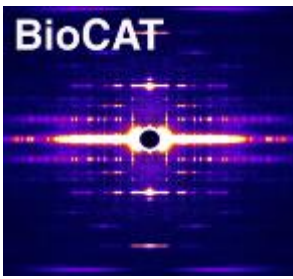


Time-Resolved SAXS

Srinivas Chakravarthy

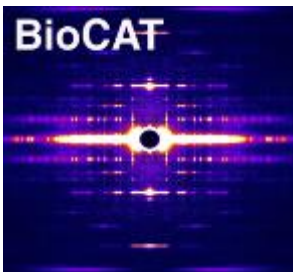
IIT, Center for Synchrotron Research & Instrumentation

Sector 18ID/BioCAT@APS

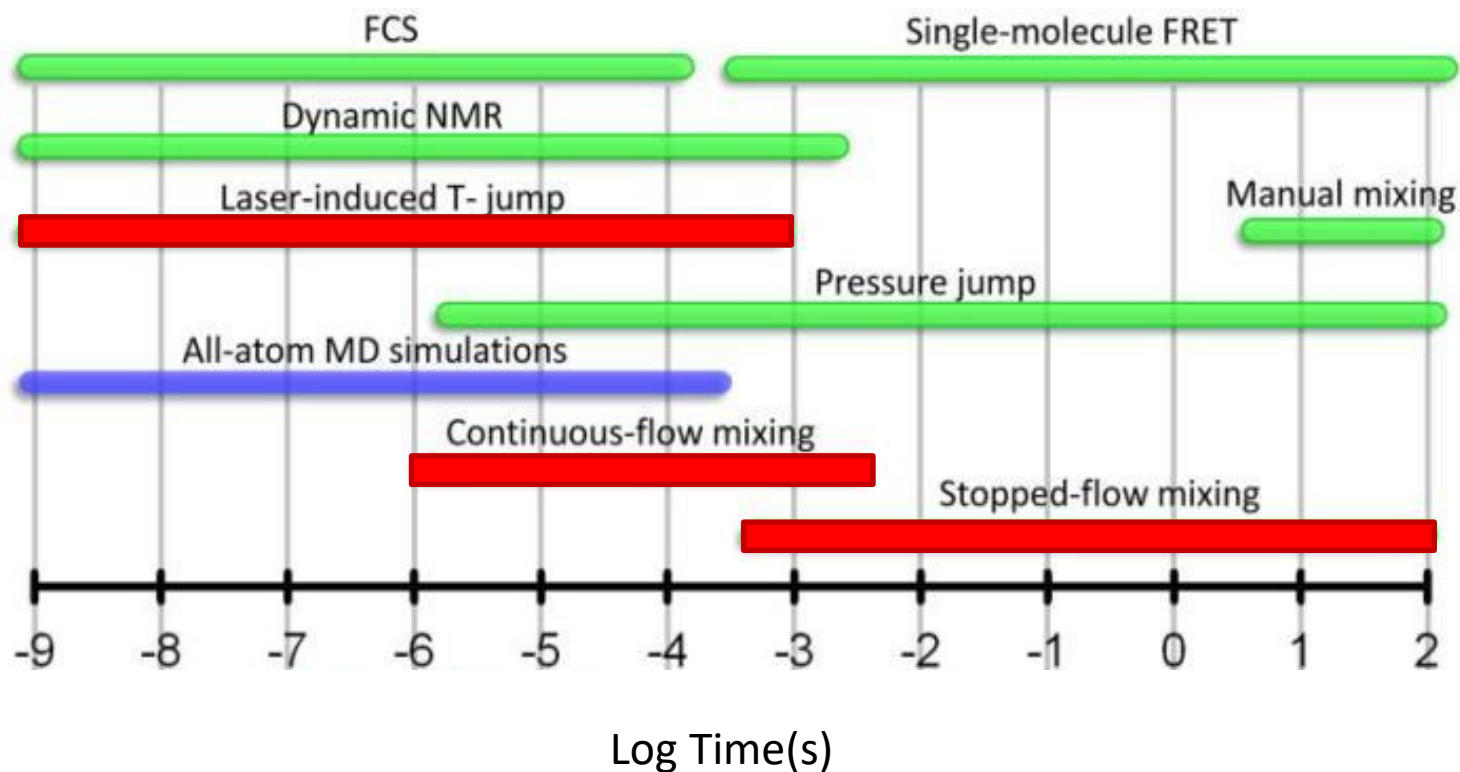


Mixer Types

- Stopped Flow SAXS
- Advanced microfluidic mixers with micro-beams for continuous flow, sub millisecond time resolution SAXS
- Kinetic SAXS with dramatically lower sample consumption



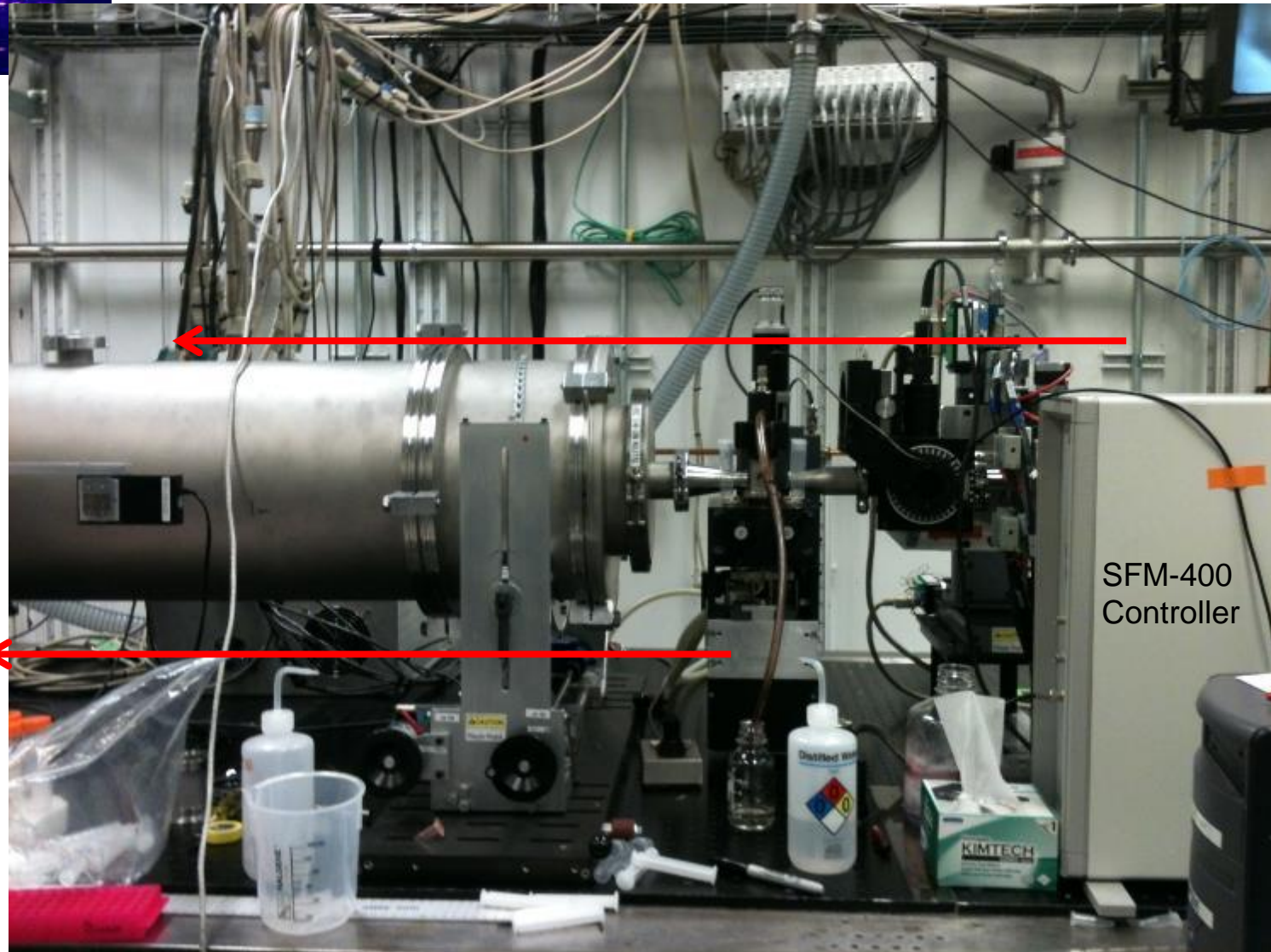
Time-Regimes with Different Techniques



Adapted from
Kathuria et al., 2011,
Biopolymers, 95(8)

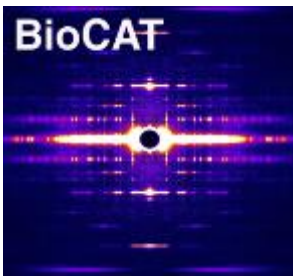
BioCAT

Stopped-Flow Setup at BioCAT



SFM-400
Controller

Stopped-
Flow Unit



Example Stopped-Flow Data

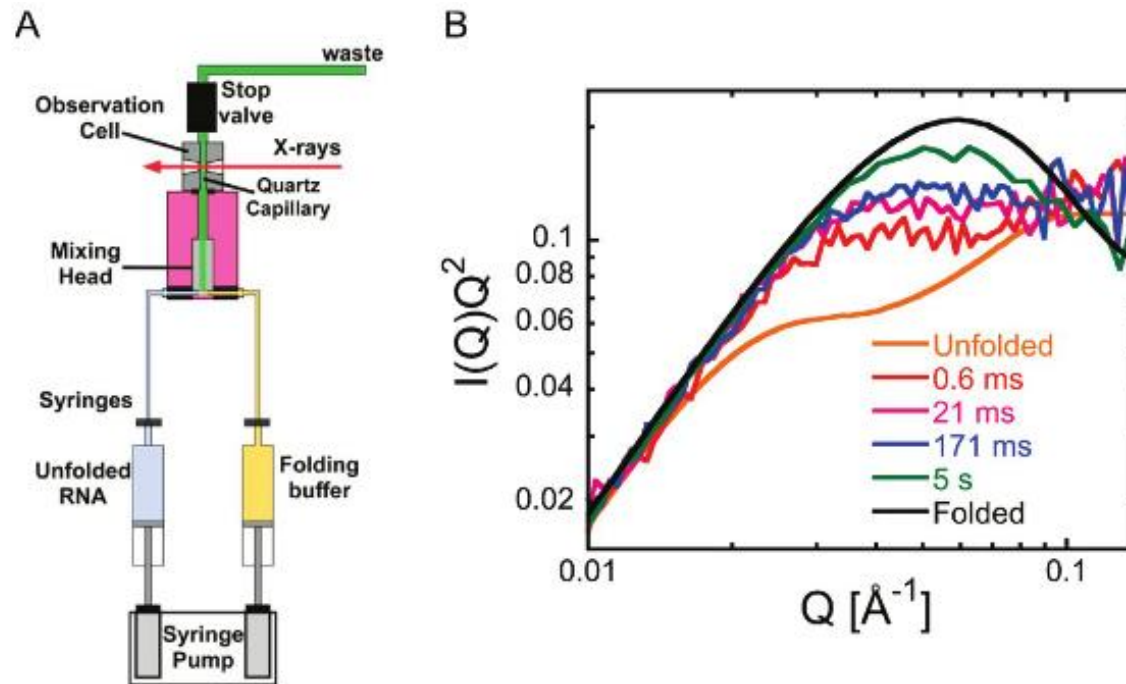
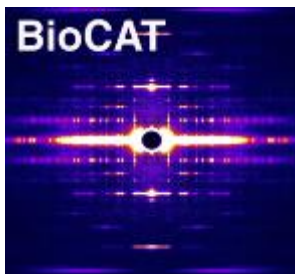


