± 5.38E-01	1.68E-02	9.83E-01	5.89E-01	6.32E-01	8.18E-01
αS-Fam ⁹ +	0M	3.84E+00 ± 1.12E-02	-	7.97E-01 ± 3.15E-02	-	1.00E+00	-	3.84E+00	3.84E+00	1.06E+00
	2M	3.89E+00 ± 1.08E-02	-	7.06E-01 ± 1.43E-02	-	1.00E+00	-	3.89E+00	3.89E+00	9.95E-01
	4M	1.87E+00 ± 2.43E-01	6.12E-01 ± 5.06E-02	1.46E-01 ± 4.68E-02	1.75E+00 ± 1.05E-01	2.02E-01	7.98E-01	7.08E-01	8.66E-01	8.82E-01
αS-Fam ²⁴ +	0M	3.96E+00 ± 1.12E-02	-	7.40E-01 ± 1.73E-02	-	1.00E+00	-	3.96E+00	3.96E+00	1.03E+00
	2M	3.98E+00 ± 1.19E-02	-	6.94E-01 ± 1.78E-02	-	1.00E+00	-	3.98E+00	3.98E+00	1.05E+00
	4M	1.09E+01 ± 3.81E+00	1.09E+00 ± 1.75E-02	2.78E-02 ± 2.99E-03	2.21E+00 ± 1.39E-01	1.12E-01	8.88E-01	1.21E+00	2.20E+00	1.06E+00
αS-Fam ⁴² +	0M	3.99E+00 ± 1.30E-02	-	7.16E-01 ± 2.35E-02	-	1.00E+00	-	3.99E+00	3.99E+00	8.63E-01
	2M	3.96E+00 ± 8.80E-03	-	6.91E-01 ± 1.12E-02	-	1.00E+00	-	3.96E+00	3.96E+00	1.15E+00
	4M	3.01E+00 ± 7.68E-03	7.91E-01 ± 9.01E-04	7.68E-02 ± 3.12E-04	1.37E+00 ± 2.59E-03	1.76E-01	8.24E-01	9.08E-01	1.18E+00	9.02E-01
αS-Fam ⁶² +	0M	4.15E+00 ± 1.32E-02	-	6.91E-01 ± 1.50E-02	-	1.00E+00	-	4.15E+00	4.15E+00	9.06E-01
	2M	4.09E+00 ± 1.01E-02	-	6.70E-01 ± 9.15E-03	-	1.00E+00	-	4.09E+00	4.09E+00	9.62E-01
	4M	2.84E+00 ± 4.19E-03	7.47E-01 ± 1.18E-03	1.55E-01 ± 3.86E-04	1.22E+00 ± 3.23E-03	3.26E-01	6.74E-01	9.84E-01	1.43E+00	1.06E+00
αS-Fam ¹¹⁴ +	0M	4.00E+00 ± 1.26E-02	-	7.22E-01 ± 1.72E-02	-	1.00E+00	-	4.00E+00	4.00E+00	1.11E+00
	2M	4.03E+00 ± 1.43E-02	-	7.52E-01 ± 2.54E-02	-	1.00E+00	-	4.03E+00	4.03E+00	9.12E-01
	4M	2.70E+00 ± 2.64E-02	8.19E-01 ± 9.15E-03	1.46E-01 ± 2.92E-03	1.12E+00 ± 1.48E-02	3.01E-01	6.99E-01	1.04E+00	1.39E+00	8.68E-01
αS-Fam ¹²³ +	0M	3.58E+00 ± 1.07E-02	-	8.99E-01 ± 3.99E-02	-	1.00E+00	-	3.58E+00	3.58E+00	1.02E+00
	2M	3.78E+00 ± 1.13E-02	-	8.35E-01 ± 3.10E-02	-	1.00E+00	-	3.78E+00	3.78E+00	1.07E+00
	4M	2.60E+00 ± 1.18E-01	7.56E-01 ± 6.47E-02	2.23E-01 ± 1.88E-02	9.39E-01 ± 2.30E-02	4.50E-01	5.50E-01	1.11E+00	1.59E+00	9.34E-01
αS-Fam ¹³⁶ +	0M	3.72E+00 ± 9.63E-03	-	8.29E-01 ± 2.93E-02	-	1.00E+00	-	3.72E+00	3.72E+00	9.76E-01
	2M	3.79E+00 ± 1.18E-02	-	9.81E-01 ± 6.79E-02	-	1.00E+00	-	3.79E+00	3.79E+00	1.05E+00
	4M	3.79E+00 ± 1.00E-03	7.55E-01 ± 5.69E-04	2.70E-01 ± 1.01E-04	8.85E-01 ± 1.25E-03	6.05E-01	3.95E-01	1.46E+00	2.59E+00	9.35E-01

Table S5. Calculated E_{FRET} values from TCSPC

Construct	Intramolecular			Construct	Intermolecular		
	TMAO	EFRET _{Amp}	EFRET _{Int}		TMAO	EFRET _{Amp}	EFRET _{Int}
$\alpha\text{S-Fam}^9\text{-Raz}^{94}$	0 M	0.18	0.18	$\alpha\text{S-Fam}^9+$ $\alpha\text{S-Raz}^{94}$	0 M	0.03	0.03
	2 M	0.33	0.27		2 M	0.02	0.02
	4 M	0.45	0.55		4 M	0.57	0.55
$\alpha\text{S-Fam}^9\text{-Raz}^{136}$	0 M	0.16	0.13	$\alpha\text{S-Fam}^{24}+$ $\alpha\text{S-Raz}^{94}$	0 M	0.01	0.01
	2 M	0.14	0.14		2 M	0.01	0.01
	4 M	0.49	0.02		4 M	0.14	-0.34
$\alpha\text{S-Fam}^{24}\text{-Raz}^{94}$	0 M	0.26	0.23	$\alpha\text{S-Fam}_{42}+$ $\alpha\text{S-Raz}^{94}$	0 M	0.01	0.01
	2 M	0.47	0.35		2 M	0.01	0.01
	4 M	0.69	0.66		4 M	0.36	0.32
$\alpha\text{S-Fam}^{42}\text{-Raz}^{94}$	0 M	0.34	0.28	$\alpha\text{S-Fam}_{62}+$ $\alpha\text{S-Raz}^{94}$	0 M	0.00	0.00
	2 M	0.58	0.39		2 M	0.01	0.01
	4 M	0.60	0.60		4 M	0.39	0.29
$\alpha\text{S-Fam}^{62}\text{-Raz}^{94}$	0 M	0.38	0.26	$\alpha\text{S-Fam}_{114}+$ $\alpha\text{S-Raz}^{94}$	0 M	0.01	0.01
	2 M	0.30	0.18		2 M	0.01	0.01
	4 M	0.82	0.40		4 M	0.50	0.37
$\alpha\text{S-Fam}^{87}\text{-Raz}^{94}$	0 M	0.64	0.34	$\alpha\text{S-Fam}_{123}+$ $\alpha\text{S-Raz}^{94}$	0 M	0.02	0.02
	2 M	0.80	0.48		2 M	0.01	0.01
	4 M	0.70	0.76		4 M	0.43	0.28
$\alpha\text{S-Fam}^{114}\text{-Raz}^{94}$	0 M	0.79	0.74	$\alpha\text{S-Fam}_{136}+$ $\alpha\text{S-Raz}^{94}$	0 M	0.01	0.01
	2 M	0.83	0.76		2 M	0.01	0.01
	4 M	0.76	0.77		4 M	0.32	0.00
$\alpha\text{S-Fam}^{123}\text{-Raz}^{94}$	0 M	0.27	0.17				
	2 M	0.38	0.21				
	4 M	0.73	0.76				
$\alpha\text{S-Fam}^{136}\text{-Raz}^{94}$	0 M	0.48	0.42				
	2 M	0.65	0.55				
	4 M	0.73	0.76				

FCS and AFM Characterization

FCS Measurements: FCS measurements were done at 20°C on a lab-built instrument based on an Olympus IX71 microscope. A continuous emission 488 nm DPSS 50 mW laser (Spectra-Physics, Santa Clara, CA) was adjusted to 4.5 μ W power just prior to entering the microscope. Fluorescence was collected through the objective and separated from the excitation laser using a Z488rdc long pass dichroic and an HQ600/200m bandpass filter (Chroma, Bellows Falls, VT). Fluorescence was focused onto the aperture of a 50 μ m optical fiber coupled to an avalanche photodiode (Perkin Elmer, Waltham, MA). 10 autocorrelation curves of 10 seconds each were taken using a digital correlator (Flex03Q-12, correlator.com, Bridgewater, NJ). Fitting was done using MATLAB (The MathWorks, Natick, MA).

Eight-well chambered coverglasses (Nunc, Rochester, NY) were cleaned by plasma treatment followed by incubation with polylysine-conjugated polyethylene glycol (PEG-PLL). PEG-PLL was prepared from a modified Pierce PEGylation protocol (Pierce, Rockford, IL). After overnight incubation with PEG-PLL, chambers were rinsed with Millipore water and stored until use for measurements. Measurements were done by first replacing the water with solutions of \sim 400 μ M wild type α -synuclein in each concentration of TMAO to prevent adsorption of labeled protein. 20 nM of Alexa Fluor 488 or labeled α -synuclein were added into a chamber, and each sample was mixed by pipetting immediately before measurement. The free dye measurements were used to obtain reference diffusion times.

The observed fluorescence fluctuations were autocorrelated and the raw autocorrelation data was fit to the equation:

$$G(\tau) = \frac{1}{N} \left(\frac{1}{1 + \frac{\tau}{\tau_{\alpha S}}} \times \left(\frac{1}{1 + \frac{s^2 \tau}{\tau_{\alpha S}}} \right)^{1/2} \right) \quad (\text{Eq. S15})$$

N is the number of molecules in the focal volume, τ is the delay time, τ_{aS} is the time spent by the sample in the focal volume, and s represents the eccentricity of the confocal volume, and is fixed to 0.2. The diffusion time of the protein, τ_{aS} , is extracted from fit.

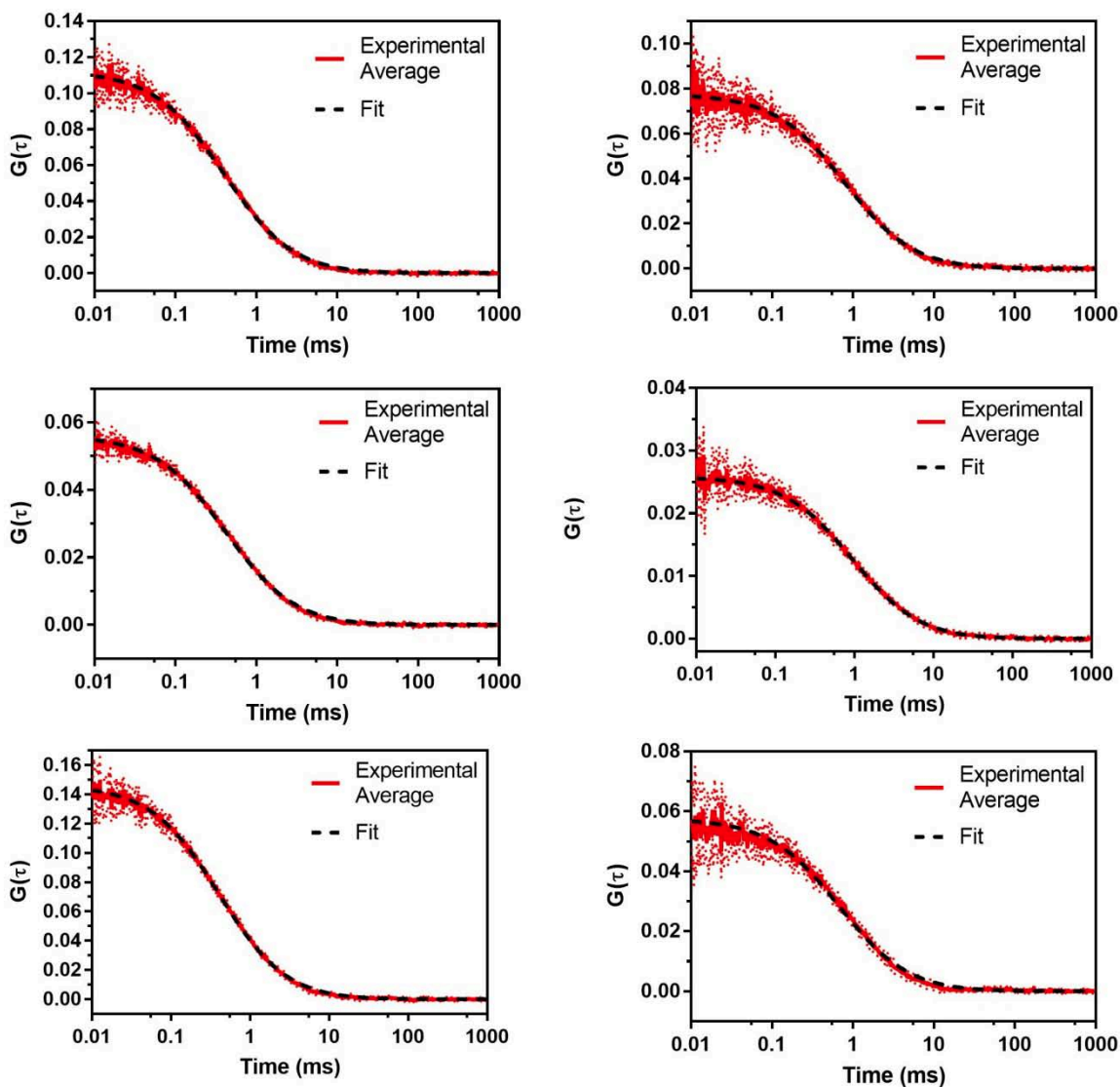


Figure S51: Autocorrelation plots from FCS Data. Plots show the average autocorrelation decays from 10 measurements (red) with fits (black-dashed) for α S-AF₄₈₈⁹ (Top), α S-AF₄₈₈¹¹⁴ (Middle) and α S-AF₄₈₈¹³⁰ (Left) in 0 M (Left) and 2 M (Right) TMAO.

