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# Fluorescence characterization of denatured proteins

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Characterization of unfolded states, while critical to a complete understanding of protein folding, is inherently difficult due to structural heterogeneity and dynamic interchange between states. The growing body of work focusing on single molecule fluorescence techniques for the study of protein folding, also highlights their potential for studies of unfolded proteins. These methods can obtain conformational information about individual subpopulations of molecules in an ensemble, and measure dynamics without the need for synchronization. The studies highlighted here demonstrate the promise of these techniques for obtaining novel information about unfolded states *in vitro* and in more physiologically relevant milieu.

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## Introduction

A complete understanding of protein folding requires not only characterization of the folded and functional forms of proteins, but also a description of the structures and dynamics of the unfolded ensemble. The earliest steps of folding likely involve intramolecular contacts formed in the denatured states, and while these contacts may be transient, they may also play a critical role in the folding process.

Unfolded states are complicated to study due to their inherent heterogeneity, lack of stable, well-defined structures, and dynamic motions of the protein chain over a range of timescales. For very fast dynamics, on the timescale of the excited-state lifetime of the probe, time-resolved luminescent methods have been applied with great success to characterize transiently populated states and dynamics of both proteins and model polypeptides (reviewed in [1–3]). NMR has also proven to be a powerful technique for the study of disordered proteins, particularly in its ability to provide information about dynamics over a range of timescales and atomic resolution characterization of regions of local structure in an otherwise disordered protein (reviewed in [4]).

Single molecule fluorescence methods are ideal for studying denatured states because they uniquely allow for independent analyses of the signals from different conformational subpopulations. Denatured populations can thus be studied in native-like conditions where signals from the folded proteins would otherwise dominate. In this review we focus on recent developments in the application of fluorescence correlation spectroscopy (FCS) and single molecule Förster resonance energy transfer (smFRET) to the characterization of the conformations and dynamics of denatured proteins. In these closely related methods, global or local structural fluctuations of a denatured protein can be quantified by observing changes in the emission of a fluorescent probe or probes attached to the protein (Box 1). We will present and briefly discuss examples where these methods have been used to measure the dimensions of denatured states, investigate the presence of residual structure, and determine the relevance of chain dynamics on a range of timescales.

## Experimental approaches

### Single molecule Förster resonance energy transfer (smFRET)

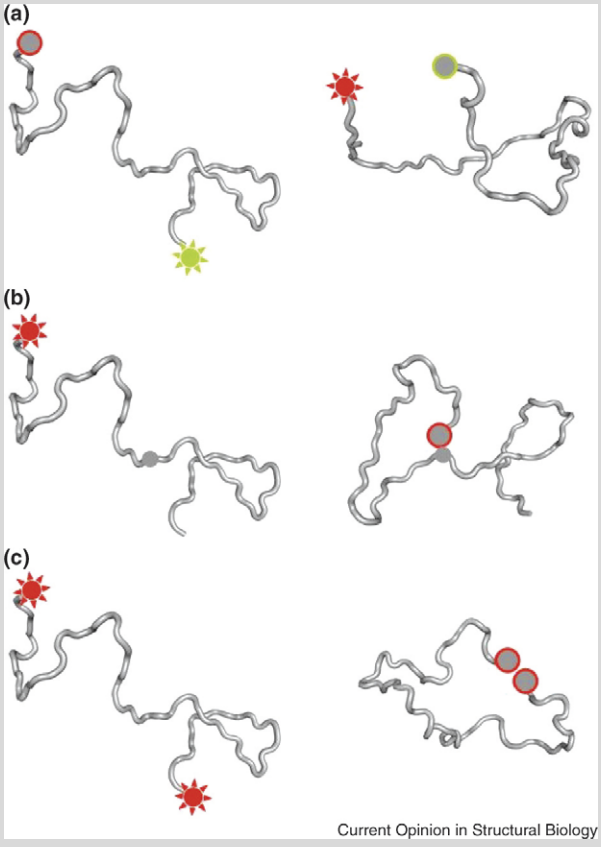
FRET occurs via a non-radiative dipole–dipole interaction between the donor and acceptor fluorophore (Box 2). The efficiency of transfer is strongly dependent upon the distance between the two fluorophores making it a powerful tool for measuring inter- and intramolecular distances [5]. Time-resolved FRET (trFRET) and smFRET share the ability to characterize heterogeneous populations and detect rapid chain motions, and the application of trFRET to protein folding and dynamics has been reviewed recently [1,2]. While the number of publications featuring smFRET is growing rapidly, the technique is still in its infancy when applied to protein folding. A number of recent reviews that focus on smFRET studies of protein folding provide excellent background to the technique and its strengths and difficulties that will not be covered in this contribution [6–8].