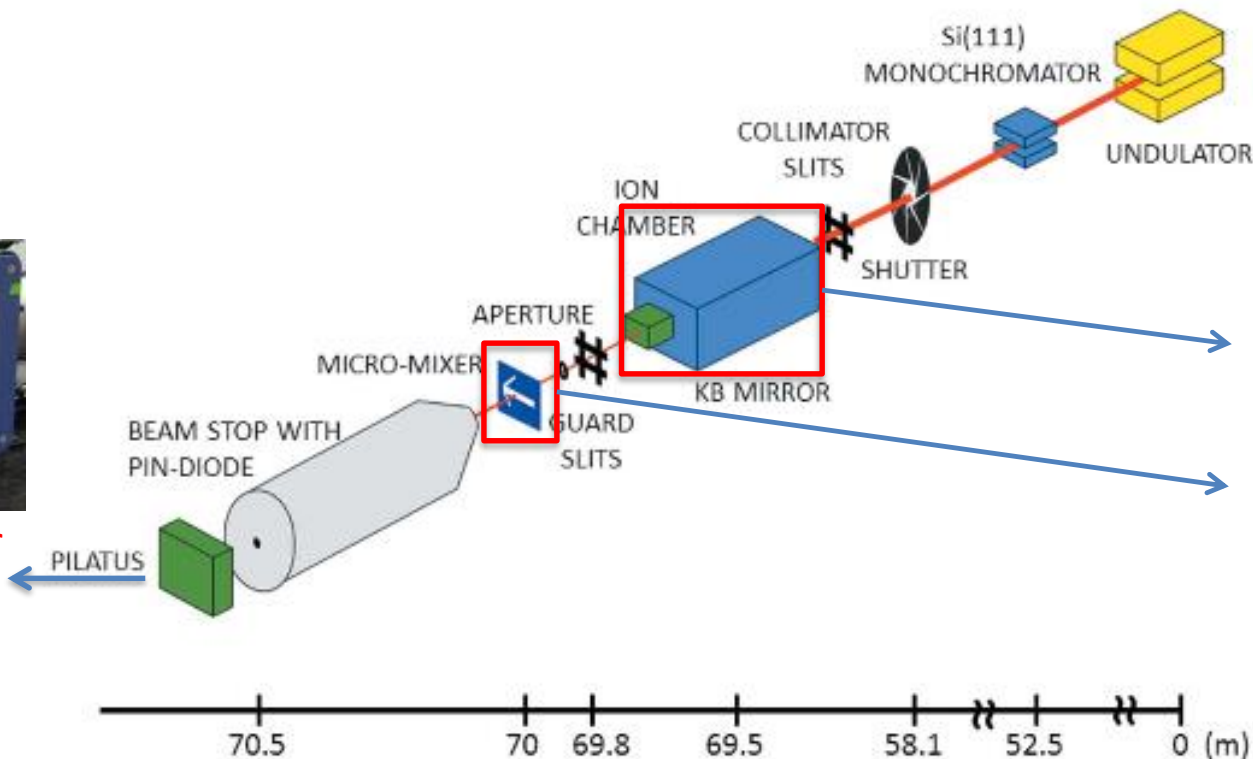
Figure 1. Time-resolved SAXS of *Azoarcus* ribozyme folding. (A) Schematic view of the stopped-flow mixer (SFM400). Syringes were loaded with unfolded RNA (1 mg/mL after mixing) in 20 mM Tris-HCl and folding buffer containing MgCl_2 . The dead time (~ 0.6 ms) was minimized by a high flow rate and short distance from the small-volume mixer to the observation point. (B) Kratky plots of real-time folding data in 1.5 mM MgCl_2 . Curve at 5 s (green) were in 5 mM MgCl_2 . For time-resolved measurements (≤ 200 ms), 15–20 identical 1 ms data sets were averaged. Scattering data up to 5 s were acquired for 50 ms and averaged over 4 shots. For unfolded RNA in 20 mM Tris-HCl (orange) and folded RNA in 5 mM MgCl_2 (black) at equilibrium, data were collected for 1.6 s (4 times).



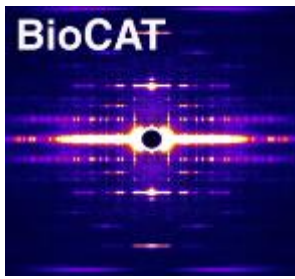
Components of the Continuous-Flow SAXS Setup



New Faster Detector

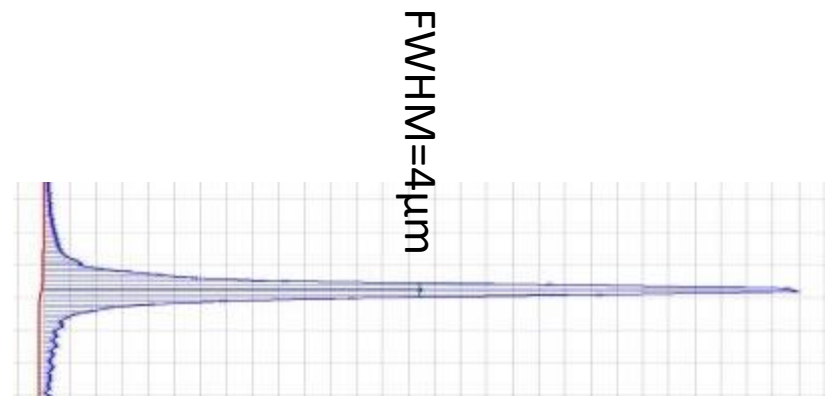
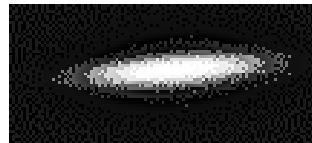
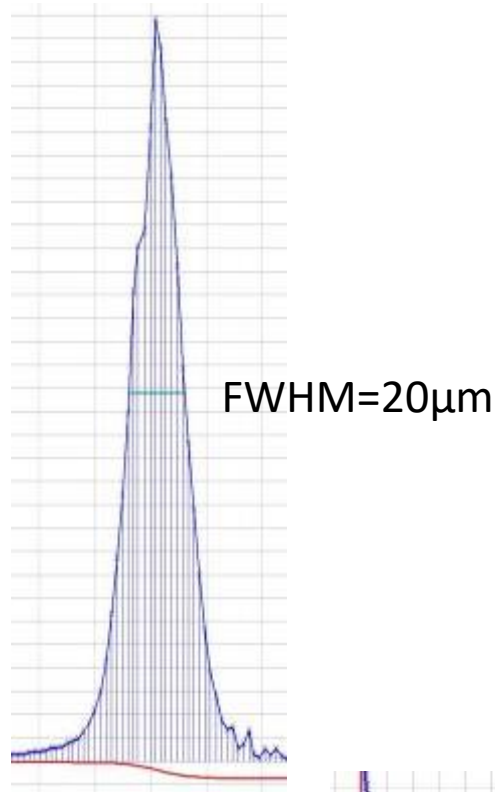


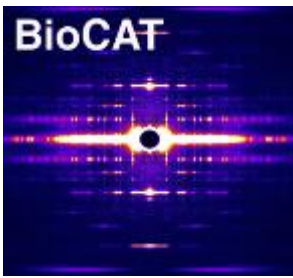
Major Foci Of Developmental Effort



Compound Refractive Lens generates smaller cleaner beams

- Focal Length = 1.88m
- Lower Divergence
- Higher flux density.



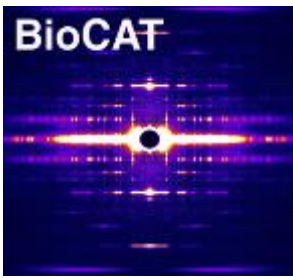


Time-Resolved SAXS Development

- **Aim 1: Advanced microfluidic mixers with micro-beams for continuous flow, sub millisecond time resolution SAXS**

- Improved optics (low divergence, cleaner profile, higher flux density)
- Higher sensitivity detector with faster readout
- More efficient mixers (mixing time and sample consumption)
- Improved fluid delivery systems.

We obtain structural information at the sub-100 μ s time-regime of a variety of macromolecular processes.