Table S6. FCS Data

Position (TMAO)	9 (0 M)	114 (0 M)	130 (0 M)
N	8.9006	17.809	6.8298
Delay Time	0.401	0.42034	0.40662
Intensity	59007	193000	51376
$\tau\alpha S$	0.43±0.027	0.418±0.010	0.411±0.010
Position (TMAO)	9 (2 M)	114 (2 M)	130 (2 M)
N	12.889	38.693	17.384
Delay Time	0.79827	0.94794	0.68422
Intensity	44824	163510	54824
$\tau\alpha S$	0.822±±0.007	0.946±0.035	0.779±0.037

AFM Measurements: Three droplets of α S solution were incubated on the mica substrate for 5 minutes. To remove the excess solution, the samples were blotted by Kimwipe, gently rinsed with 2 mL Milli-Q water, and then dried using a weak nitrogen stream. During the sample preparation, the substrate was tilted 30-45° to prevent the solution from flowing backwards. The samples were imaged in tapping mode using a Keysight 5500 AFM instrument (Keysight Technologies) equipped with a closed-loop scanner. Rotated silicon probes with aluminum reflex coating (BudgetSensors, Tap-300G, resonance frequency \sim 300 kHz, tip radius $<$ 10 nm, force constant 40 N/m) were used to record topographic, amplitude, and phase images with 512 x 512 pixel resolution. The AFM images were analyzed by Gwyddion package. A third-order polynomial was used to flatten the background for topographic images. The volumetric analyses of globular structures were performed using Gwyddion software.

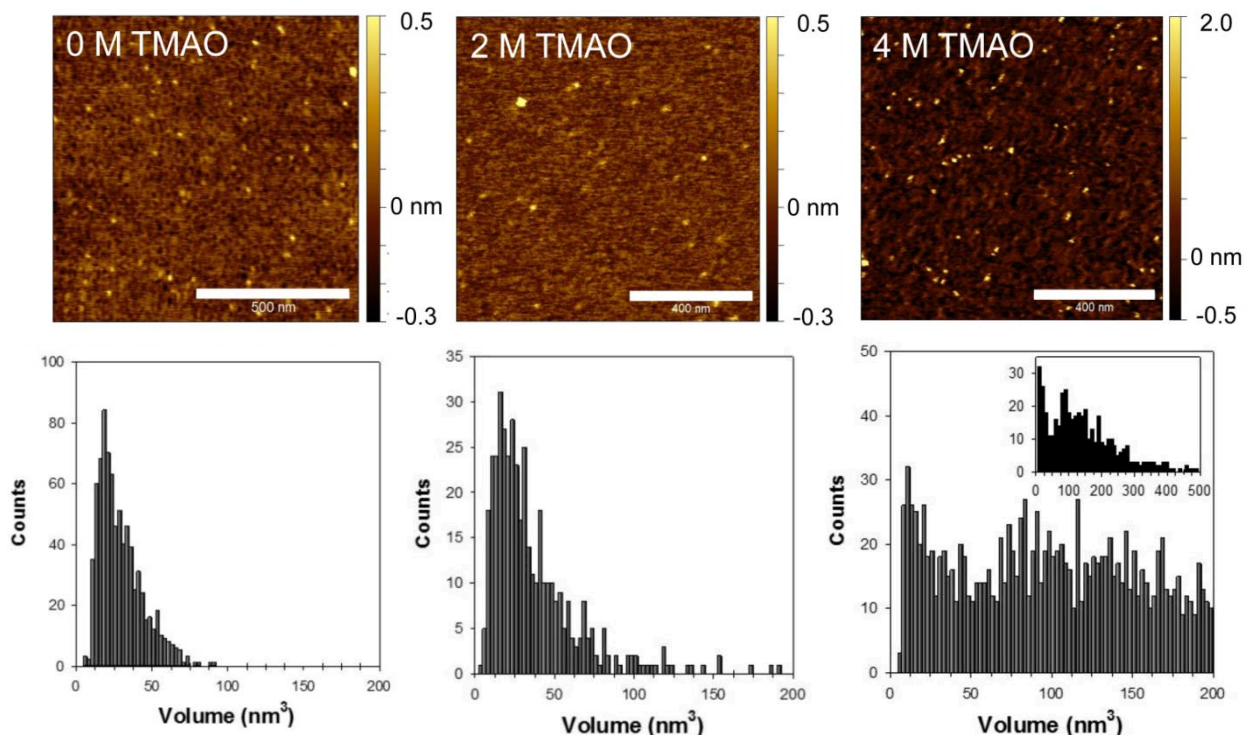


Figure S52: The effect of TMAO on the aggregation of wild type α S. Top: AFM images of the dried samples. Bottom: corresponding volume histograms of the α S aggregates. The α S concentration used for these samples are (left) 10 μ M, (middle) 0.1 μ M, and (right) 0.5 μ M. Each volume histogram was obtained with accumulated data from more than five AFM images screened at various area on the sample. No α S fibrillization is observed, which is consistent with the fact that α S fibrillization in solution can't occur in low concentrations and under static conditions, and no agitation or shaking of the solution were performed here.

Computational Modeling Procedures

Semi-Empirical Probability Distribution: This simulation was run utilizing the same format as the 0 M TMAO unconstrained simulation where the score function at each step was replaced simply with a single repulsive van der Waals term. For the combined set of outputs, all segments which are spaced by the same number of residues in the primary sequence were fit to a normal distribution. From this an empirical relationship was constructed between the distance spanning any two parts of the protein and the average breadth, σ .

PyRosetta Simulations: All simulations were performed in PyRosetta on the University of Pennsylvania School of Arts and Science General Purpose Cluster. The simulation format followed a basic simulated annealing procedure detailed in the general script attached. In short, the initial protein structure was randomized by sampling random backbone ϕ/ψ angles against a score function consisting solely of repulsive van der Waals energy terms while the protein was represented in the course-grained centroid model. Following randomization, constraints were applied with continued sampling of ϕ/ψ angles in centroid model while increasing the score function complexity from score0-score3 ending with the score3 function as well as long and short range hydrogen bonding terms (hbond_srbb and hbond_lrbb). Lastly, the protein representation was switched into full-atom and backbone torsion angles were sampled along with side-chain χ angles while the 'beta' score function was applied with the addition of constraints. Acceptance for a set of moves was determined as usual in a Monte Carlo simulation by comparing the sum of the score energy and constraint energy of the previous structure to the newly generated structure. The lowest energy structure generated from each of these steps was retained and used as the starting structure for the next simulation step. The final output structure as determined from the sum of the total 'beta' score function energy and the constraint energy.

Several different methods were employed in an attempt to capture the influence of TMAO on the protein structure in addition to applying constraints. Simulations were performed where the solvation term (fa_sol) was removed from the score function since solvation effects from TMAO introduction could not be accounted for directly. Moreover, fragment insertions was employed alongside ϕ/ψ angle sampling in an effort to incorporate the possible formation of significant secondary structure. Fragment libraries were prepared from the primary sequence of wild-type α S using Robetta, a protein prediction server which uses Rosetta software and was developed and

supported by the Rosetta Commons and the Baker Lab (9). Modifications to the base method for each simulation for the 2 M TMAO data set are detailed with the reported results.

In order to produce a simulation which accurately represented the FRET data, each distance constraint was implemented with a "knowledge" weighting factor, γ . This knowledge weighting factor, γ , is inversely proportional to the standard deviation in the calculated distance, based on a given version of the polymer-scaled Förster equation.

$$\gamma(E_{FRET}) = \frac{N}{S_{DA} \times \delta x(E_{FRET}) / \delta E_{FRET}} \quad (\text{Eq. S16})$$

$$N = \left(\int_{E_{FRET}} \frac{1}{S_{DA} \times \delta x(E_{FRET}) / \delta E_{FRET}} d(E_{FRET}) \right)^{-1} \quad (\text{Eq. S17})$$

Constraints were applied using one of two functions based on the probability distribution functions used to interpret the FRET data. The Gaussian chain (Eq. 3) and normal distribution (Eq. 4) functions were transformed into Eq. S18 and S19 respectively to generate potential energy functions termed the Gaussian chain constraint and the harmonic constraint functions respectively.

$$U_1(r) = a \times \gamma \times \log \left(4\pi \left(\frac{3}{2\pi \langle r^2 \rangle} \right)^{3/2} \exp \left(-\frac{3}{2} \frac{r^2}{\langle r^2 \rangle} \right) \right) \quad (\text{Eq. S18})$$

$$U_2(r) = a \times \gamma \times \log \left(\frac{1}{\sqrt{2\pi\sigma^2}} \exp \left(-\frac{1}{2} \frac{(r - \langle r^2 \rangle)^2}{\sigma^2} \right) \right) \quad (\text{Eq. S19})$$

The additional term, a , in both equations represents the "thermodynamic" weighting factor of the constraints with respect to the Rosetta score function. The optimal thermodynamic weighting factor was determined empirically by running trial simulations with a set to values from 0.25-5. The breadth, σ for the harmonic restraints (Eq. S19) was determined from a relationship between the average distance and the normal distribution breadth derived from the simulated structures used to generate the semi-empirical constraints. The optimal weight (a) was determined to be 1,

since at that value constraints were satisfied but the resulting ensembles were not overconstrained so as to yield physically unreasonable conformations. For each interpretation of the data, both constraint functions were applied to determine the efficacy of the constraining method and the data interpretation. Simulations assessing the constraint weight and the method of constraining generated 120 outputs, where the lowest 100 structures were used in analysis due to the lack of differences in energy in the outputs. Final simulations of the Gaussian chain interpreted and Gaussian chain constrained data produced 1020 structures where the lowest 1000 were used for interpretation.

Simulation Output Analysis: HydroPro was used to calculate the diffusion coefficients and radii of gyration. For analysis of 2 M simulated structures in HydroPro, the input viscosity was multiplied by the ratio of the 0 M:2 M diffusion times of AlexaFluor488. This effectively accounted for the increase in viscosity in 2 M versus 0 M TMAO. Following determination in HydroPro, the diffusion coefficient was converted for comparison with the diffusion coefficient determined from FCS using a previously published conversion equation detailed in Eq. S20, while the diffusion coefficient was determined from the measured diffusion time via Eq. S21 (10).

$$D_{Calibrated} = \frac{D_{HydroPro} + 0.582}{1.08} \quad (\text{Eq. S20})$$

$$D_p = D_{AF488} \frac{\tau_{AF488}}{\tau_p} \quad (\text{Eq. S21})$$

In these equations, D_p , D_{AF488} , $D_{HydroPro}$ and $D_{Calibrated}$ are the diffusion coefficients of the protein of interest, AlexaFluor488, as determined from literature, from HydroPro and the experimentally calibrated diffusion coefficient for comparison to D_p respectively. Additionally, τ_{AF488} and τ_p are the experimentally derived diffusion times of AlexaFluor488 and the protein of interest respectively.

All distances required for Flory scaling plots, heat maps and comparisons to experimental FRET data were extracted from C-alpha to C-alpha distances for each residue using Python or BioPython and all calculations were performed using NumPy. For E_{FRET} determination, distance values from each member of a given ensemble were transformed into E_{FRET} values using the classical Förster equation prior to averaging to capture the explicit distance probability distribution

of the ensemble. The same method was used to calculate comparisons to paramagnetic relaxation enhancement data, where distances were extracted from C-alpha to amide proton distance. Distances converted into intensity ratios as described by Piana *et. al.* and the intensity ratios were averaged to calculate the final values (11).