### Fluorescence correlation spectroscopy (FCS)

Though it was experimentally realized nearly 40 years ago [9,10], FCS only became a powerful tool for biochemical and biophysical characterization when technological advances produced stable lasers and single photon detectors. As the theory and experimental implementation of

**Box 1** Mechanisms for detecting chain dynamics in denatured proteins

Fluorescence studies of chain dynamics probe conformationally sensitive fluorophores whose quantum efficiencies are mediated by their surrounding environments. The two main mechanisms for conformational sensitivity are FRET and quenching. (A) In FRET, the efficiency of transfer is dependent upon the distance between the donor and acceptor molecules to the sixth power [66]. Typically both the donor and the acceptor molecules are fluorescent, but it is also possible to use a non-fluorescent molecule as the acceptor. Quenching can occur by two primary mechanisms: (B) In photo-induced electron transfer (PET) the excited fluorophore donates or accepts an electron from the quencher, and returns to the ground state without emission of a photon. For the FCS experiments described here, the quenching molecule is a single residue or a number of residues in the protein chain, which may or may not be identifiable [67]. (C) Self-quenching occurs due to intermolecular interactions that take place when two identical fluorophores come into close proximity [68]. Quenching requires almost van der Waals contact of the fluorophore and quencher, whereas FRET occurs over longer distances, typically 10–100 Å with commonly used fluorophores. In a completely denatured protein where close contact between probe molecules may be too transient or infrequent for a significant fluctuation signal to be detected, FRET can measure dynamics that may not result in contact between two molecules. Lastly, FRET acceptor emission intensity is dependent on its separation from the donor, whereas quenching involves complete absence of photon emission.




FCS have already been covered in detail in many thorough reviews [7,11–13], we will only briefly review key features. In FCS, the temporal autocorrelation of spontaneous fluctuations in fluorescence intensity yields quantitative parameters associated with concentration, diffusion, and chemical or photophysical kinetics of the observed species (Box 3). It is commonly used to analyze diffusion behavior, which is dependent upon the hydrodynamic radius, $R_h$, of the diffusing species. This approach has been used to study protein folding, where the transition from the extended denatured state to a compact native one results in a decrease in the diffusion time [13–15]. However, conformational changes that do not result in significant changes in $R_h$ can also be detected if they modulate emission of the fluorophore via FRET or quenching mechanisms (Box 1). Measurements of both the diffusion time and chain dynamics are of interest for studies of disordered proteins by FCS [13]. While FCS is not strictly a single molecule technique, it can be carried out at the single molecule level and is most sensitive for studies of $<100$ molecules.

## Dimensions of the denatured state

The predominant model for describing the denatured states of proteins has long been the Gaussian random coil; based on this model, the radius of gyration, $R_g$, of a denatured protein is expected to scale with the number of residues, $N$, as follows: $R_g = R_0 N^{0.588}$ [16]. Experimental support for this theoretical model comes largely from small-angle X-ray scattering (SAXS) experiments that show that dimensions of a denatured protein agree with those predicted for a similarly sized random coil [16]; historically, this has been interpreted as the absence of residual structure. In recent years, however, convincing spectroscopic experiments, primarily from NMR, show that some denatured proteins display considerable amounts of secondary structure [17–20]. These findings are supported by computational studies that demonstrate that the presence of local, native-like structure is not incompatible with end-to-end distances and $R_g$ predicted by the random-coil model [21–24]. The existence of residual structure in denatured proteins is of extreme interest to the protein folding community, as the presence of even transient residual structure in the denatured state could have a profound effect on protein folding rates.

### Interpretation of the peak shift of the unfolded population

A shared feature of the proteins studied thus far by smFRET is a shift of the average FRET efficiency of the unfolded peak (Box 2, panel A) to higher values (smaller distances) with decreasing denaturant concentrations (Box 2, panel B) [15,25–30]. This behavior may not be unexpected, as both experimental evidence and predictions from computer simulations indicate that upon transfer from a good solvent (high denaturant concentration) to a poor solvent (low denaturant concentration or buffer), a protein chain will collapse from a random coil to a more compact albeit denatured structure. An open question is whether this transition is separate from or concurrent with the actual folding transition, particularly

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