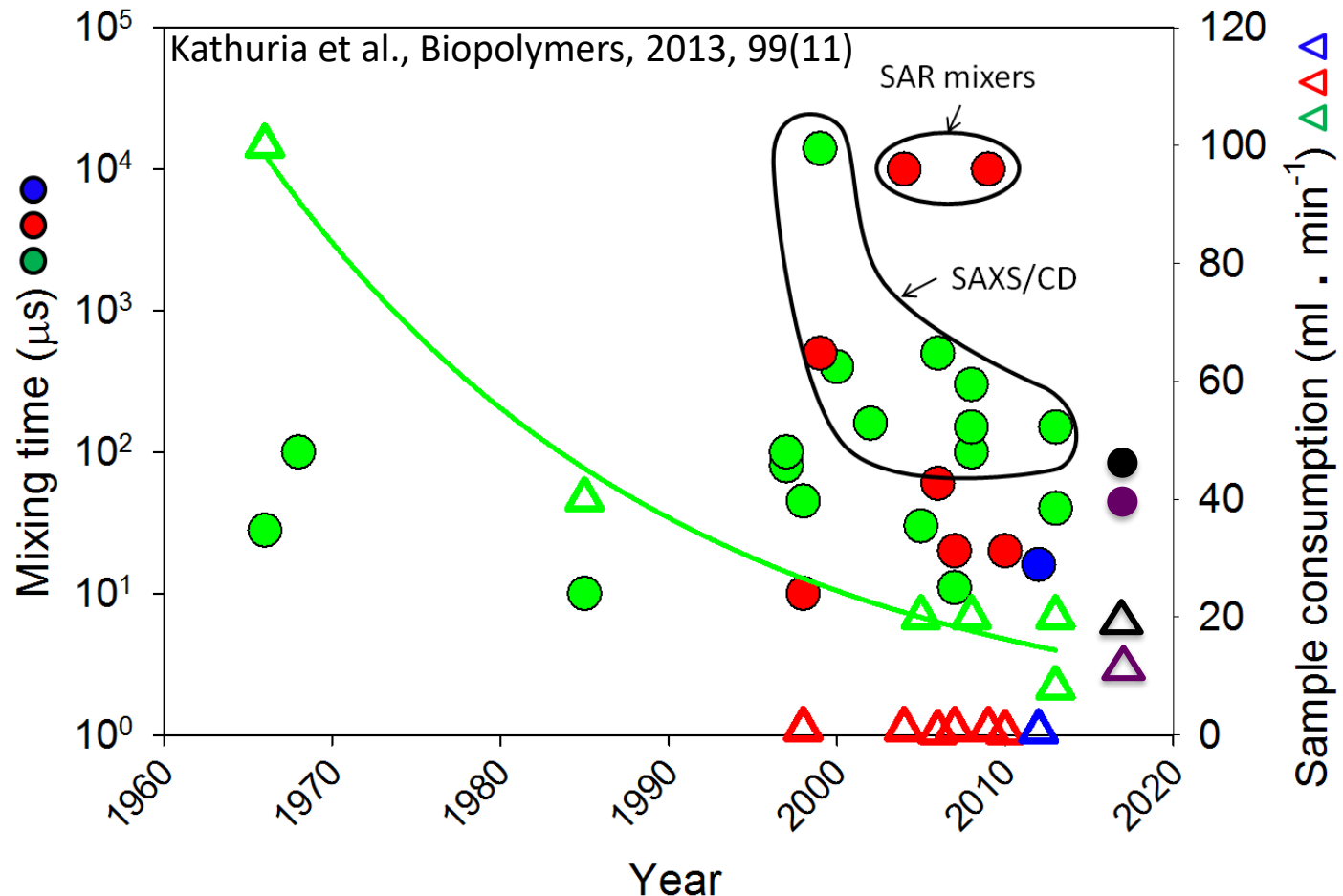


Mixer Evolution

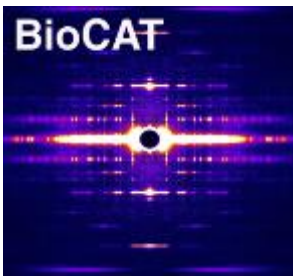
2013 Design



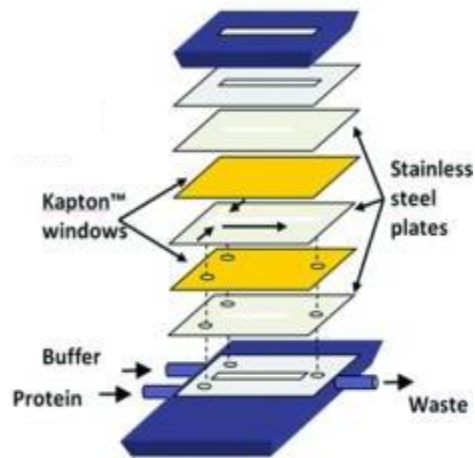
2014 Design



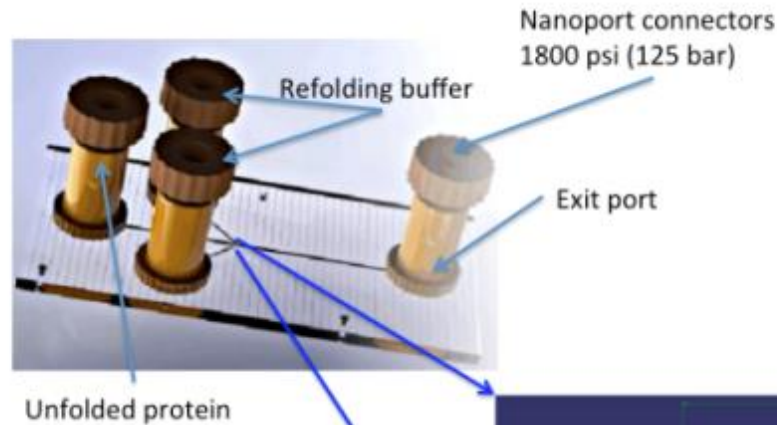
Mixing times of turbulent mixers (green filled circles), chaotic mixers (blue filled circles), and laminar mixers (red filled circles). Improvements in sample consumption (open triangles).



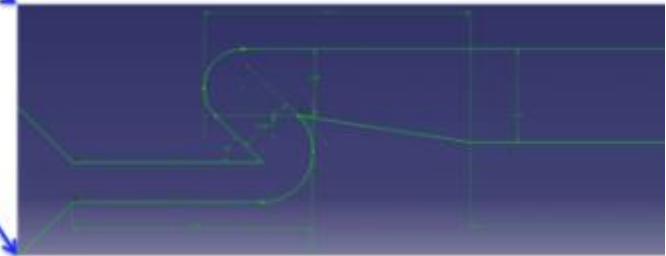
Mixer Evolution



2013 - Mixer

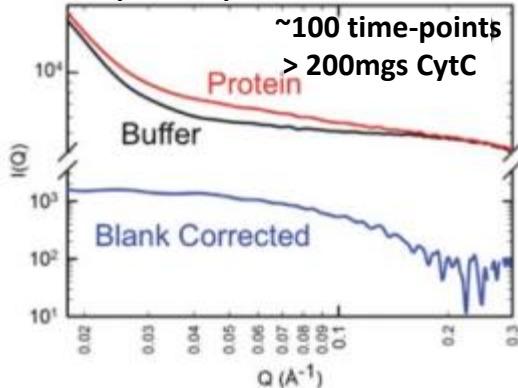


2015 - Mixer

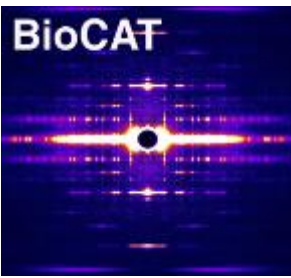


100 μ s time point :

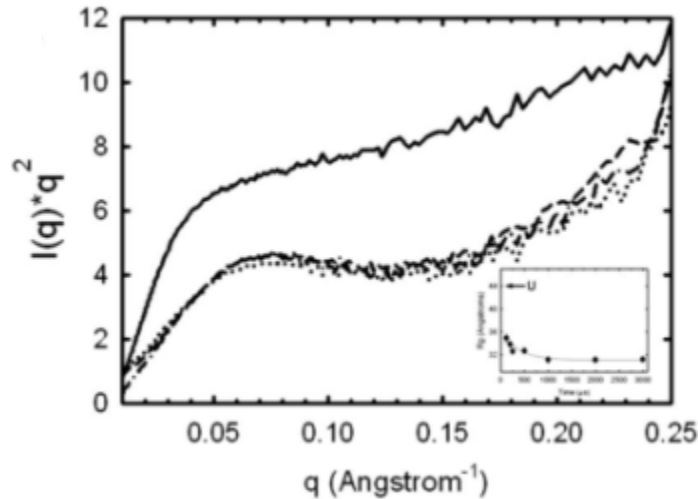
~100 time-points
> 200mgs CytC



Mixer design – Quartz chips manufactured by Translume using 2-photon etching and hydrofluoric acid. Y format 3-channel mixer, 0.25 mm depth, with S-bend – expanding from 30 μ m to 70 μ m over 0.5 mm. Three layers fused by heat. The top and bottom layers are thinned to ~ 50 μ m over the observation channel.

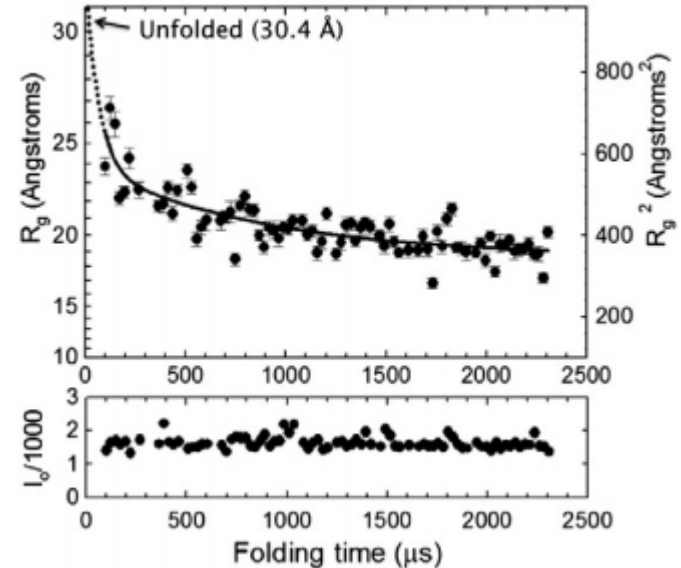


CF-SAXS Setup Evolution



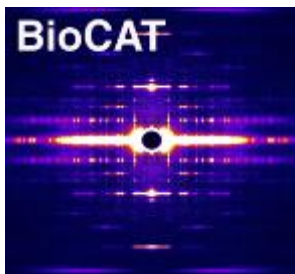
- ~ 150 μ mX50 μ m beam
- Bigger mixers
- 10-20 ml/min flow rates
- Took ~ 3gms of protein and multiple trips to obtain 12 time points
- Earliest time point = 150 μ s

[Wu et al., Proc Natl Acad Sci U S A. 2008 Sep 9;105\(36\):13367-72.](#)

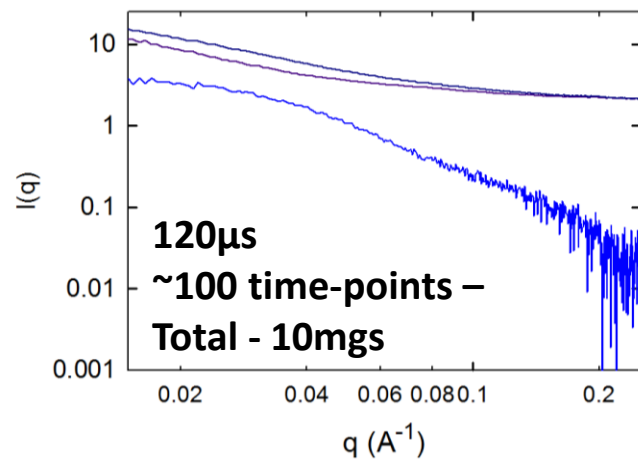


- 20 μ mX5 μ m beam using KB mirror
- Smaller more efficient mixers
- 4-10 ml/min flow rates
- Took ~ 2mgs of protein / time point
- ~ 100 time points in a single trip.
- Earliest time point = 80 μ s

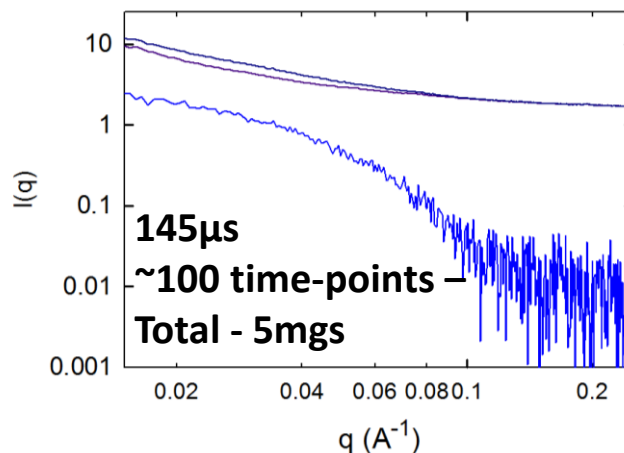
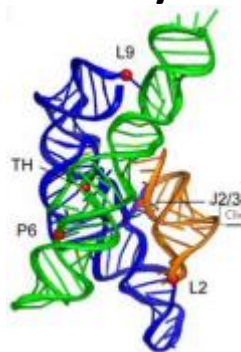
[Kathuria et al., J Mol Biol. 2014 May 1;426\(9\):1980-94.](#)



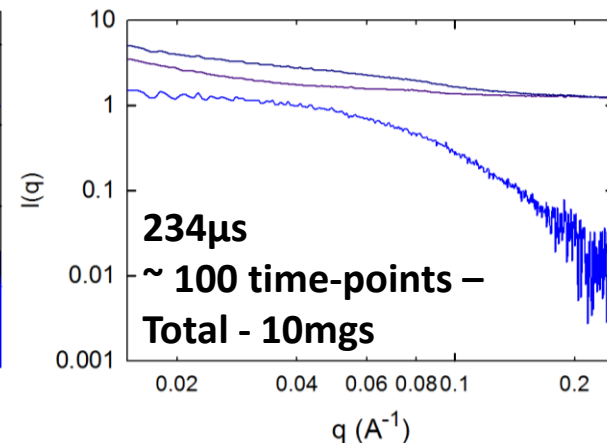
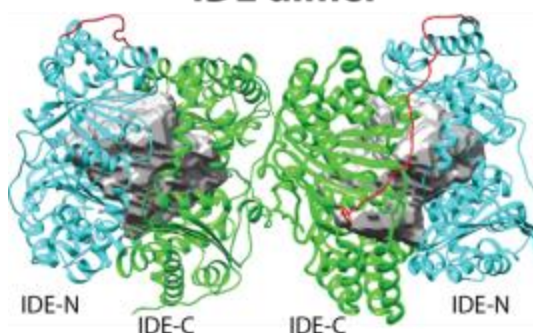
Feasibility Studies with New CF-SAXS Setup