Impact of the Thermodynamic Constraint Weight: Assessment of the thermodynamic constraint weight effects were assessed prior to application of the knowledge constraint weight. This was done in order to assess the maximum impact of the constraint set. Knowledge constraints applied to the full simulation were scaled to a maximum value determined by the thermodynamic constraint as shown in Eq. S18 and Eq. S19, which is why determination of the maximum impact of the constraint set was crucial. Experimentally constrained simulations were run as described below with the omission of side chain rotamer packing using constraints where the distances were determined by using the Gaussian chain version of the polymer scaled Förster equation and were applied using the Gaussian chain derived constraining function.

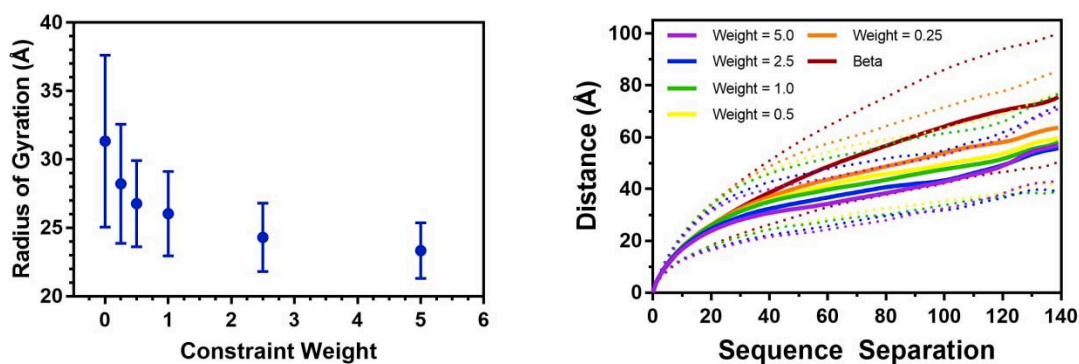


Figure S53: Determination of Optimal Thermodynamic Constraint Value. Radius of gyration (left) and average Flory scaling behavior (right) of simulated structures with varying values of the thermodynamic constraint weight, a .

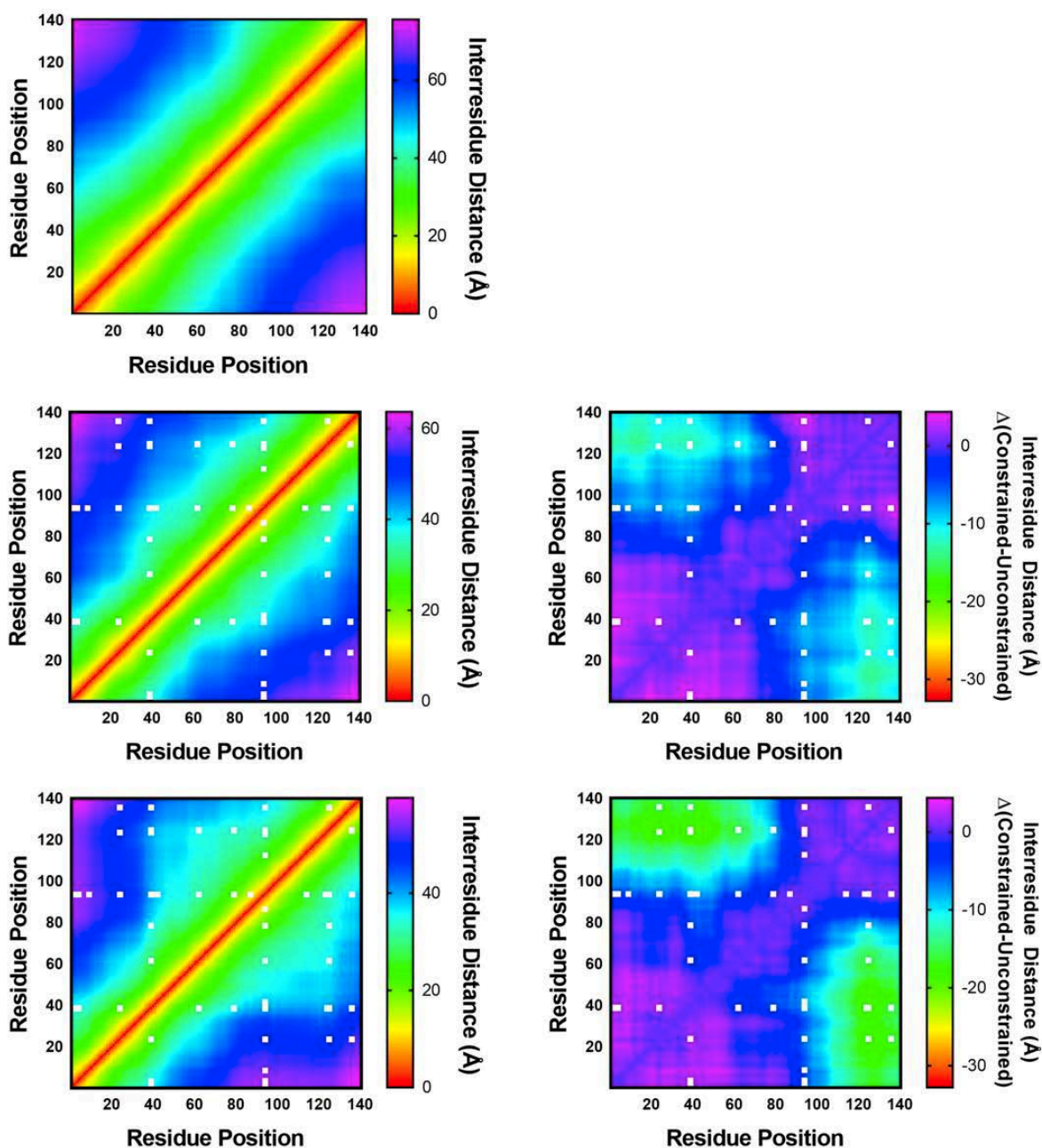


Figure S54: Heat maps depicting the average interresidue distance for each thermodynamic constraint weight, a , (left) and average difference in interresidue distance between constrained and unconstrained simulations (right). Heat maps depict averages from an unconstrained simulation (top row) and simulations with thermodynamic constraint weights set to 0.25 (middle row) and 0.5 (bottom row).

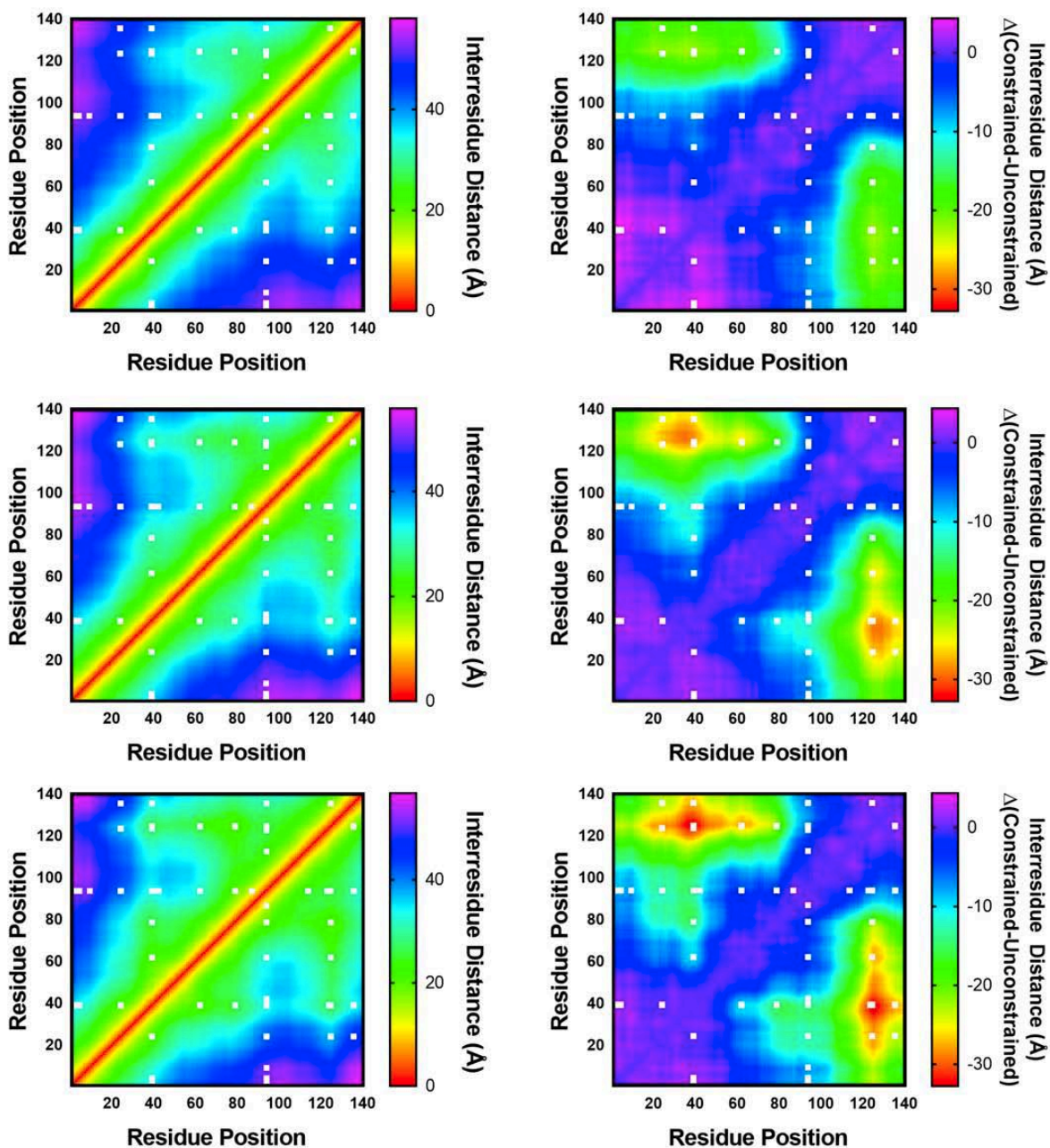


Figure S55: Heat maps depicting the average interresidue distance for each thermodynamic constraint weight, a , (left) and average difference in interresidue distance between constrained and unconstrained simulations (right). Heat maps depict averages from simulations with thermodynamic constraint weights set to 1.0 (top row), 2.5 (middle row) and 5.0 (bottom row).

Constraints for Utilized each Constraining Method:

0 M Gaussian-Chain Distance - Gaussian Chain Constraint

Res 1	Res 2	r	γ
39	24	39.061	0.25
39	62	36.48	0.308
39	79	52.301	0.097
39	94	44.112	0.171
94	24	74.119	0.025
94	39	44.823	0.146
94	62	34.425	0.332
94	79	32.21	0.401
125	24	48.945	0.084
125	39	45.722	0.106
125	62	42.349	0.137
125	79	46.02	0.104
125	94	39.257	0.175
136	24	66.266	0.045
136	39	47.473	0.141
136	94	45.153	0.167
136	125	46.607	0.15
9	94	69.478	1.105
24	94	60.531	1.393
42	94	51.785	1.573
62	94	48.25	1.619
87	94	34.824	1.335
114	94	25.412	0.588
123	94	45.228	1.625
136	94	39.352	1.512
9	136	142.742	0.184

0 M Semi-Empirical Distance - Harmonic Constraint

Res 1	Res 2	r	γ	σ
39	24	26.436	0.315	5.382
39	62	25.231	0.385	5.035
39	79	32.576	0.122	7.206
39	94	28.788	0.218	6.075
94	24	42.495	0.03	10.267
94	39	28.73	0.188	6.058
94	62	23.936	0.416	4.661
94	79	22.903	0.502	4.367
125	24	29.619	0.111	6.32
125	39	28.202	0.138	5.902
125	62	26.699	0.178	5.458
125	79	28.334	0.136	5.941
125	94	25.322	0.227	5.061
136	24	39.473	0.055	9.318
136	39	30.523	0.179	6.588
136	94	29.452	0.212	6.271
136	125	30.123	0.191	6.469
9	94	59.77	1.261	16.254
24	94	54.252	1.574	14.318
42	94	48.236	1.755	12.189
62	94	45.915	1.77	11.392
87	94	36.143	1.291	8.295
114	94	28.375	0.474	5.953
123	94	43.364	1.763	10.544
136	94	39.686	1.542	9.384
9	136	108.103	0.124	33.291

0 M Gaussian Chain Distance - Harmonic Constraint

Res 1	Res 2	r	γ	σ
39	24	39.061	0.25	9.19
39	62	36.48	0.308	8.399
39	79	52.301	0.097	13.627
39	94	44.112	0.171	10.791
94	24	74.119	0.025	20.898
94	39	44.823	0.146	11.028
94	62	34.425	0.332	7.768
94	79	32.21	0.401	7.096
125	24	48.945	0.084	12.438
125	39	45.722	0.106	11.327
125	62	42.349	0.137	10.221
125	79	46.02	0.104	11.427
125	94	39.257	0.175	9.251
136	24	66.266	0.045	18.37
136	39	47.473	0.141	11.924
136	94	45.153	0.167	11.138
136	125	46.607	0.15	11.626
9	94	69.478	1.105	19.401
24	94	60.531	1.393	16.51
42	94	51.785	1.573	13.443
62	94	48.25	1.619	12.194
87	94	34.824	1.335	7.89
114	94	25.412	0.588	5.087
123	94	45.228	1.625	11.163
136	94	39.352	1.512	9.28
9	136	142.742	0.184	30

