Timescales in protein folding accessible with single molecule spectroscopy. Single molecule fluorescence methods, including fluorescence correlation spectroscopy (FCS), cover more than fifteen orders of magnitude in time and allow a wide range of processes relevant for protein folding to be investigated. Essentially all timescales above the lower limit set by the photophysics of the fluorophores can be probed with the available range of experiments and analysis methods on immobilized and/or freely diffusing molecules. The approximate time ranges accessible with different techniques are indicated as horizontal bars. Recent examples for the development and application of these methods are shown at the bottom: (a) Autocorrelation and crosscorrelation functions for FRET-labeled unfolded cyclophilin at 1.5 M GdmCl obtained with ns-FCS, from which the chain reconfiguration dynamics can be determined [25,28,52]. (b) Intramolecular times between species can be obtained from the analysis of transfer efficiency histograms. The solid lines are fits to simulated data according to the theory of Gopich and Szabo [36]. (Figure adapted from [39].) (c) Schematic of a microfluidic mixing device with a dead time of 200 µs for single-molecule detection designed by Gambin et al. [81], (Figure taken from [81*].) (d) Example of a FRET efficiency trajectory of immobilized adenylate kinase, a multi-domain protein whose folding dynamics were investigated by Pichl et al. [73]. (Data from [73*].) (e) Transfer efficiency histogram of a refolded 127 tandem repeat with 5% misfolded molecules at high transfer efficiencies (red box) that convert back to the native species with a half-life of about one week [98], (Figure adapted from [98*]).


By combining single-molecule FRET, ns-FRET-FCS, and concepts from polymer physics, the contribution of internal friction to the dynamics of unfolded and disordered proteins could be quantified for the first time. Surprisingly, internal friction exceeds solvent friction by almost one order of magnitude in the absence of denaturant, suggesting that internal friction in the unfolded state may affect the folding rates of ultra-fast-folding proteins.


73. Pirchi M, Ziv G, Riven I, Cohen SS, Zohar N, Barak Y, Haran G:
- **Single-molecule fluorescence spectroscopy maps the folding landscape of a large protein.** *Nat Commun* 2011, **2**:493.

The authors investigated the folding of the multi-domain protein adenylate kinase by analyzing thousands of single-molecule trajectories with Markov-state models. The results suggest a complex energy landscape for adenylate kinase folding, with about six meta-stable states whose connectivity is strongly denaturant-dependent.

81. Gambin Y, Vandelinder V, Ferreon AC, Lemke EA, Groisman A,
- **Deniz AA: Visualizing a one-way protein encounter complex by ultrafast single-molecule mixing.** *Nat Methods* 2011, **8**:239-241.

An elegant microfluidic mixing device for non-equilibrium single-molecule kinetics was designed with a dead time of 200 μs. The authors used this device to investigate the SDS-induced folding of α-synuclein, an intrinsically disordered protein, and observed the rapid population of transient structures in the formation of the α-synuclein-SDS complex.

98. Borgia MB, Borgia A, Best RB, Steward A, Nettels D,

In a combination of protein engineering and single-molecule spectroscopy, the authors provide evidence for an evolutionary mechanism to prevent misfolding in multi-domain proteins. Tandem arrays of immunglobulin-like domains only showed a misfolded population if adjacent domains had a high sequence identity. The misfolded species was shown to convert to folded proteins with a half-life of 100 hours, demonstrating that single-molecule FRET can be used to investigate very slow conformational changes.