Ribozyme

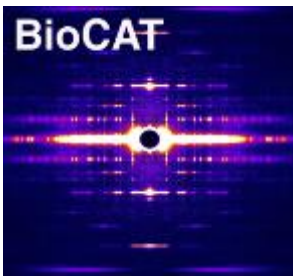


IDE dimer

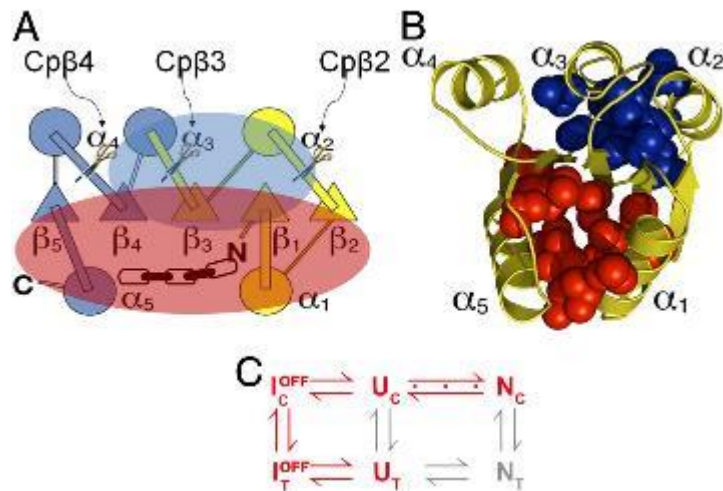


Calmodulin

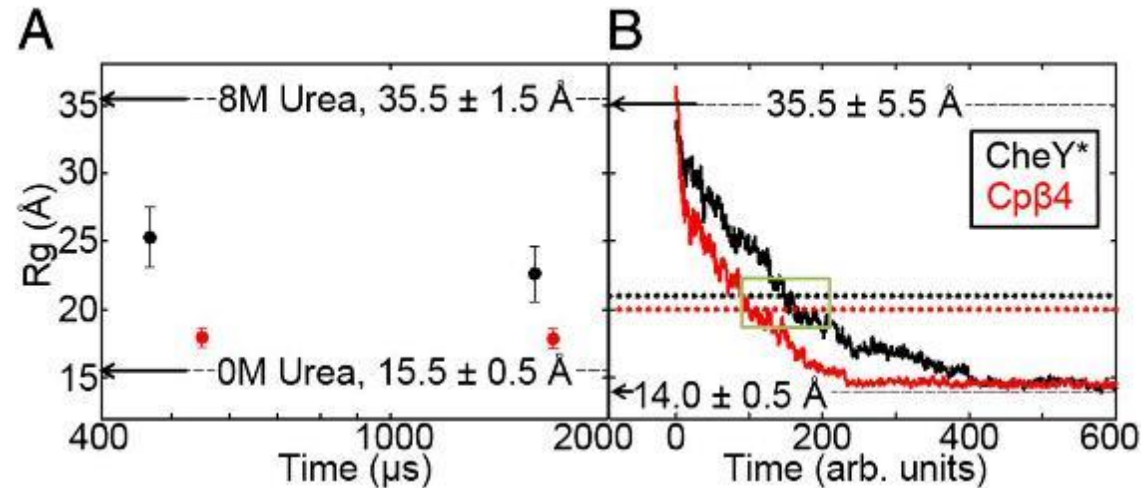




CF-SAXS Applications – Case 1

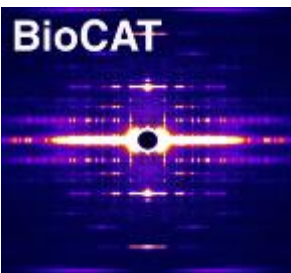


(A) Topology diagram of CheY. The N-terminal folding subdomain is highlighted in yellow, and the C-terminal folding subdomain is highlighted in blue. (B) Clusters of ILV residues are superimposed on the crystal structure of CheY (Protein Data Bank ID code: 3CHY). (C) The folding mechanism of WT CheY. The major pathway is highlighted in red. The $U_C \rightarrow N_C$ step, involving the on-pathway intermediate, is designated by the triple dots.

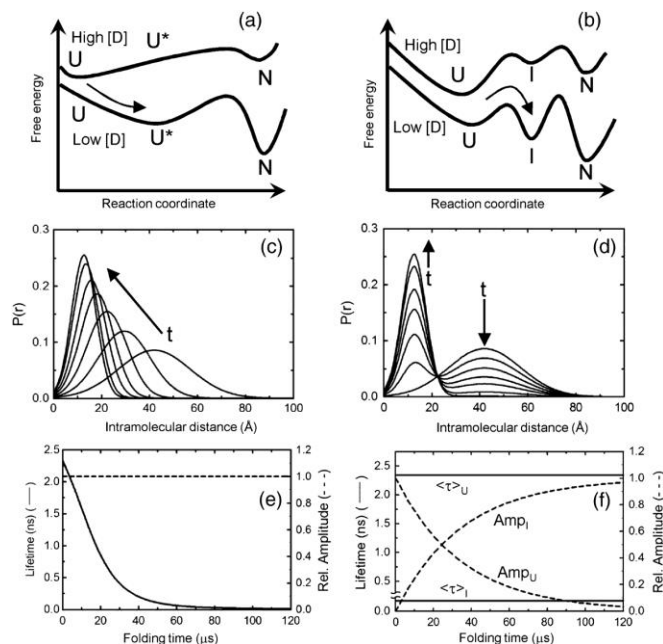


Dimensional analysis of CheY* and Cpβ4 during folding by SAXS and simulations. The radius of gyration for CheY* (black) and Cpβ4 (red) from CF-SAXS (A) and the average R_g from Gō-model simulations (CheY*: $n = 46$; Cpβ4: $n = 32$) in which the intermediate was observed (B) as a function of folding time.

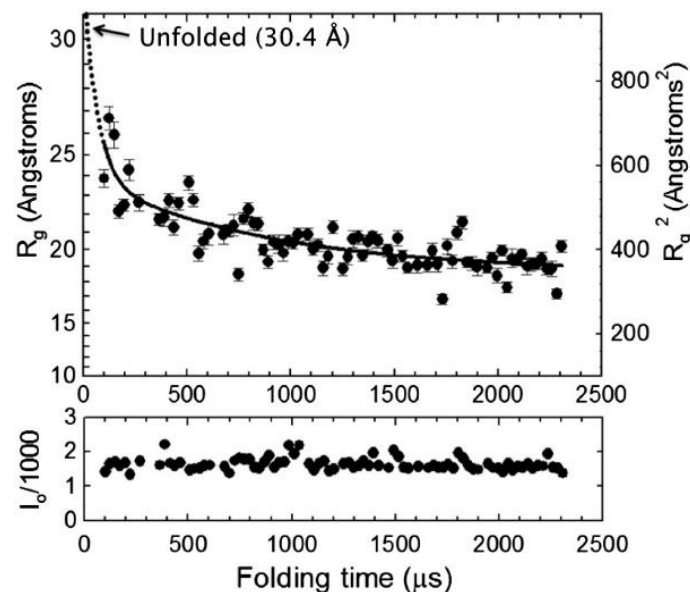
Modulation of frustration in folding by sequence permutation Nobrega et al., PNAS July 22, 2014 111 (29) 10562-10567;



CF-SAXS Applications – Case 2

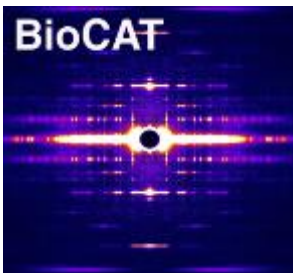


Schematic of continuous and two-state barrier-limited collapse scenarios. (a) continuous-collapse model (b) the barrier-limited collapse model, The collapse transition in (a) and (b) is indicated by the arrow. (c) Expected Trp-heme distance distributions corresponding to the continuous-collapse Model. (d) In the barrier-limited collapse model, there are two distance distributions, corresponding to U and I.

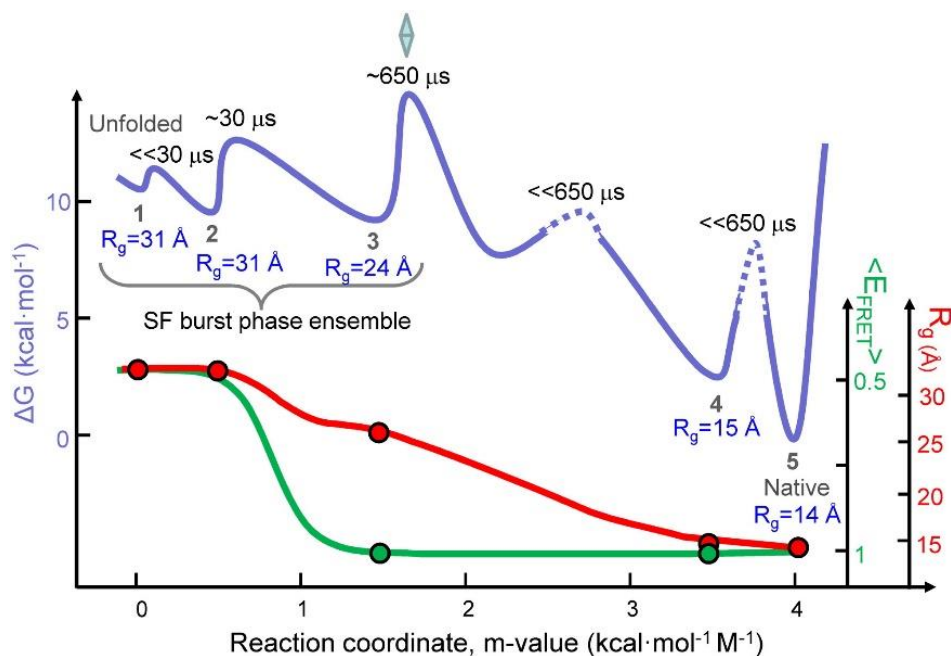


SAXS of cytochrome c during sub-millisecond refolding. (a) The radius of gyration, R_g (circles), during refolding from 4.5 M GdnHCl to 0.45 M GdnHCl at pH 7.0 in the presence of 0.2 M Imidazole. (b) The zero-angle scattering intensity obtained from the Guinier analysis in (a) shows that cytochrome c is monomeric throughout folding.