0 M Semi-Empirical Distance - Gaussian Chain Constraint

Res 1	Res 2	r	γ
39	24	26.436	0.315
39	62	25.231	0.385
39	79	32.576	0.122
39	94	28.788	0.218
94	24	42.495	0.03
94	39	28.73	0.188
94	62	23.936	0.416
94	79	22.903	0.502
125	24	29.619	0.111
125	39	28.202	0.138
125	62	26.699	0.178
125	79	28.334	0.136
125	94	25.322	0.227
136	24	39.473	0.055
136	39	30.523	0.179
136	94	29.452	0.212
136	125	30.123	0.191
9	94	59.77	1.261
24	94	54.252	1.574
42	94	48.236	1.755
62	94	45.915	1.77
87	94	36.143	1.291
114	94	28.375	0.474
123	94	43.364	1.763
136	94	39.686	1.542
9	136	108.103	0.124

2 M Gaussian Chain Distance - Gaussian Chain Constraint

Res 1	Res 2	r	γ
39	24	31.254	0.323
39	62	27.808	0.45
39	79	34.562	0.239
39	94	33.201	0.27
94	24	58.374	0.039
94	39	37.018	0.184
94	62	24.762	0.587
94	79	27.224	0.458
125	24	28.271	0.334
125	39	36.222	0.155
125	62	32.75	0.214
125	79	31.391	0.244
125	94	30.104	0.277
136	24	48.748	0.078
136	39	40.401	0.147
136	94	36.77	0.199
136	125	43.522	0.115
9	94	49.257	1.551
24	94	45.782	1.618
42	94	41.443	1.611
62	94	42.748	1.616
87	94	33.392	1.37
114	94	23.095	0.541
123	94	39.607	1.596
136	94	33.74	1.338
9	136	82.725	0.729

2 M Semi-Empirical Distance - Harmonic Constraint

Res 1	Res 2	r	γ	σ
39	24	21.447	0.419	3.963
39	62	19.904	0.577	3.541
39	79	22.919	0.31	4.372
39	94	22.314	0.351	4.202
94	24	33.203	0.05	7.395
94	39	23.855	0.241	4.638
94	62	18.41	0.747	3.155
94	79	19.516	0.591	3.439
125	24	19.316	0.446	3.387
125	39	22.711	0.208	4.313
125	62	21.233	0.286	3.904
125	79	20.651	0.325	3.743
125	94	20.1	0.369	3.593
136	24	29.21	0.104	6.199
136	39	25.544	0.193	5.125
136	94	23.937	0.259	4.662
136	125	26.918	0.15	5.522
9	94	45.599	1.732	11.286
24	94	43.544	1.774	10.603
42	94	40.651	1.695	9.686
62	94	43.045	1.718	10.441
87	94	34.585	1.333	7.817
114	94	26	0.425	5.255
123	94	39.151	1.669	9.218
136	94	35.057	1.296	7.961
9	136	67.873	0.841	18.885

2 M Gaussian Chain Distance - Harmonic Constraint

Res 1	Res 2	r	γ	σ
39	24	31.254	0.323	6.808
39	62	27.808	0.45	5.785
39	79	34.562	0.239	7.81
39	94	33.201	0.27	7.394
94	24	58.374	0.039	15.78
94	39	37.018	0.184	8.564
94	62	24.762	0.587	4.899
94	79	27.224	0.458	5.613
125	24	28.271	0.334	5.922
125	39	36.222	0.155	8.319
125	62	32.75	0.214	7.258
125	79	31.391	0.244	6.849
125	94	30.104	0.277	6.463
136	24	48.748	0.078	12.369
136	39	40.401	0.147	9.608
136	94	36.77	0.199	8.488
136	125	43.522	0.115	10.596
9	94	49.257	1.551	12.549
24	94	45.782	1.618	11.348
42	94	41.443	1.611	9.935
62	94	42.748	1.616	10.347
87	94	33.392	1.37	7.452
114	94	23.095	0.541	4.422
123	94	39.607	1.596	9.359
136	94	33.74	1.338	7.558
9	136	82.725	0.729	23.729

2 M Semi-Empirical Distance - Gaussian Chain Constraint

Res 1	Res 2	r	γ
39	24	21.447	0.14
39	62	19.904	0.267
39	79	22.919	0.077
39	94	22.314	0.098
94	24	33.203	0.002
94	39	23.855	0.046
94	62	18.41	0.447
94	79	19.516	0.28
125	24	19.316	0.16
125	39	22.711	0.035
125	62	21.233	0.066
125	79	20.651	0.085
125	94	20.1	0.11
136	24	29.21	0.009
136	39	25.544	0.03
136	94	23.937	0.054
136	125	26.918	0.018
9	94	45.599	2.105
24	94	43.544	2.209
42	94	40.651	2.015
62	94	43.045	2.071
87	94	34.585	1.248
114	94	26	0.127
123	94	39.151	1.956
136	94	35.057	1.179
9	136	67.873	0.497

Assessment of the Constraint Functional Form and Sampling Method: Simulations were performed utilizing distances from the Gaussian chain (Eq. 3) and semi-empirical (Eq. 4) FRET distribution analyses in conjunction with either the Gaussian chain (Eq. S18) or harmonic (Eq. S19) constraints. This was done to assure that no bias was introduced by selecting a particular constraining method. No significant differences in the resulting ensembles were observed between simulations run with different constraint data or constraining functions when comparing the results in Flory-scaling plots (Fig. S54), or plots of computed E_{FRET} values (Fig. S55-S56), diffusion coefficients (Fig. S57), or “heat maps” of global structural analyses (Fig. S58-S59).

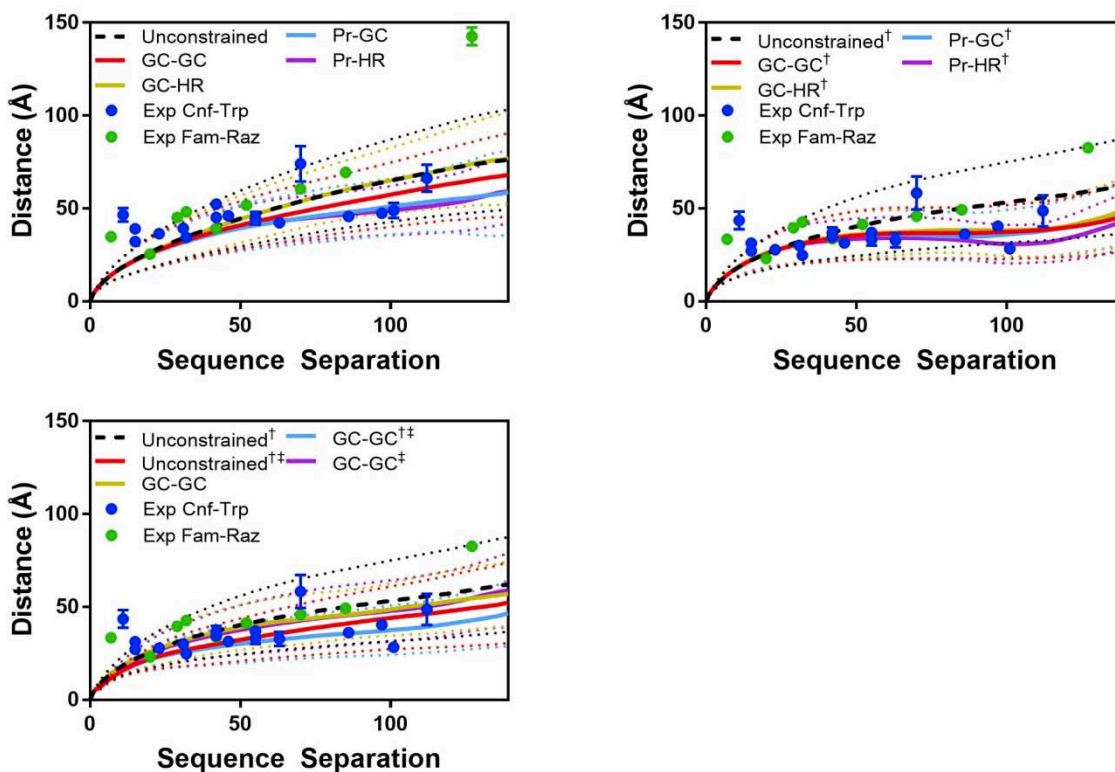


Figure S56: Flory Scaling Plots. Plots of Flory scaling determined from simulated ensembles constrained with distance constraints from measurements in 0 M (top left) and 2 M (top right/bottom left) TMAO. Names in the legends represent the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) followed by the constraining function employed (Gaussian-chain = GC or harmonic potential = HR). Simulations marked with [†] indicate that the weight of the solvation term (fa_{sol}) was set to 0 while simulations marked with [‡] indicated that fragment insertion was incorporated within the search.

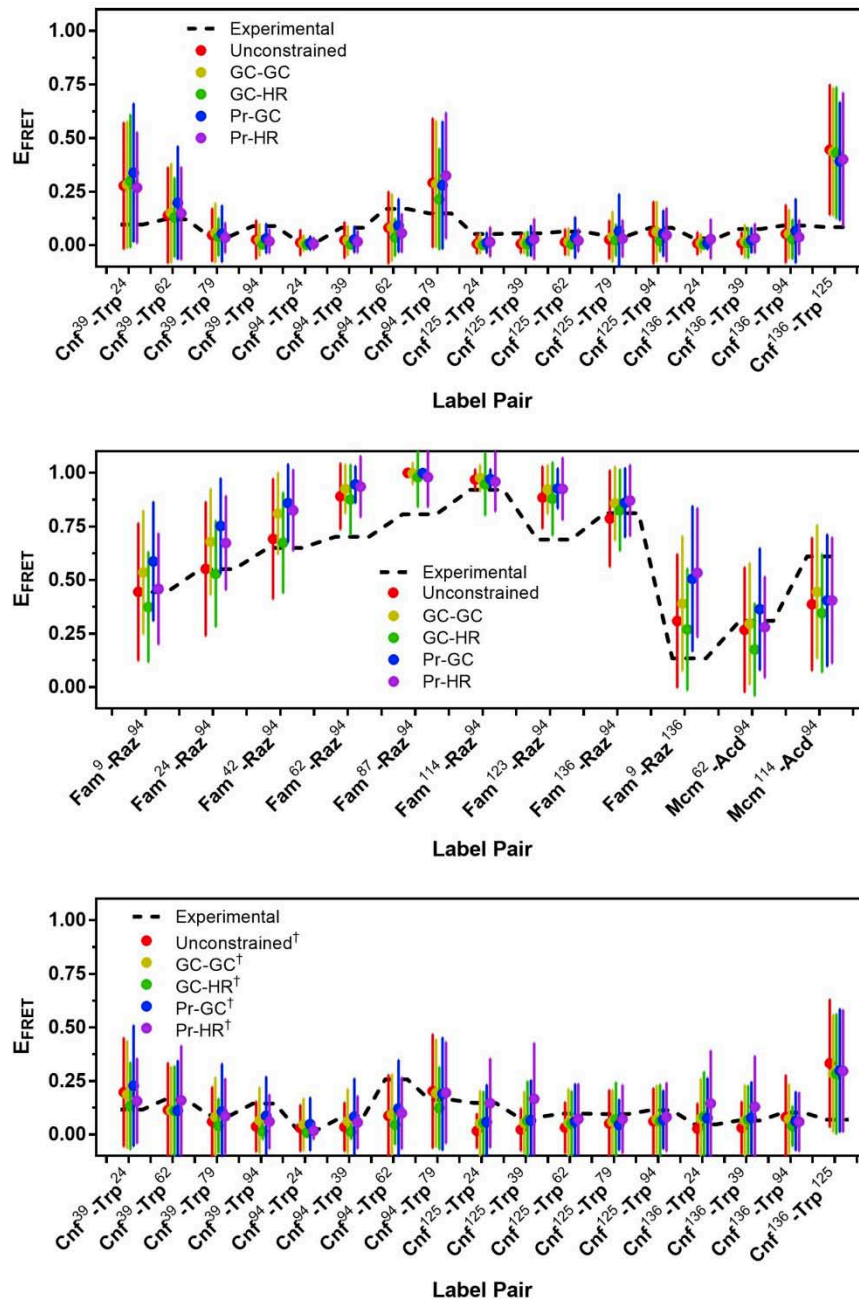


Figure S57: E_{FRET} Plots. Plots show the average and standard deviation of the E_{FRET} values determined from simulated ensembles constrained with distance constraints from measurements in 0 M (top/middle) and 2 M (bottom) TMAO. Names in the legends represent the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) followed by the constraining function employed (Gaussian-chain = GC or harmonic potential = HR). Simulations marked with † indicate that the weight of the solvation term (f_{a_sol}) was set to 0 while simulations marked with ‡ indicated that fragment insertion was incorporated within the search.

