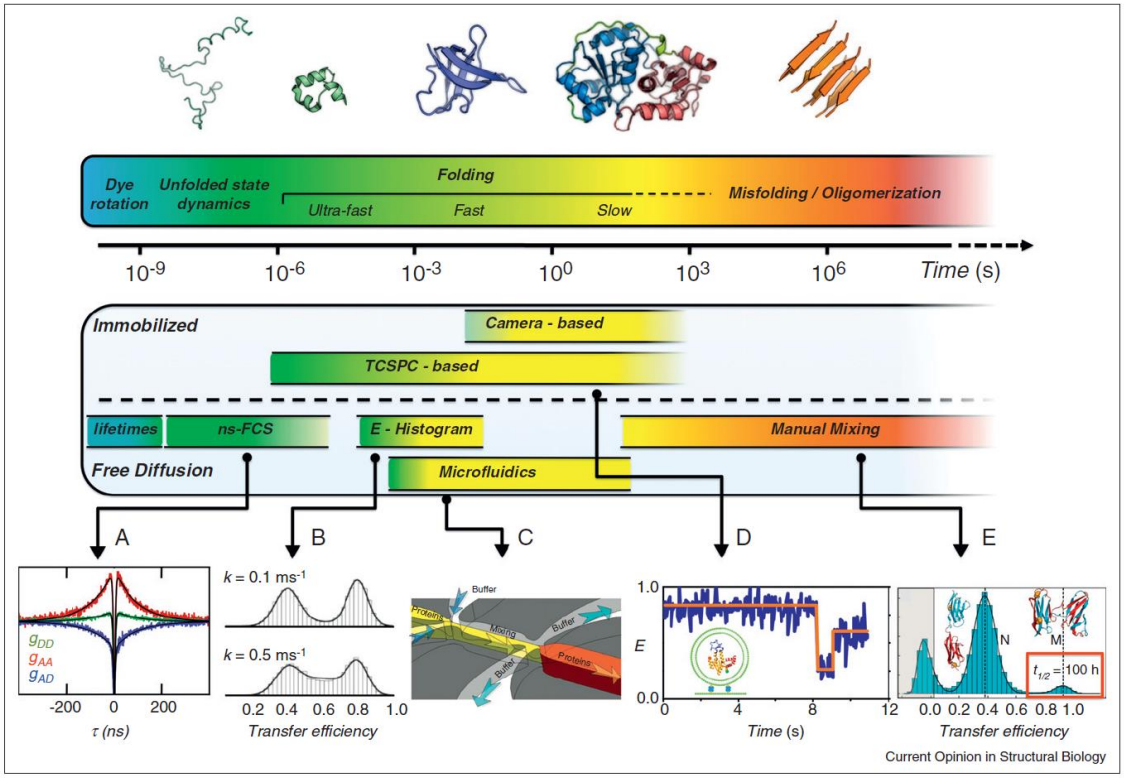
Figure 1



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)** Interconversion 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 [30]. (Figure adapted from [30].) **(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 Pirchi *et al.* [73•]. (Data from [73•].) **(e)** Transfer efficiency histogram of a refolded I27 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•].)

25. • Soranno A, Buchli B, Nettels D, Müller-Späth S, Cheng RR, Pfeil SH, Hoffmann A, Lipman EA, Makarov DE, Schuler B: **Quantifying internal friction in unfolded and intrinsically disordered proteins with single molecule spectroscopy**. *Proc Natl Acad Sci USA* 2012 http://dx.doi.org/10.1073/pnas.1117368109. early edition.

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.

28. Gopich IV, Nettels D, Schuler B, Szabo A: **Protein dynamics from single-molecule fluorescence intensity correlation functions**. *J Chem Phys* 2009, **131**:095102.

30. Gopich IV, Szabo A: **FRET efficiency distributions of multistate single molecules**. *J Phys Chem B* 2010, **114**:15221-15226.

52. Nettels D, Gopich IV, Hoffmann A, Schuler B: **Ultrafast dynamics of protein collapse from single-molecule photon statistics**. *Proc Natl Acad Sci USA* 2007, **104**:2655-2660.

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, Wunderlich B, Schuler B, Clarke J: **Single-molecule fluorescence reveals sequence-specific misfolding in multidomain proteins**. *Nature* 2011, **474**:662-665.

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 immunoglobulin-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.