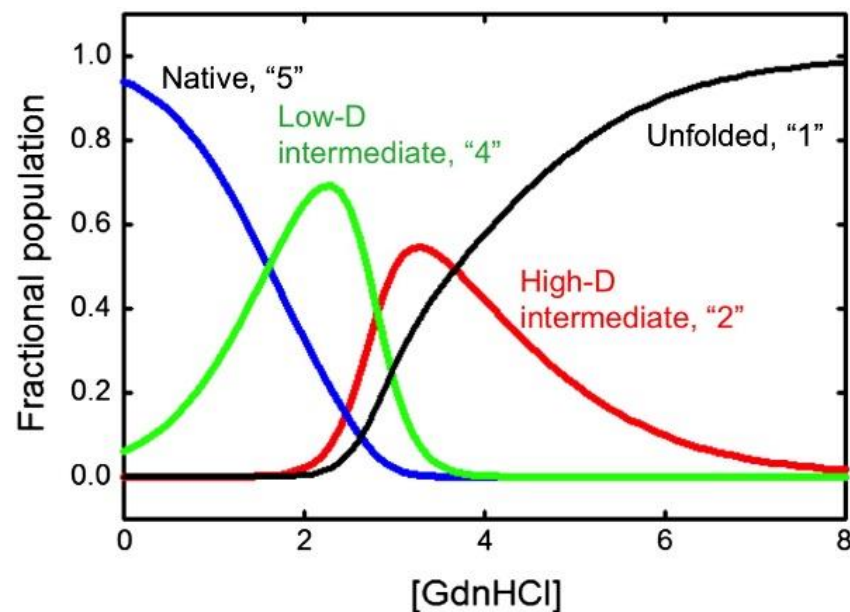
Microsecond Barrier-Limited Chain Collapse Observed by Time-Resolved FRET and SAXS. Kathuria et al., [JMB](#). 2014 May 1;426(9):1980-94.



CF-SAXS Applications – Case 2

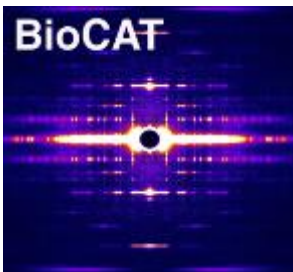


Kinetic model for cytochrome c folding.

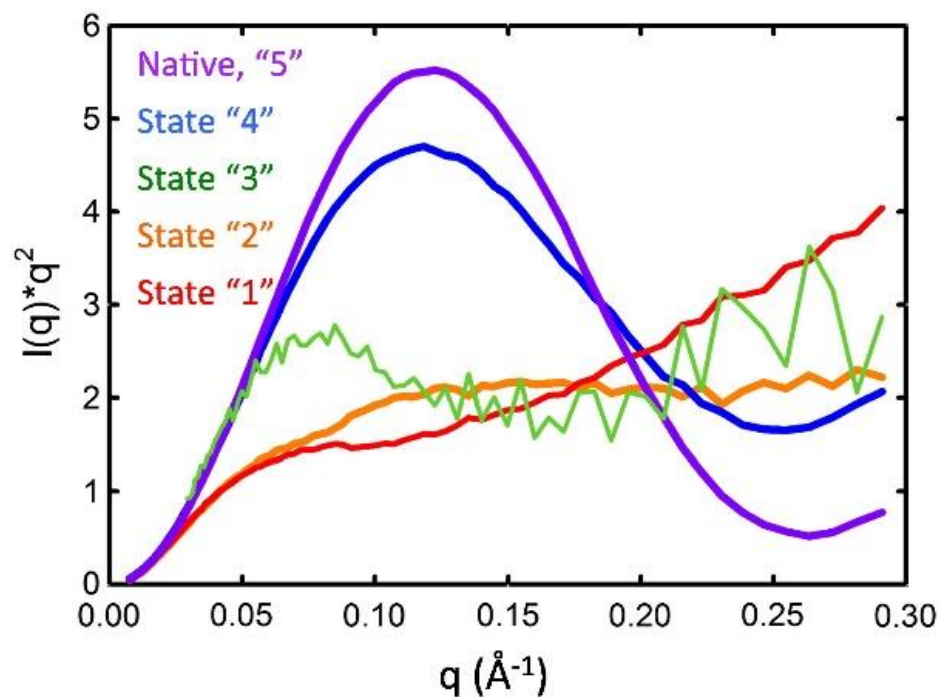


Species populations predicted from fits of equilibrium unfolding experiments

Microsecond Barrier-Limited Chain Collapse Observed by Time-Resolved FRET and SAXS. Kathuria et al., [JMB](#). 2014 May 1;426(9):1980-94.

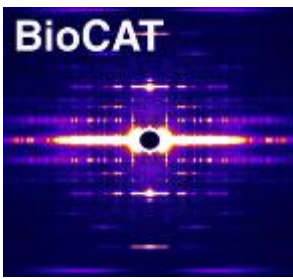


CF-SAXS Applications – Case 2

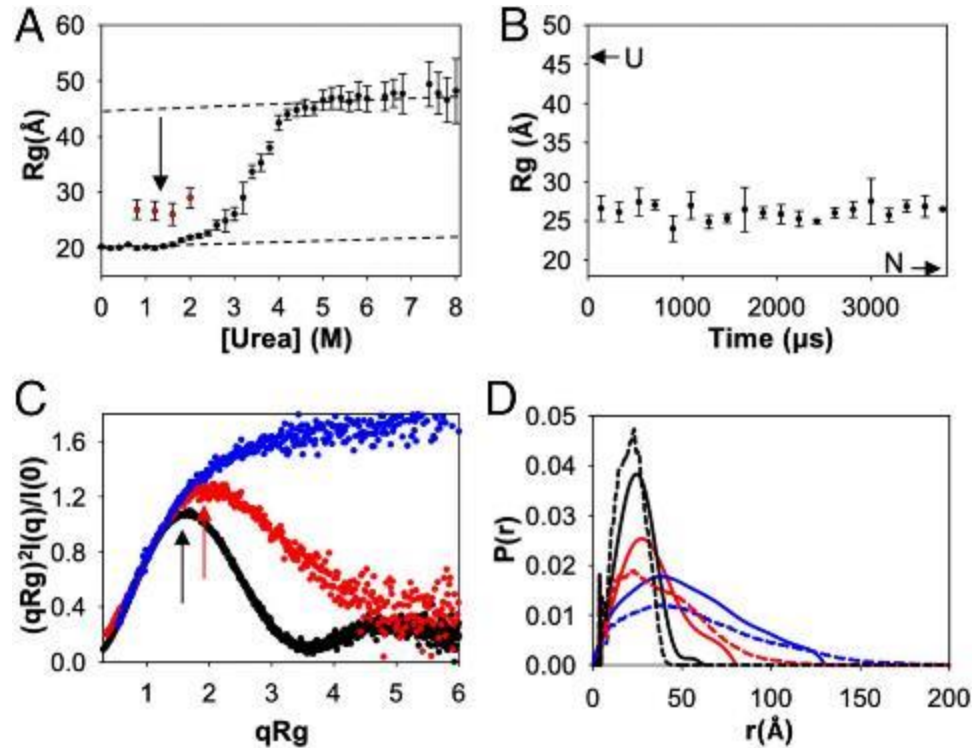


Kratky spectra of kinetic and equilibrium species.

Microsecond Barrier-Limited Chain Collapse Observed by Time-Resolved FRET and SAXS. Kathuria et al., [JMB](#). 2014 May 1;426(9):1980-94.

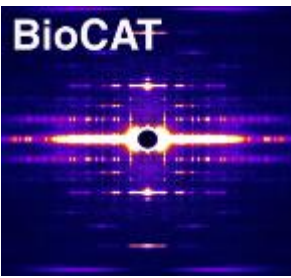


CF-SAXS Applications – Case 3

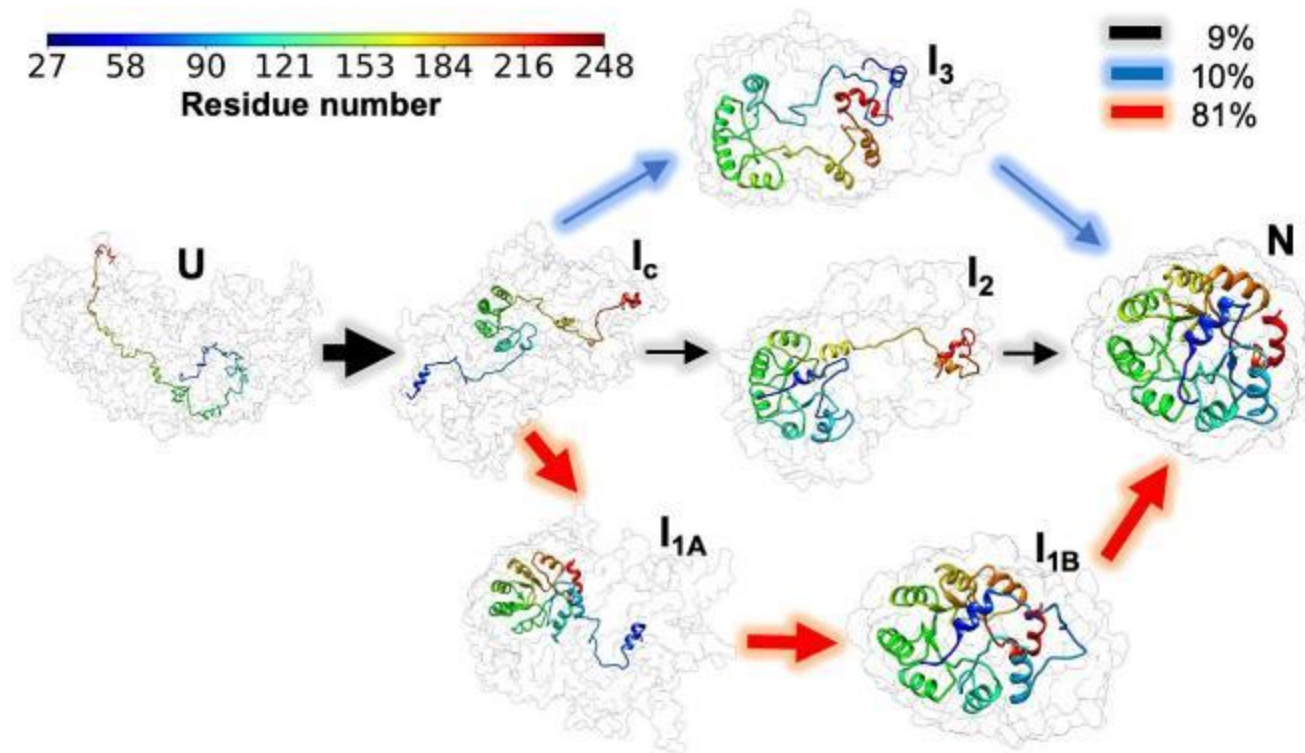


(A) R_g as a function of urea concentration for the unfolding of SsIGPS (black circles). The estimated R_g after 150 μ s of refolding at several final urea concentrations in the native baseline region (red circles). (B) R_g as a function of folding time at 0.8 M urea. (C) Dimensionless Kratky plots of the unfolded (blue), I_{BP} intermediate (red), and native state (black). (D) The $P(r)$ of the unfolded (blue), I_{BP} intermediate (red), and native states (black). The dashed lines represent the $P(r)$ for these states calculated from the simulations.