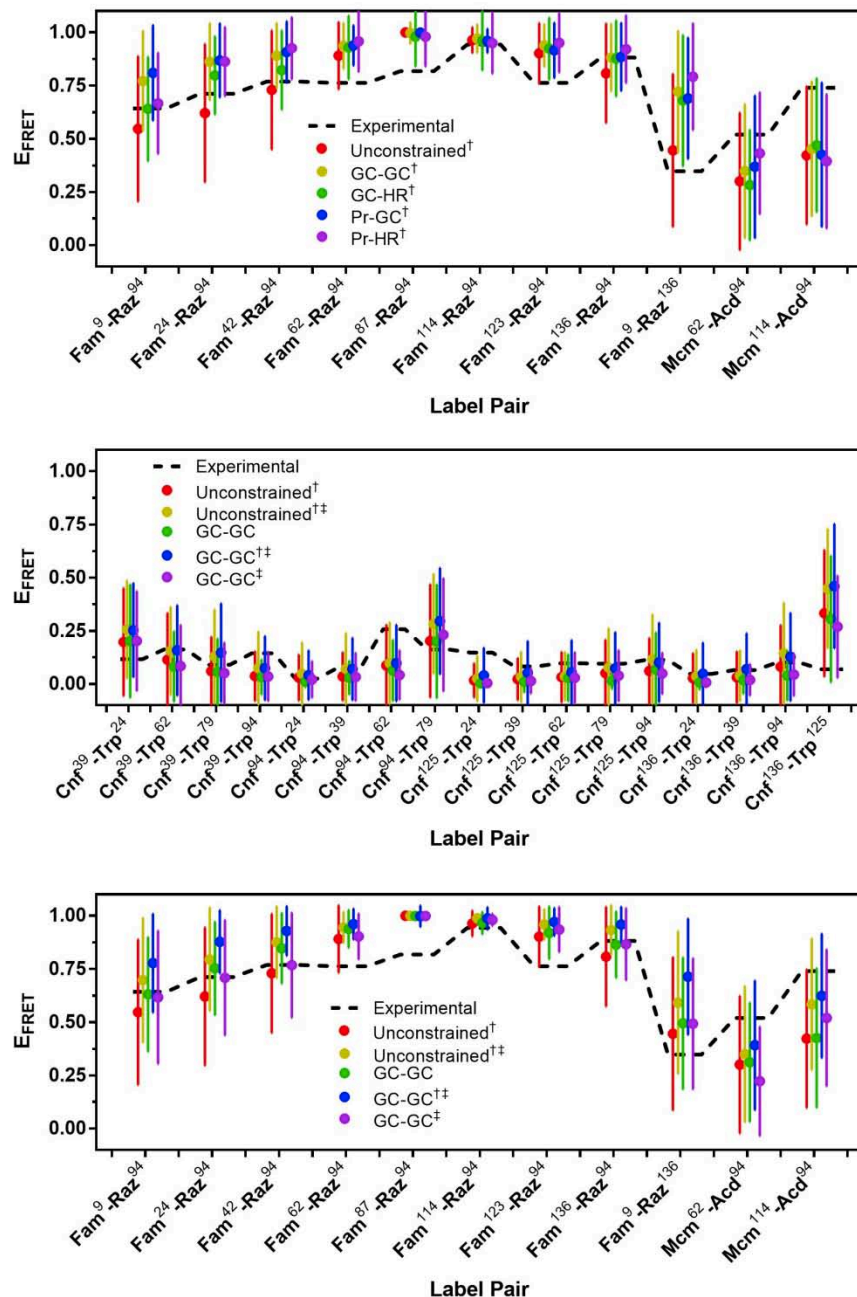


Figure S58: E_{FRET} Plots. Plots show the average and standard deviation of the E_{FRET} values determined from simulated ensembles constrained with distance constraints from measurements in 2 M TMAO. Names in the legends represent the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) followed by the constraining function employed (Gaussian-chain = GC or harmonic potential = HR). Simulations marked with [†] indicate that the weight of the solvation term (fa_{sol}) was set to 0 while simulations marked with ^{††} indicated that fragment insertion was incorporated within the search.

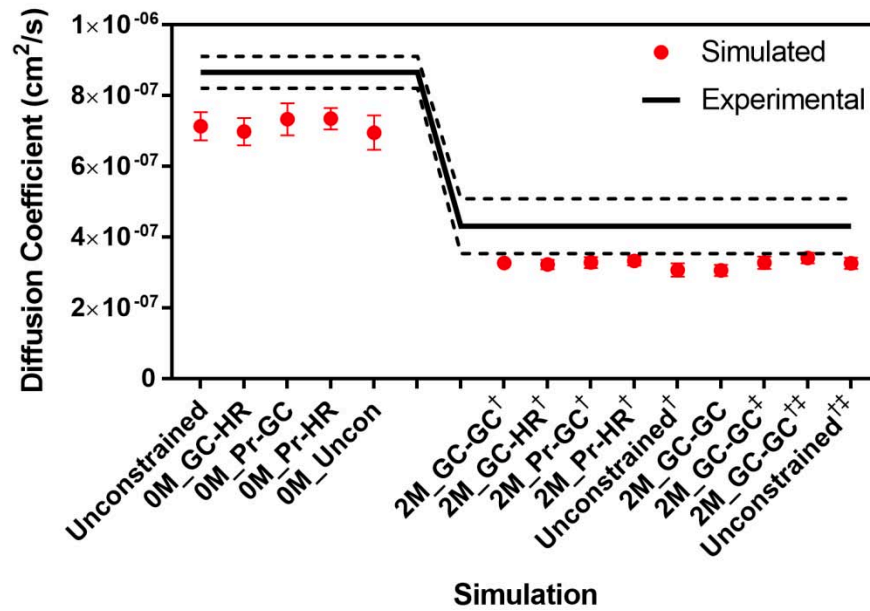


Figure S59: Diffusion Coefficients. Plots show the average diffusion coefficient determined from FCS data (black line) and from various simulations (red points). Simulation names represent the concentration of TMAO in which measurements were taken, the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) and the constraining function employed (Gaussian-chain = GC or harmonic potential = HR), respectively. Simulations marked with [†] indicate that the weight of the solvation term (f_{a_sol}) was set to 0 while simulations marked with [‡] indicated that fragment insertion was incorporated within the search.

Analysis of Simulated Structure Ensembles

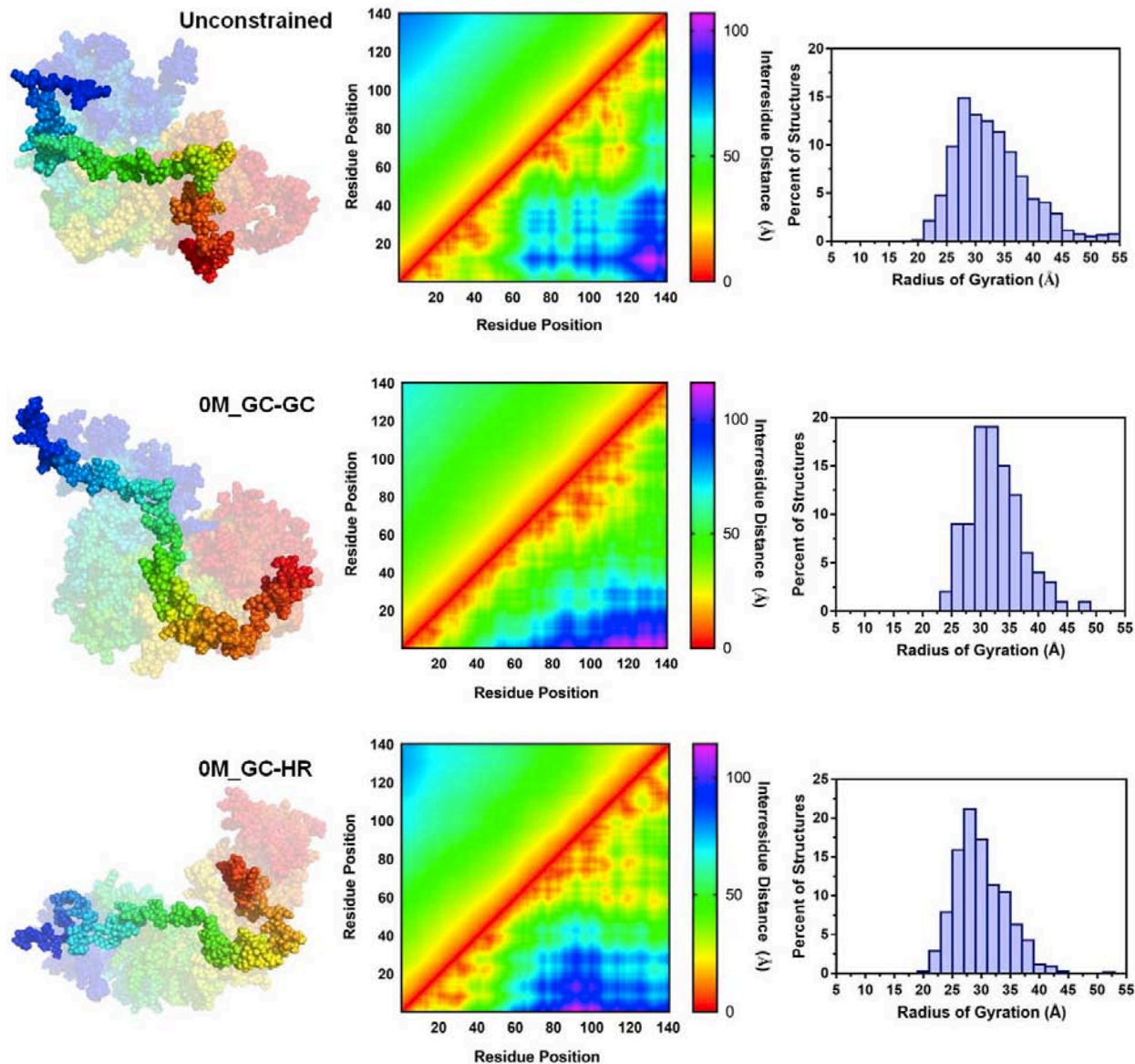


Figure S60: Global structural summary. Plots are derived from unconstrained (top set), 0M_GC-GC (middle set) and 0M_GC-HR (bottom set) simulations. Simulation names represent the concentration of TMAO in which measurements were taken, the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) and the constraining function employed (Gaussian-chain = GC or harmonic potential = HR), respectively. Structures (left) show the 10 lowest energy structures faded with the single lowest energy structure darkened (blue - red, N-term - C-term). Heat map (middle) shows the average inter-residue distances from all output structures (above diagonal) and the inter-residue distances from the single lowest energy structure (below diagonal) as a function of residue pair. Histogram (right) of the percent of output structures for a given radius of gyration with bin widths of 2 Å.

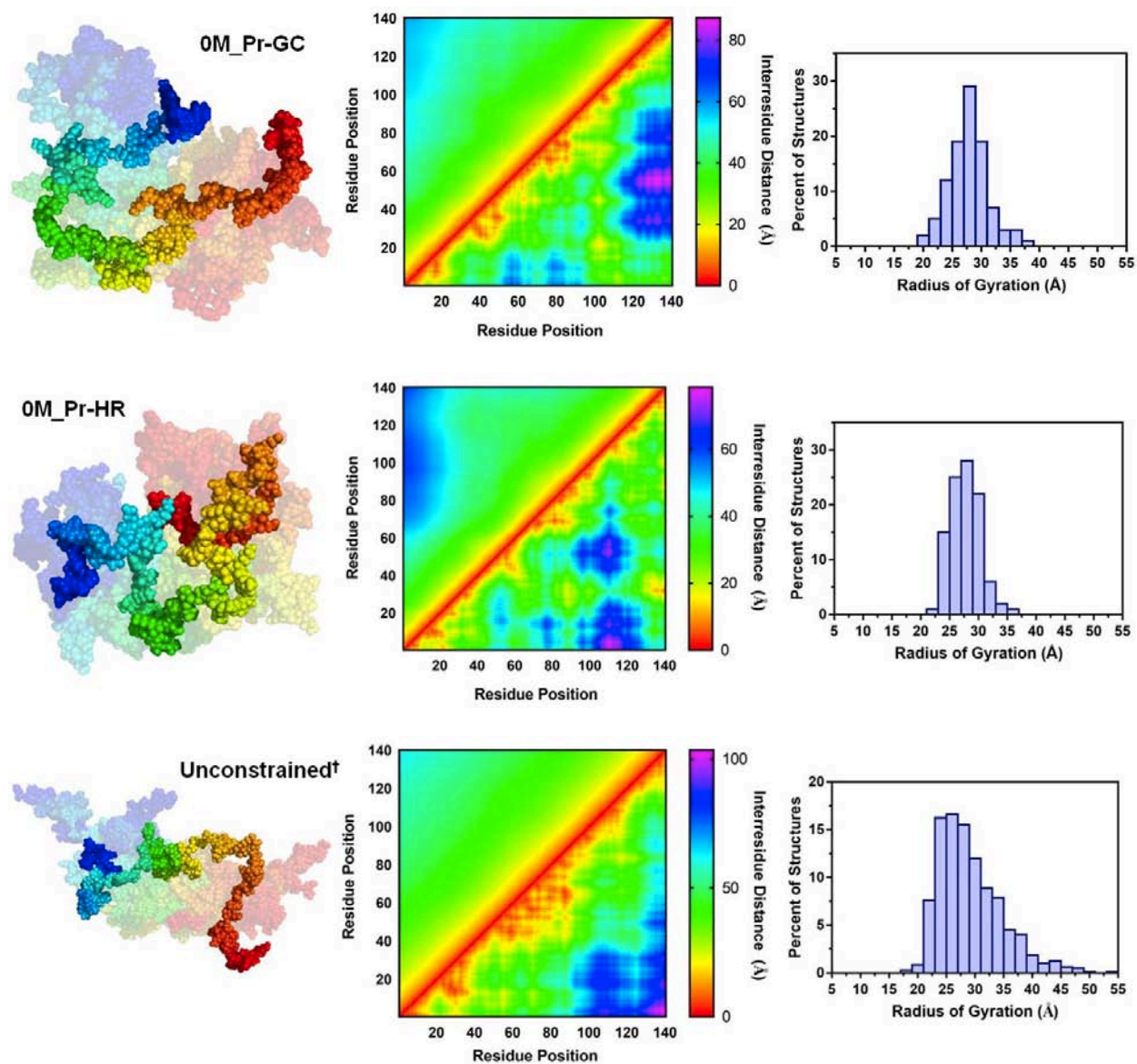


Figure S61: Global structural summary. Plots are derived from 0M_Pr-GC (top set), 0M_Pr-HR (middle set) and unconstrained[†] (bottom set) simulations. Simulation names represent the concentration of TMAO in which measurements were taken, the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) and the constraining function employed (Gaussian-chain = GC or harmonic potential = HR), respectively. Structures (left) show the 10 lowest energy structures faded with the single lowest energy structure darkened (blue - red, N-term - C-term). Heat map (middle) shows the average inter-residue distances from all output structures (above diagonal) and the inter-residue distances from the single lowest energy structure (below diagonal) as a function of residue pair. Histogram (right) of the percent of output structures for a given radius of gyration with bin widths of 2 Å. Simulations marked with [†] indicate that the weight of the solvation term (*fa_sol*) was set to 0.

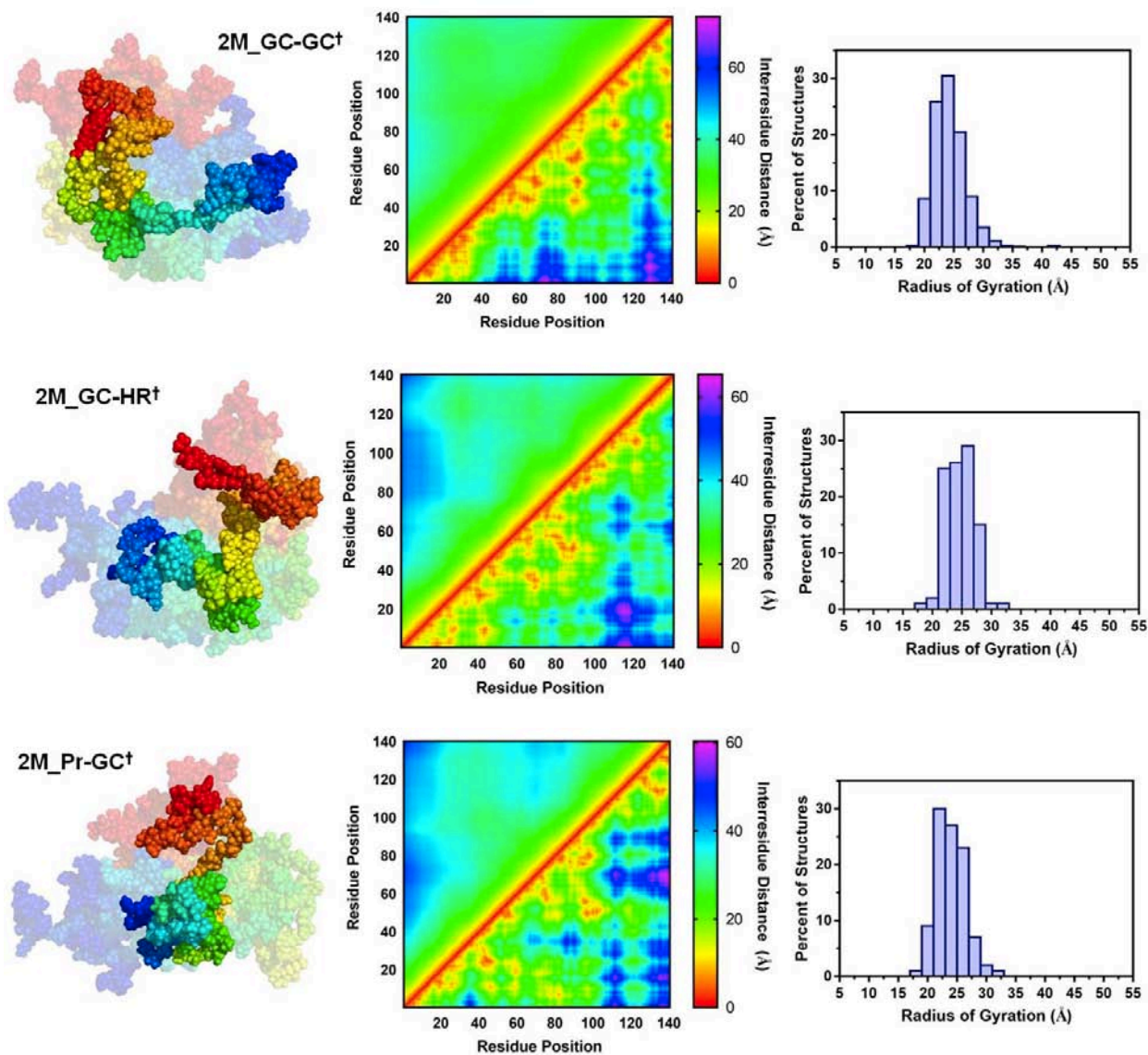


Figure S62: Global structural summary. Plots are derived from 2M_GC-GC[†] (top set), 2M_GC-HR[†] (middle set) and 2M_Pr-GC[†] (bottom set) simulations. Simulation names represent the concentration of TMAO in which measurements were taken, the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) and the constraining function employed (Gaussian-chain = GC or harmonic potential = HR), respectively. Structures (left) show the 10 lowest energy structures faded with the single lowest energy structure darkened (blue - red, N-term - C-term). Heat map (middle) shows the average inter-residue distances from all output structures (above diagonal) and the inter-residue distances from the single lowest energy structure (below diagonal) as a function of residue pair. Histogram (right) of the percent of output structures for a given radius of gyration with bin widths of 2 Å. Simulations marked with [†] indicate that the weight of the solvation term (fa_{sol}) was set to 0.

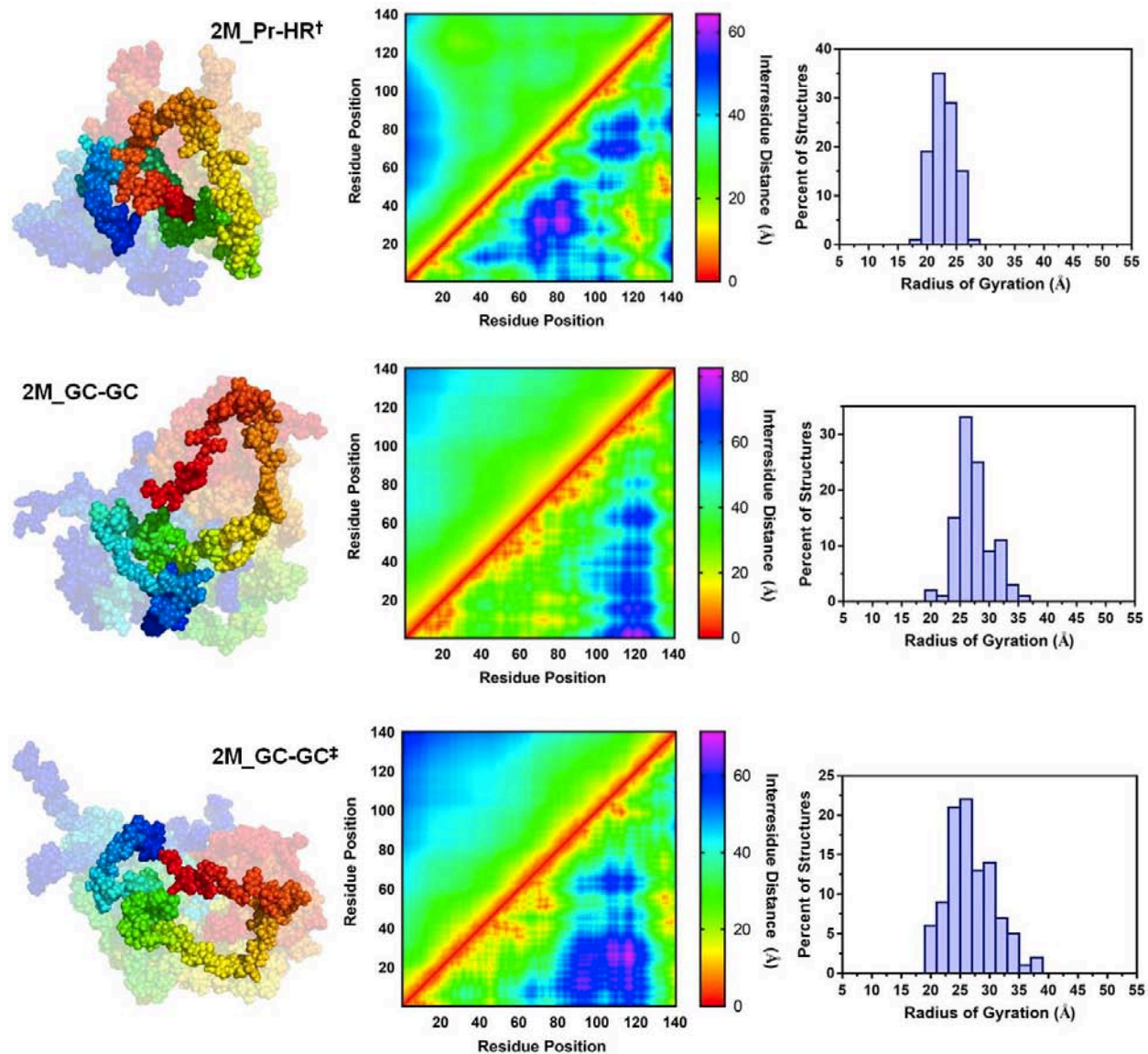


Figure S63: Global structural summary. Plots are derived from 2M_Pr-HR[†] (top set), 2M_GC-GC (middle set) and 2M_GC-GC[‡] (bottom set) simulations. Simulation names represent the concentration of TMAO in which measurements were taken, the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) and the constraining function employed (Gaussian-chain = GC or harmonic potential = HR), respectively. Structures (left) show the 10 lowest energy structures faded with the single lowest energy structure darkened (blue - red, N-term - C-term). Heat map (middle) shows the average inter-residue distances from all output structures (above diagonal) and the inter-residue distances from the single lowest energy structure (below diagonal) as a function of residue pair. Histogram (right) of the percent of output structures for a given radius of gyration with bin widths of 2 Å. Simulations marked with [†] indicate that the weight of the solvation term (*fa_sol*) was set to 0 while simulations marked with [‡] indicated that fragment insertion was incorporated within the search.