Frustration and folding of a TIM barrel protein Halloran et al., PNAS August 13, 2019 116 (33) 16378-16383; first published July 25, 2019

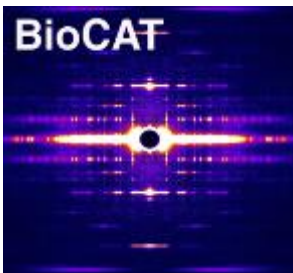


CF-SAXS Applications – Case 3



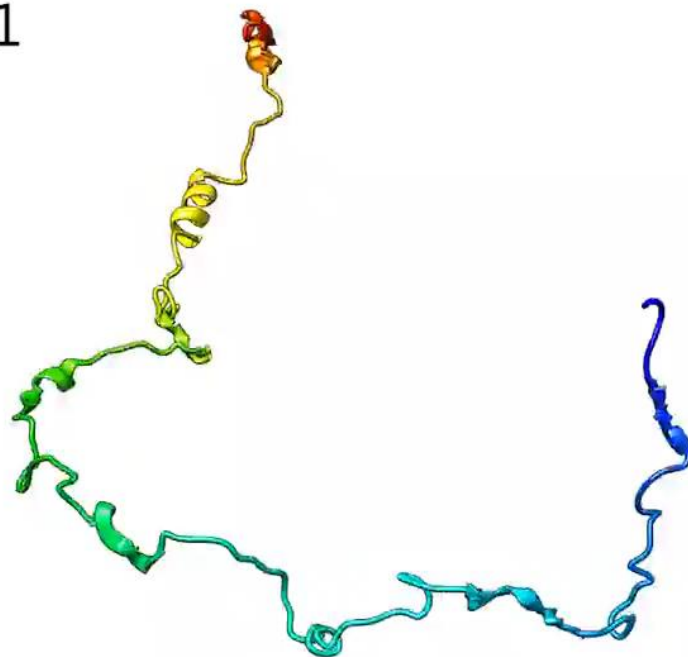
Multiple folding pathways discovered by simulations, the upper right legend shows the transition probabilities from I_c to I_{1A} , I_2 , and I_3 . The gray contours show the overlay of ~50 protein conformations, sampled from the corresponding states.

Frustration and folding of a TIM barrel protein Halloran et al., PNAS August 13, 2019 116 (33) 16378-16383; first published July 25, 2019

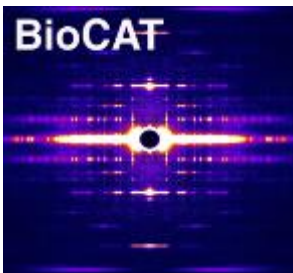


CF-SAXS Applications – Case 3

I_1

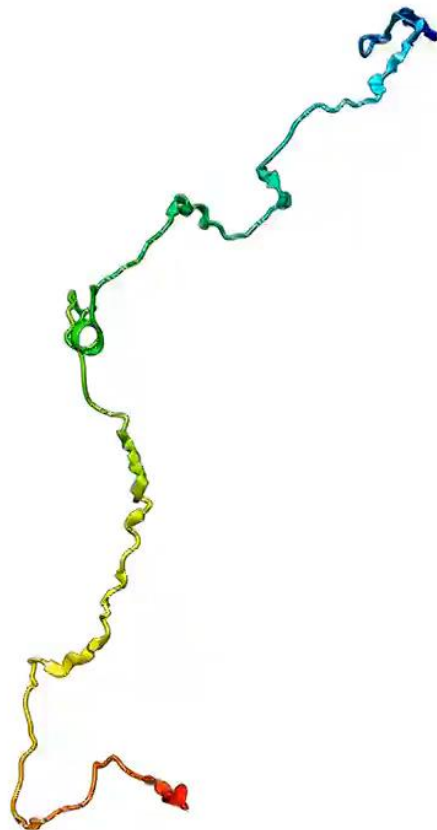


Frustration and folding of a TIM barrel protein Halloran et al., PNAS August 13, 2019 116 (33) 16378-16383; first published July 25, 2019

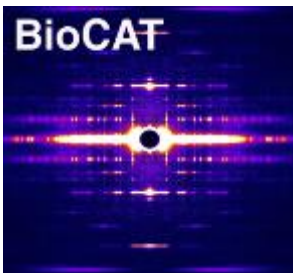


CF-SAXS Applications – Case 3

I_2

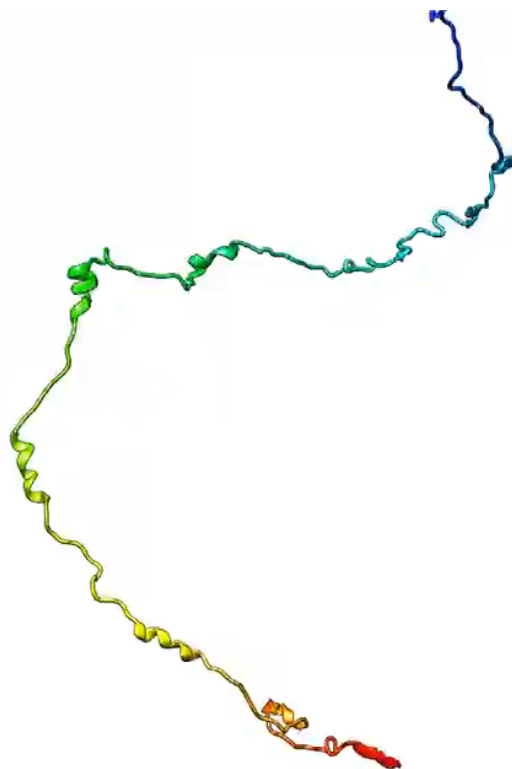


Frustration and folding of a TIM barrel protein Halloran et al., PNAS August 13, 2019 116 (33) 16378-16383; first published July 25, 2019



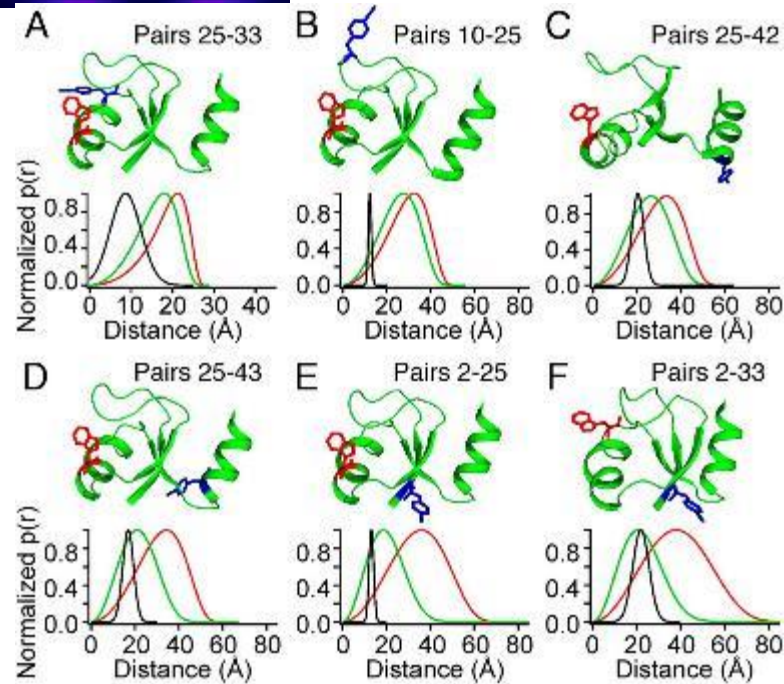
CF-SAXS Applications – Case 3

I_3

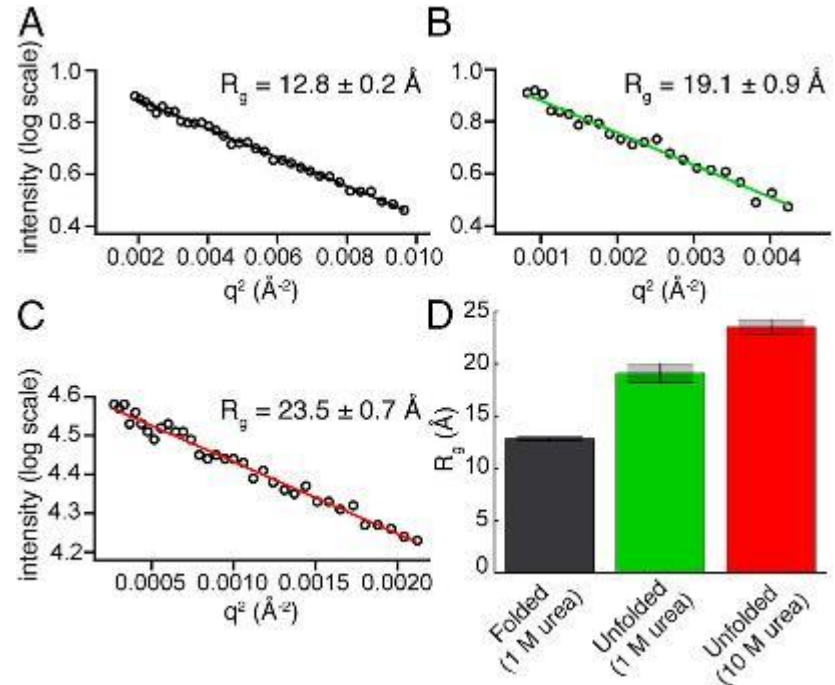


Frustration and folding of a TIM barrel protein Halloran et al., PNAS August 13, 2019 116 (33) 16378-16383; first published July 25, 2019

CF-SAXS Applications – Case 4

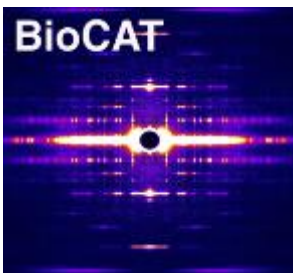


(A–F) FRET provides evidence for compaction. Ribbon diagrams illustrating the location of the FRET pairs are shown together with the distance distributions. Red, unfolded state in 10 M urea; green, unfolded state in 1 M urea; black, folded state in 1 M urea.

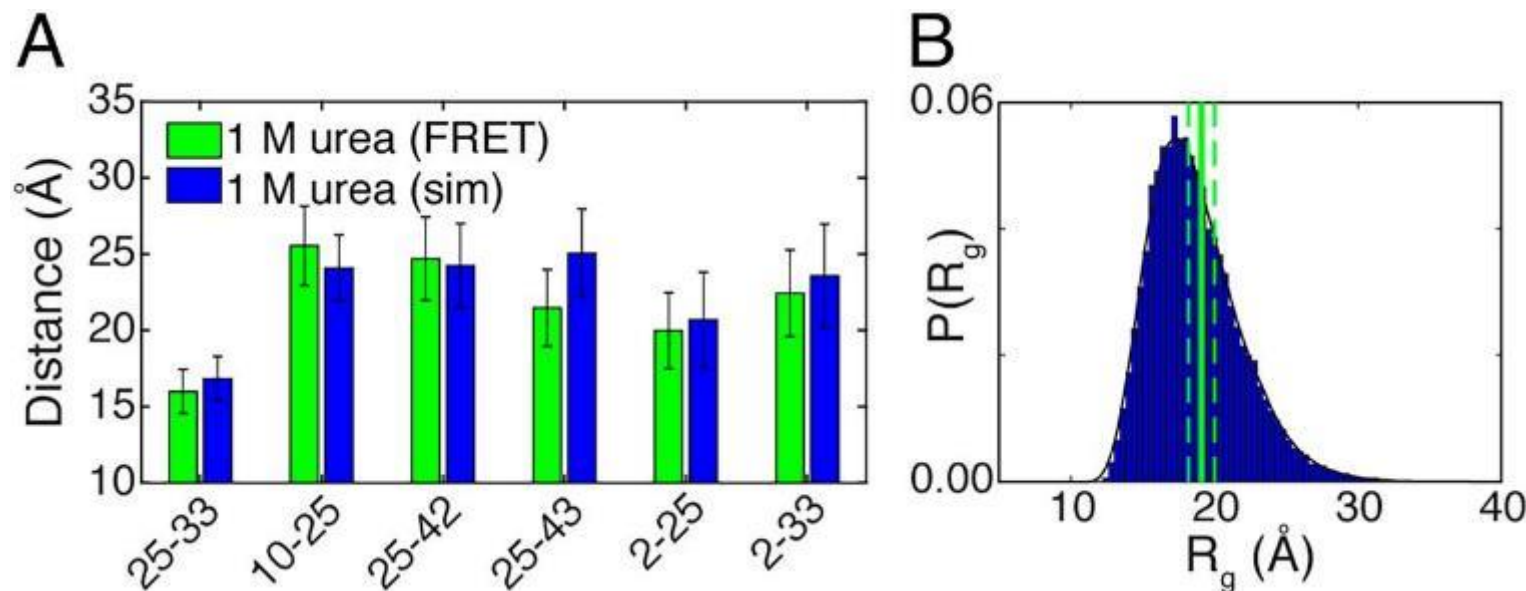


Guinier analysis of SAXS data. (A) Continuous-flow data for the native state in 1 M urea. (B) the unfolded state in 1 M urea. (C) Equilibrium data for the unfolded state in 10 M urea. (D) Comparison of average radii of gyration across the folded and unfolded states. Error bars are those calculated from the Guinier fits.