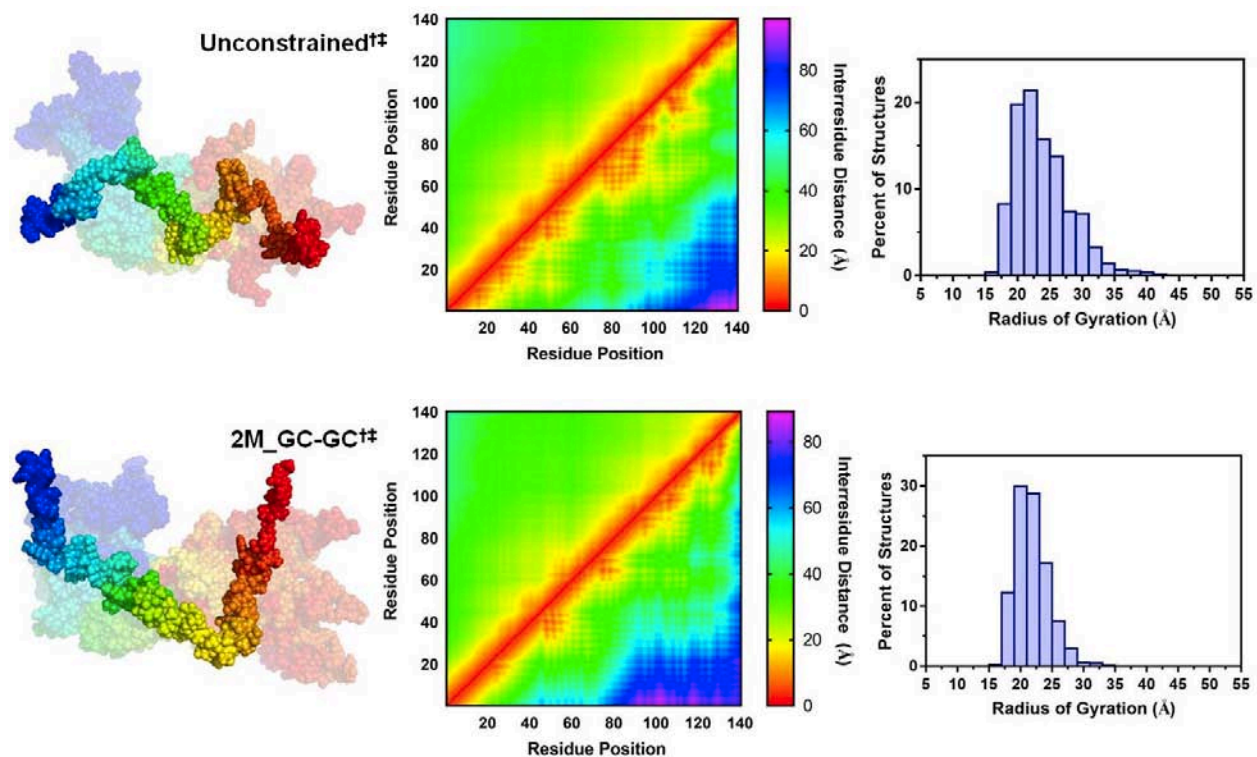


Figure S64: Global structural summary. Plots are derived from unconstrained^{†‡} (top set) and 2M_GC-GC^{†‡} (bottom set) simulations. Simulation names represent the concentration of TMAO in which measurements were taken, the probability distribution used in the polymer-scaled Förster equation for obtaining distances (Gaussian-chain = GC or semi-empirical = Pr) and the constraining function employed (Gaussian-chain = GC or harmonic potential = HR), respectively. Structures (left) show the 10 lowest energy structures faded with the single lowest energy structure darkened (blue - red, N-term - C-term). Heat map (middle) shows the average inter-residue distances from all output structures (above diagonal) and the inter-residue distances from the single lowest energy structure (below diagonal) as a function of residue pair. Histogram (right) of the percent of output structures for a given radius of gyration with bin widths of 2 Å. Simulations marked with [†] indicate that the weight of the solvation term (f_{a_sol}) was set to 0 while simulations marked with [‡] indicated that fragment insertion was incorporated within the search.

Comparison of 0 M GC-GC Model to Literature Experiments and Simulations:

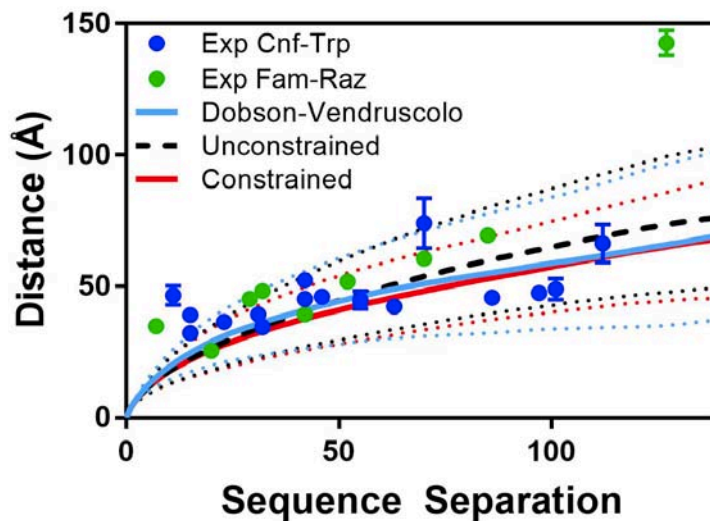


Figure S65: Flory Scaling Plot. Plot shows the average distance as a function of sequence separation for simulated ensembles (PED9AAC) and unconstrained and constrained simulations reported herein (12, 13). Distance constraints were derived from data acquired in 0 M TMAO and interpreted using the Gaussian-chain polymer scaled Förster equation. Constraints were implemented using the Gaussian-chain constraint function.

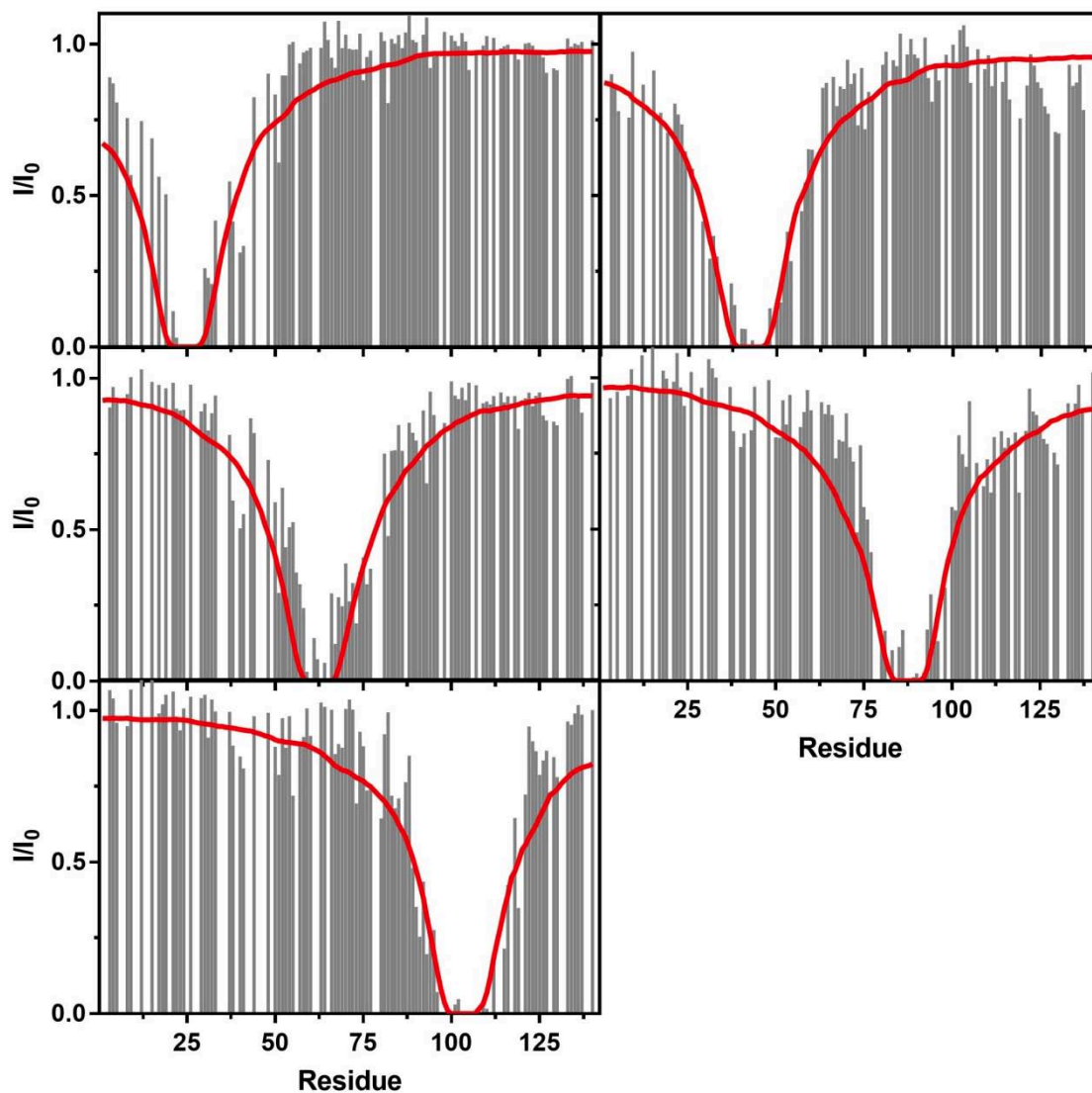


Figure S66: Intensity ratios from PRE measurements. Each plot shows the measured intensity ratio from PRE data obtained by Allison et. al. (grey bars) and the calculated average intensity ratio from the constrained simulation (red lines) were derived from data acquired in 0 M TMAO and interpreted using the Gaussian-chain polymer scaled Förster equation (12). Plots show data for spin-label placement at residues 24 (top left), 42 (top right), 62 (middle left), 87 (middle right) and 103 (bottom right).

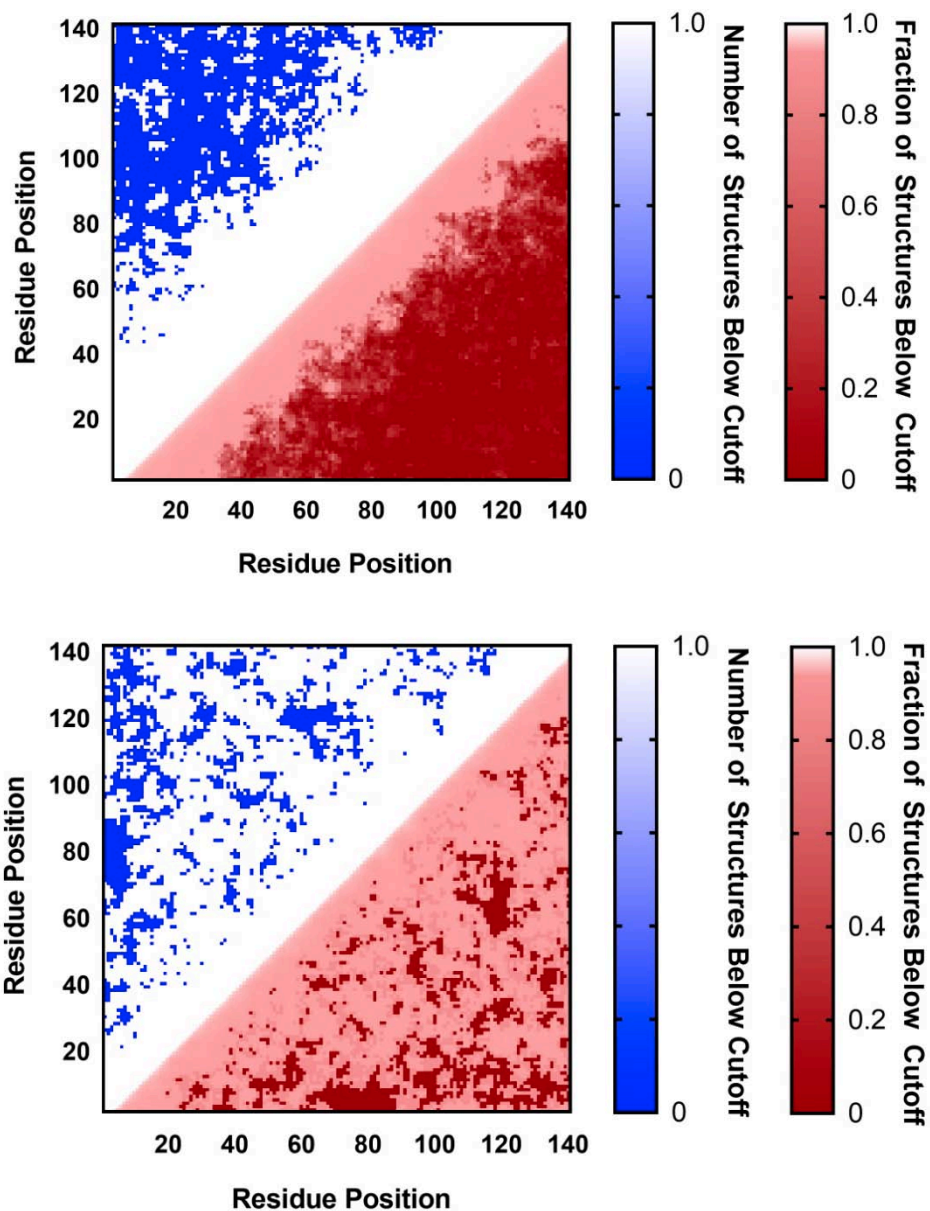


Figure S67: Contact maps from the 0M_GC-GC (top) and 2M_GC-GC[†] (bottom) ensembles. A cutoff distance of 10 Å was set as a contact. Each half of each heat map are analyses of the same ensemble and show all residue pairs which make contact in one or more structures from the ensemble as white while residue pairs which do not make contact are shown in blue (top left). The fraction of structures of the ensemble which make contact are shown over the full fractional scale with a red/white gradient (bottom right).

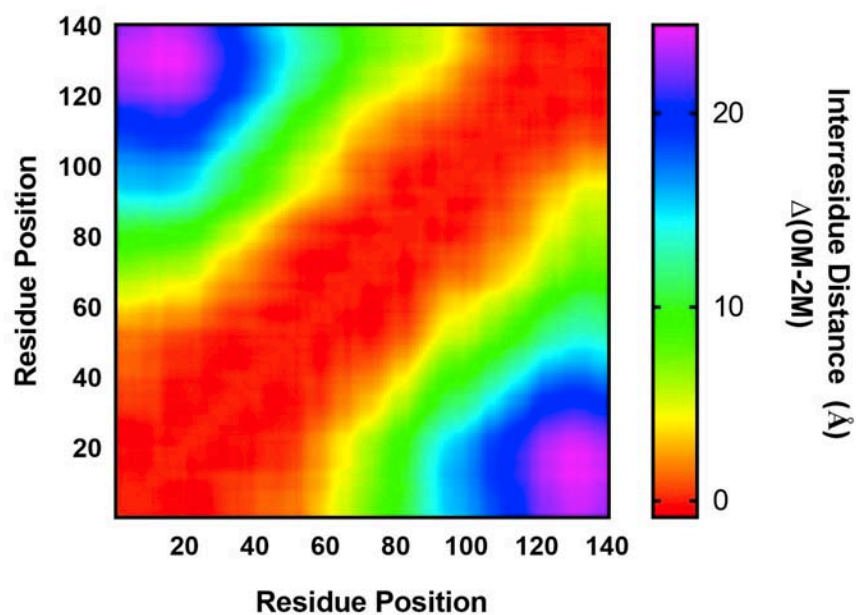


Figure S68: Heat map showing the average difference in interresidue distance between the 0M_GC-GC and 2M_GC-GC[†] ensembles.

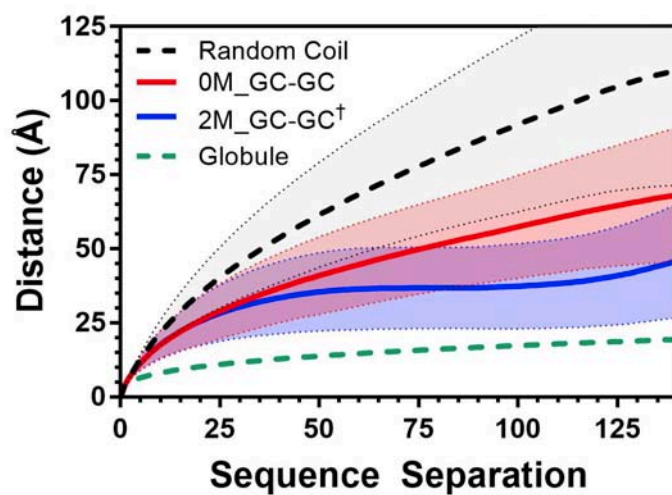


Figure S69: Flory scaling plots of 0M_GC-GC (red) and 2M_GC-GC[†] (blue) simulated ensembles along with the random coil simulation (black) used for the construction of $P_2(r,x)$ and a curve representing the scaling of a globule protein (green) (14).

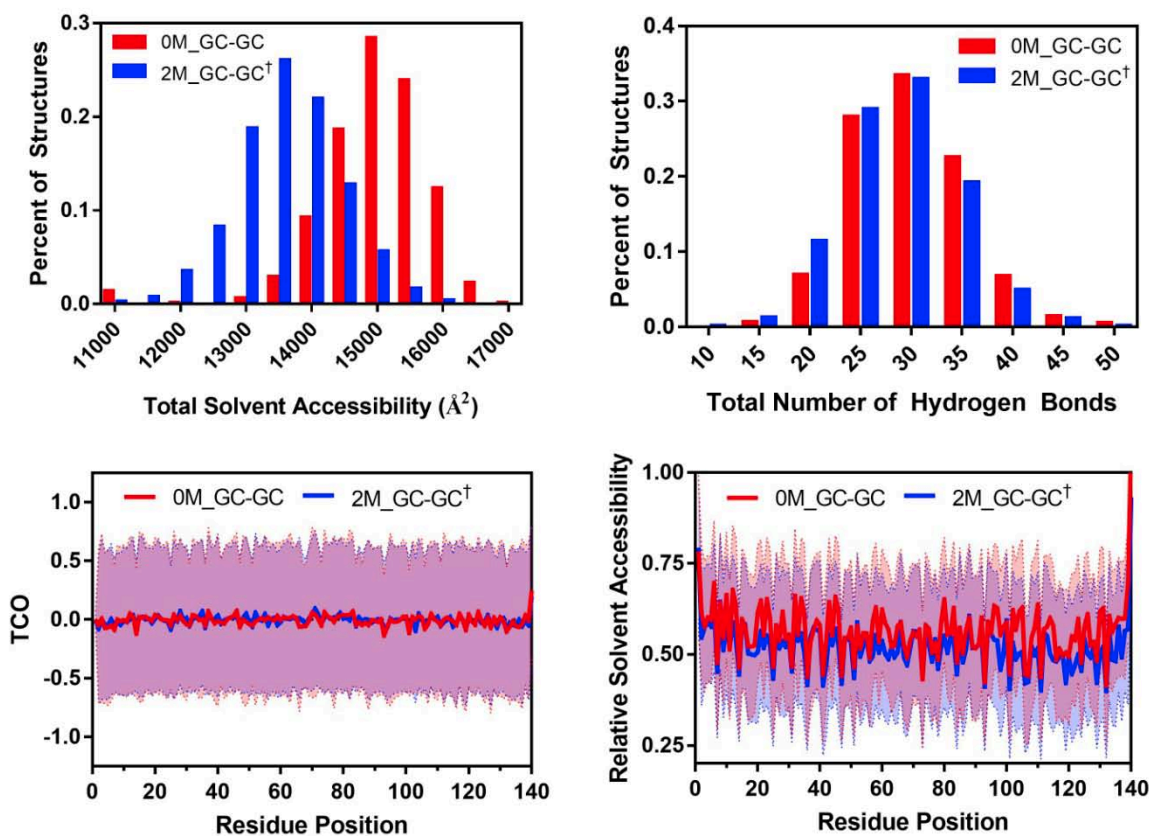


Figure S70: DSSP analysis of 0M_GC-GC (red) and 2M_GC-GC[†] (blue) ensembles for comparison. (Top Left) Histogram showing the percent of structures binned based on the total solvent accessible surface area of a given structure (\AA^2). (Top Right) Histogram showing the percent of structures binned based on the total number of intramolecular hydrogen bonds present in a given structure. (Bottom Left) Plot of the average value of the cosine of the angle between the backbone carbonyl of the current residue and the carbonyl of the previous residue (TCO) for each residue for each structure within a given ensemble. (Bottom Right) Plot of the average value relative solvent accessibility for each residue for each structure within a given ensemble.

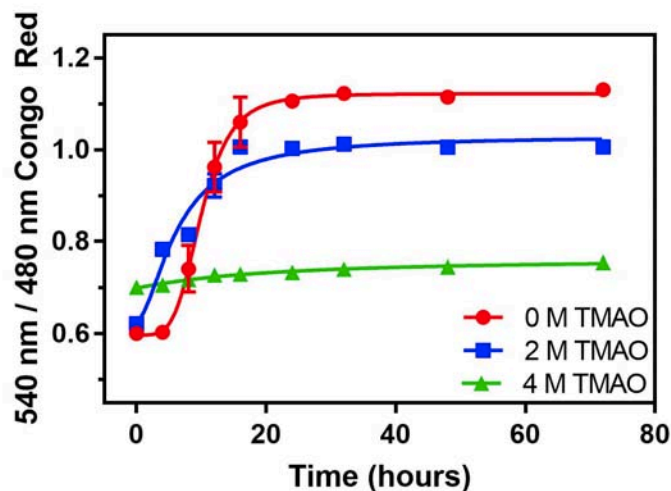


Figure S71: Aggregation kinetics of α S monitored by Congo Red. Aggregation was performed with 100 μ M wild-type α S in 20 mM Tris 100 mM NaCl pH 7.5. TMAO was added to each buffer and the pH was readjusted. Samples were agitated in an orbital shaker at 1500 rpm at 37 $^{\circ}$ C. At each timepoint, 10 μ L of sample was removed and added to 140 μ L of 20 μ M Congo Red in water and incubated for 20 minutes at room temperature prior to measurement. Absorption spectra (230-700 nm) were measured in a 96-well black CoStar clear bottom plate on a Tecan M1000 plate reader. Kinetics were fit to Eq. S22 in Prism with the detailed values in Table S7.

$$Y = A + (B - A) / (1 + (C/x)^D) \quad (\text{Eq. S22})$$

Table S7: Values from fits of Congo Red aggregation kinetics.

	0 M TMAO	2 M TMAO	4 M TMAO
A	0.5977	0.6289	0.7003
B	1.123	1.03	0.7656
C	9.989	6.397	19.72
D	4.357	1.708	1.104
R²	0.9855	0.9526	0.9062

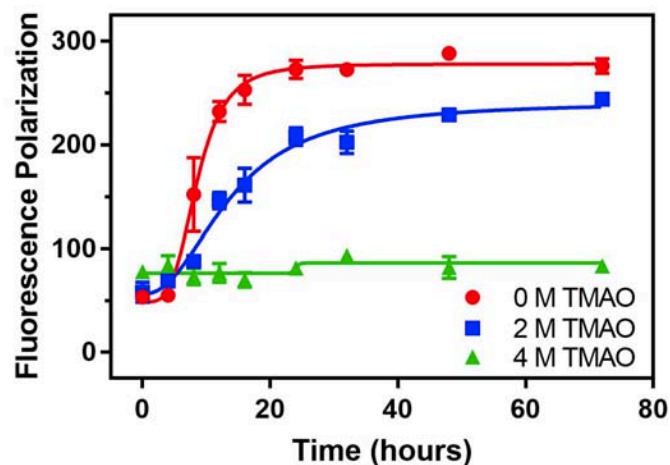


Figure S72: Aggregation kinetics of α S monitored by fluorescence polarization. Aggregation was performed with 100 μ M α S-Fam₁₃₆ in 20 mM Tris 100 mM NaCl pH 7.5. TMAO was added to each buffer and the pH was readjusted. Samples were agitated in an orbital shaker at 1500 rpm at 37 °C. At each timepoint, 10 μ L of sample was removed and added to 90 μ L of 20 mM Tris 100 mM NaCl pH 7.5. Fluorescence polarization measurements were taken in a 96-well black CoStar clear bottom plate on a Tecan F200 plate reader. Kinetics were fit to Eq. S22 in Prism with the detailed values in Table S8.

Table S8: Values from fits of fluorescence polarization aggregation kinetics.

	0 M TMAO	2 M TMAO	4 M TMAO
A	48.65	58.28	76.27
B	278.1	240.9	86.22
C	8.495	13.74	23.99
D	3.82	2.248	80.41
R ²	0.9806	0.9734	0.2733

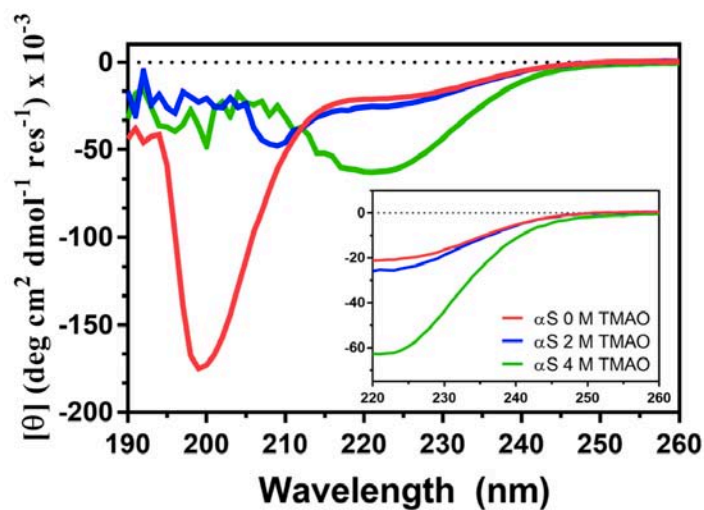


Figure S73: CD spectra of α S in varying concentrations of TMAO. Full plot and insert show the same data set, where the insert is adjusted to show difference in spectra above 220 nm. Measurements were taken on an Aviv model 410 circular dichroism spectrometer in 1 mm path length quartz cuvettes and were performed with 20 μ M wild-type α S in 20 mM Tris 100 mM NaCl pH 7.5. TMAO was added to each buffer and the pH was readjusted at 25 $^{\circ}$ C. Scans from 190-260 nm were performed with a 1 nm bandwidth, 1 nm step size and an averaging time of 10 seconds.

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