Peran et al., Unfolded states under folding conditions accommodate sequence-specific conformational preferences with random coil-like dimensions. *Proc Natl Acad Sci U S A.* 2019 Jun 18;116(25):12301-12310.

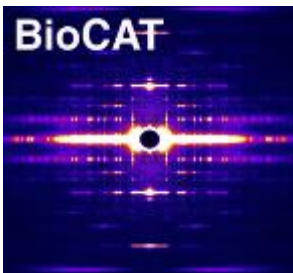


CF-SAXS Applications – Case 4



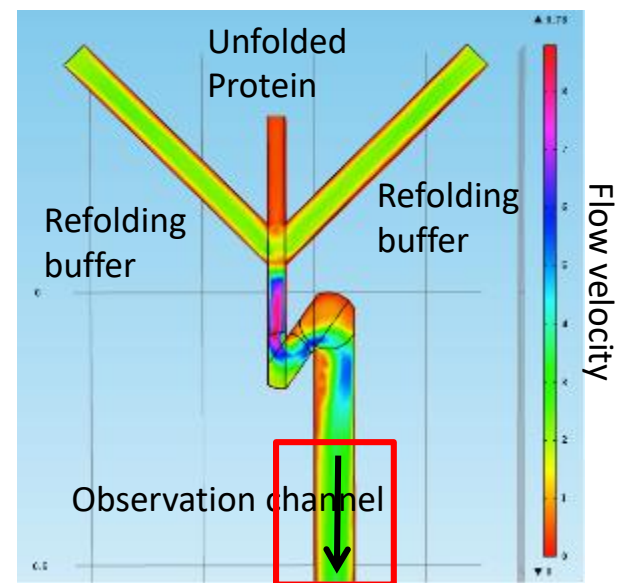
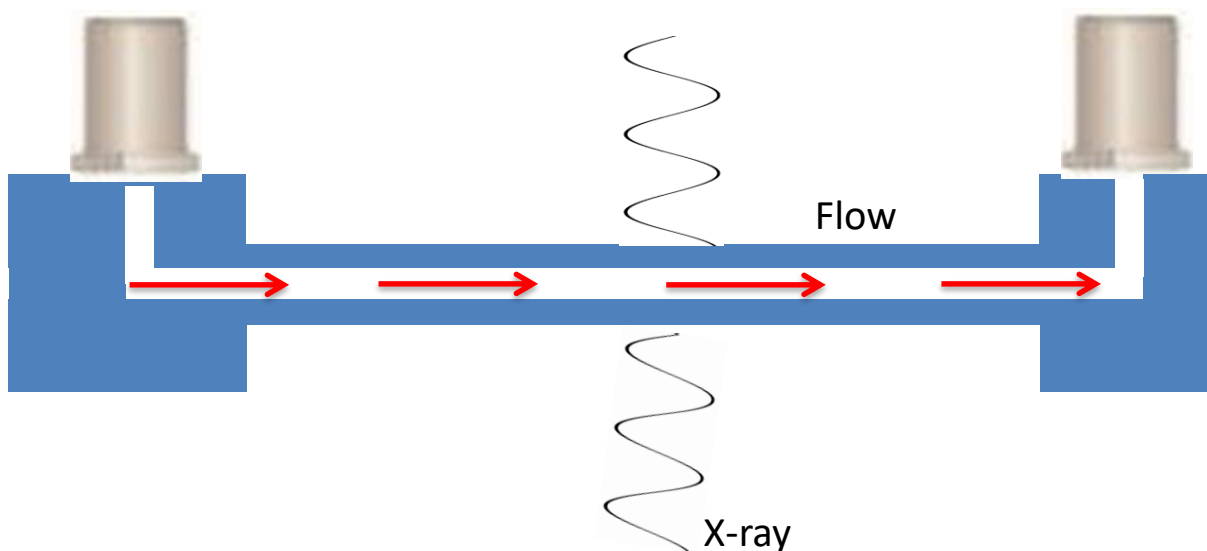
Comparison between experimental results from the 1-M urea unfolded state and the 375-K reweighted ensemble (unfolded-state ensemble in 1 M urea). (A) Intrachain distances obtained from FRET experiments (green) compared with the C_β - C_β distance extracted from the unfolded ensemble (blue). The error bars reflect the SDs of the underlying distribution associated with each measurement. (B) Radius of gyration obtained from SAXS with error bars compared with the distribution of radii of gyration obtained from simulation. The simulation data in both A and B were extracted from the same ensemble, demonstrating that FRET, SAXS, and simulation are mutually compatible.

Peran et al., Unfolded states under folding conditions accommodate sequence-specific conformational preferences with random coil-like dimensions. [Proc Natl Acad Sci U S A](#). 2019 Jun 18;116(25):12301-12310.

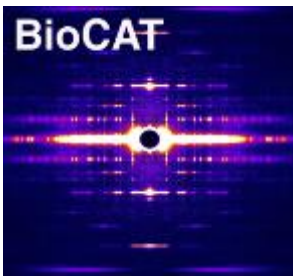


Construction of quartz mixer for continuous-flow SAXS

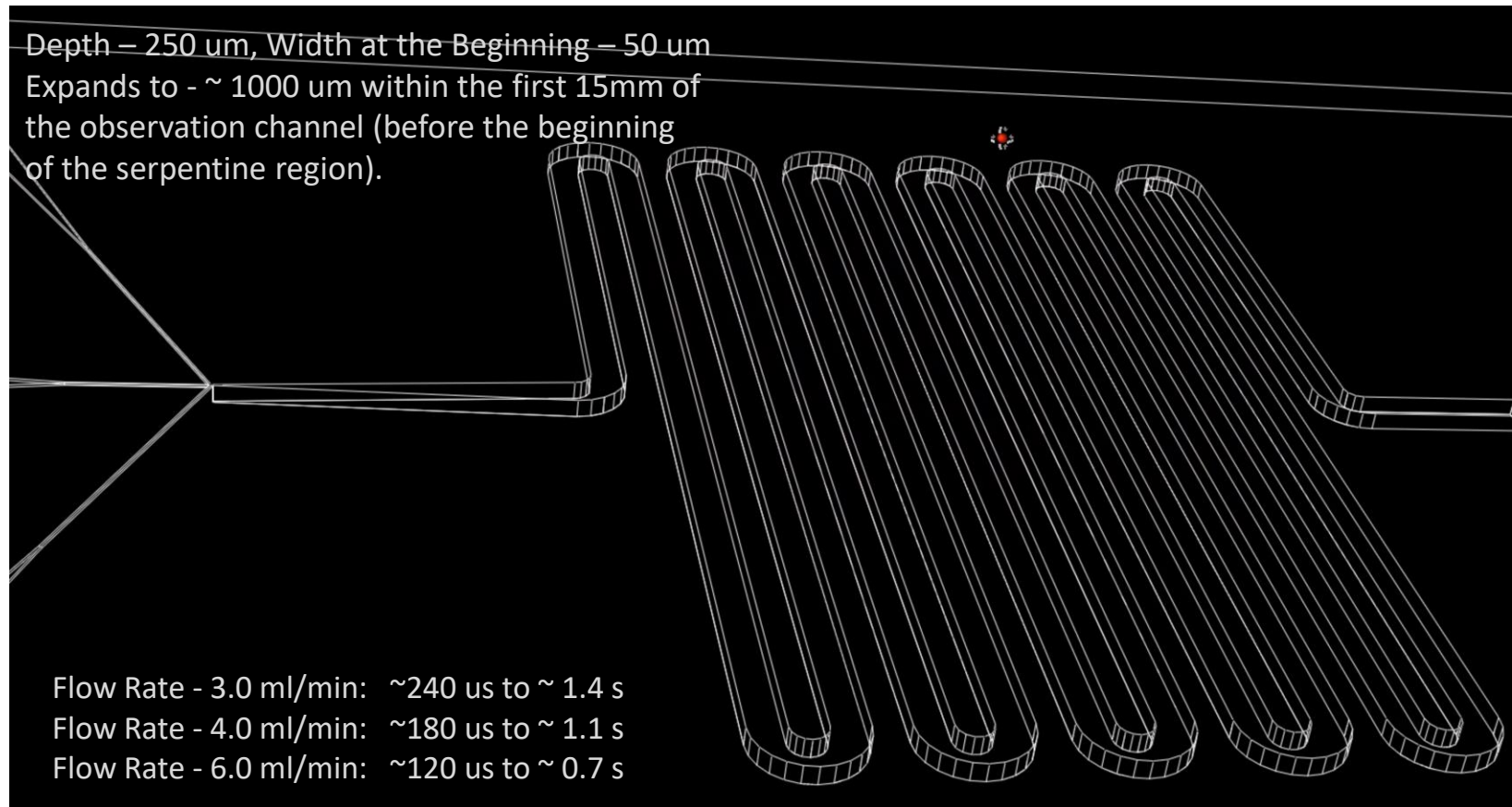
- Minimize x-ray absorption by quartz
- Windows thinned to 50 μm in observation region
- Standard 1.5 mm thickness otherwise
- Guided by CFD simulation



We can now lower flow rates to 3-5 mL/min while maintaining dead times of $\sim 30\text{-}50\mu\text{s}$ for fluorescent experiments and $\sim 150\mu\text{s}$ for SAXS experiments

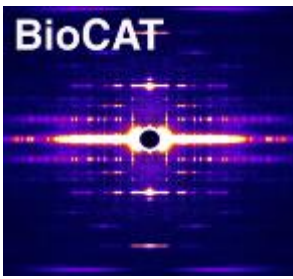


Serpentine design can be extended to achieve wider dynamic range



Accurate synchronization between scan motors, camera and beam monitors critical

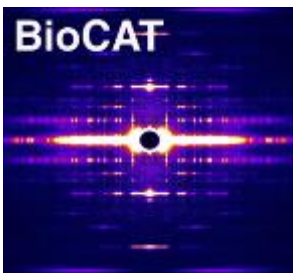
Sagar Kathuria



Low Sample Consumption Kinetic SAXS

- **Aim 2: Kinetic SAXS with 5-fold lower sample consumption**
 - Slower time regimes (millisecond – seconds) need alternative mixers (laminar flow and stopped flow).
 - Required Enhancements - sample consumption and signal to noise ratios.

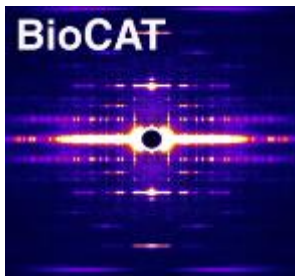
In Combination with the CF-SAXS program we can cast a wide net at time-regimes of important biological processes.



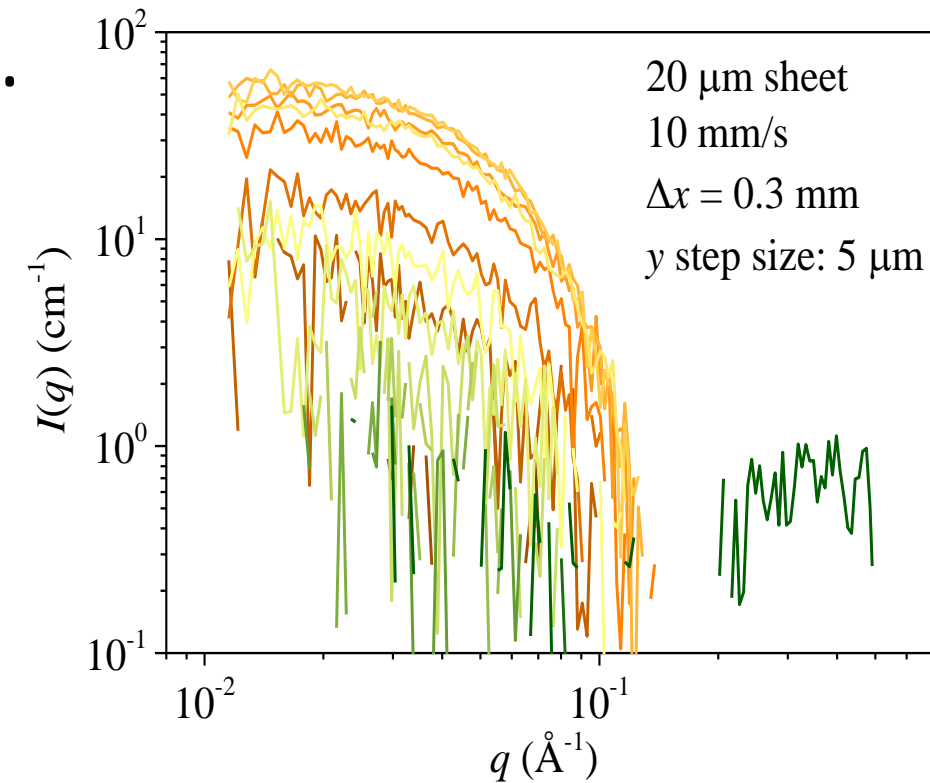
Laminar Flow SAXS Mixer Development



- Low sample volume laminar flow mixer
- Collaboration with Lise Arleth Univ. of Copenhagen
- The exposure channel is 250 μm wide; while the focused jet of protein is 20 μm wide with a typical flow velocity of 10 mm/s. The protein consumption is about 7 $\mu\text{L}/\text{min}$.
- Accessible time range 20-200 ms.
- First experiments performed in 2014-3 SAXS run.



Feasibility Study with the Laminar Flow SAXS Setup

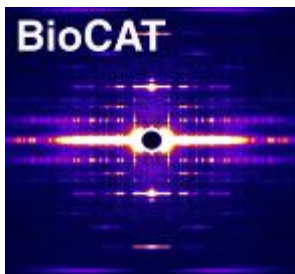


Flow velocities up to 20 mm/s were tested.
Shown here - flow rate of 10mm/s.

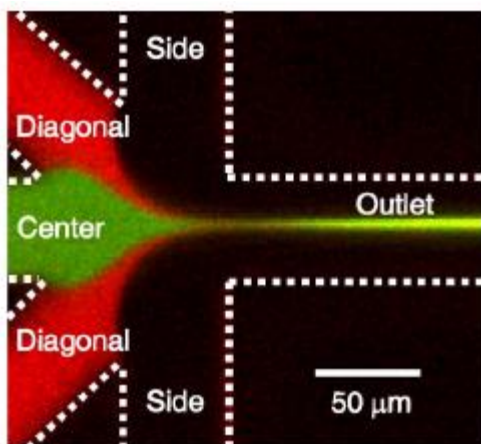
Beam focused using a CRL along with
white beam slits to 11 μm (h) X 6 μm (v).

Protein sheets of 20, 10 and 5 μm –
successfully created. Shown here – 20 μm sheet.

***Consumption of protein only 1.8, 3.6 and
7.2 $\mu\text{L}/\text{min}$ (for the three sheet thicknesses),
respectively.***



Using Laminar flow SAXS for studying self assembly of nanoparticle drug delivery systems.



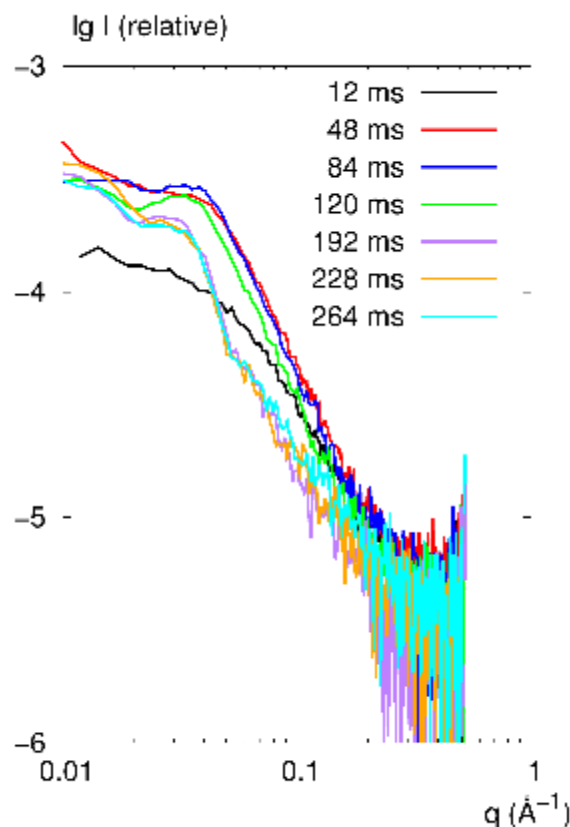
Advantages of Laminar flow SAXS:

- Very low sample consumption

Disadvantages compared to chaotic flow mixers:

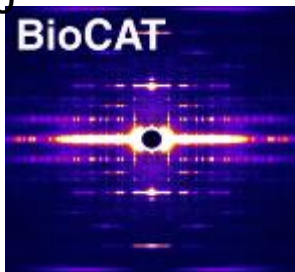
- Much worse signal to noise

- Cannot access as early first time points

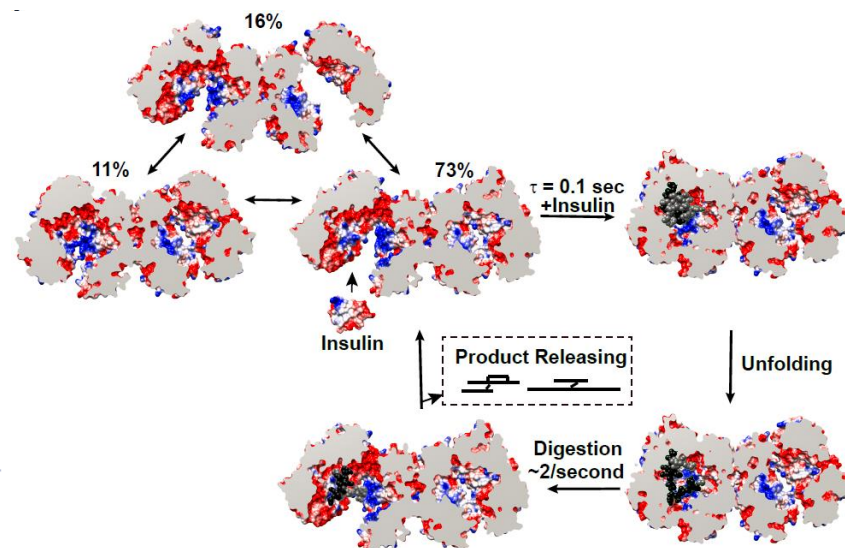
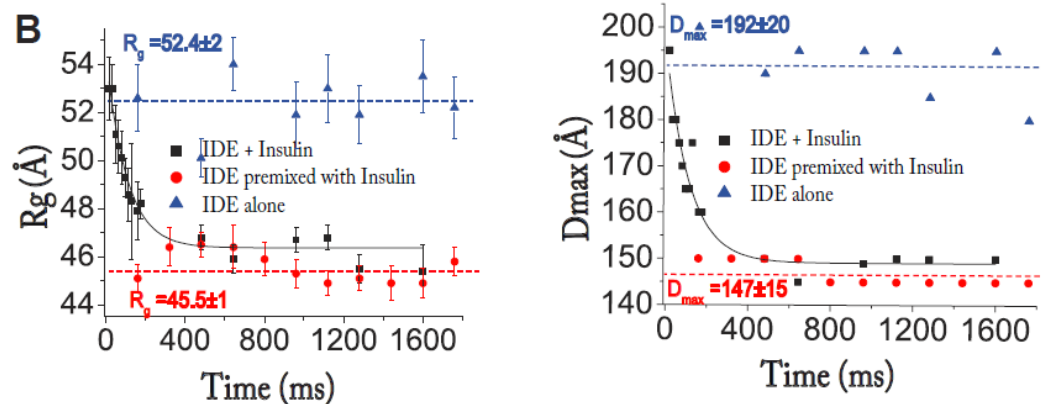
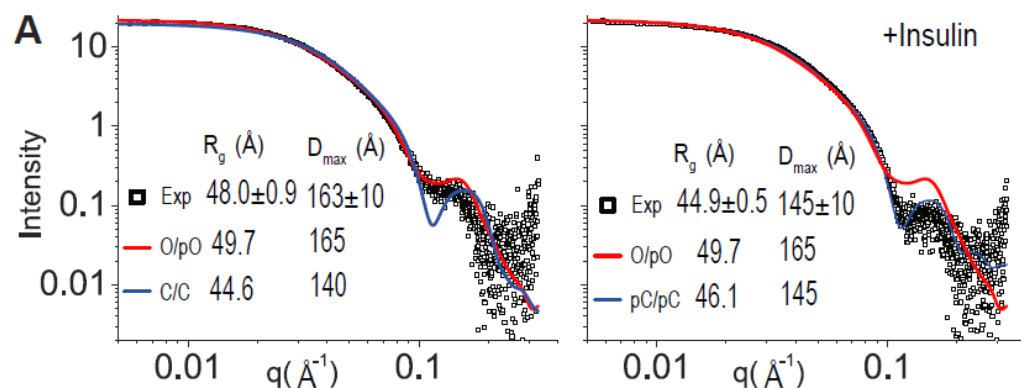


Can follow development of structure starting around 50 ms continuing through 250 ms

Courtesy Ying Liu. UIC unpublished



Example - Laminar-Flow SAXS



Zhang et al., Ensemble cryoEM elucidates the mechanism of insulin capture and degradation by human insulin degrading Enzyme. [Elife](#). 2018 Mar 29;7. pii: e33572. doi: 10.7554/eLife.33572.