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Determining Serpin Conformational Distributions with Single Molecule Fluorescence

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Abstract

Conformational plasticity is key to inhibitory serpin function, and this plasticity gives serpins relatively easy access to alternative, dysfunctional conformations. Thus, a given serpin population may contain both functional and dysfunctional proteins. Single molecule fluorescence (SMF), with its ability to interrogate one fluorescently labeled protein at a time, is a powerful method for elucidating conformational distributions and monitoring how these distributions change over time. SMF and related methods have been particularly valuable for characterizing serpin polymerization. Fluorescence correlation spectroscopy experiments have revealed a second lag phase during *in vitro* α_1 -antitrypsin polymerization associated with the formation of smaller oligomers that then condense to form longer polymers [Purkayastha, P., Klemke, J. W., Lavender, S., Oyola, R., Cooperman, B. S., and Gai, F. (2005). Alpha 1-antitrypsin polymerization: A fluorescence correlation spectroscopic study. *Biochemistry* **44**, 2642–2649.]. SMF studies of *in vitro* neuroserpin polymerization have confirmed that a monomeric intermediate is required for polymer formation while providing a test of proposed polymerization mechanisms [Chiou, A., Hägglöf, P., Orte, A., Chen, A. Y., Dunne, P. D., Belorgey, D., Karlsson-Li, S., Lomas, D., and Klennerman, D. (2009)]. Probing neuroserpin polymerization and interaction with amyloid-beta peptides using single molecule fluorescence. *Biophys. J.* **97**, 2306–2315.]. SMF has also been used to monitor protease–serpin interactions. Single pair Förster resonance energy transfer studies of covalent protease–serpin complexes suggest that the extent of protease structural disruption in the complex is protease dependent [Liu, L., Mushero, N., Hedstrom, L., and Gershenson, A. (2006). Conformational distributions of protease-serpin complexes: A partially translocated complex. *Biochemistry* **45**, 10865–10872.]. SMF techniques are still evolving and the combination of SMF with encapsulation methods has the potential to provide more detailed information on the conformational changes associated with serpin polymerization, protease–serpin complex formation, and serpin folding.

1. Introduction

Serpin structural remodeling is required for inhibition of target proteases. This functionally necessary structural plasticity allows serpins relatively easy access to multiple significantly different conformations only one of which is active resulting in conformational heterogeneity ranging from productive mixtures of active serpins and protease–serpin covalent complexes to dysfunctional mixtures of serpin oligomers. The difficulties inherent in differentiating between conformations were recently illustrated for the inhibitory serpin α_1 -antichymotrypsin where an inactive state assumed to be the latent state, with full insertion of the intact reactive center loop into β sheet A, turned out to be the δ conformation, with only partial loop insertion (Pearce *et al.*, 2010). Thus, methods that can monitor serpin conformational distributions and how these distributions change over time would aid our understanding of serpin function, dysfunction, and folding.

While most experimental methods report on the average conformation of a heterogeneous protein sample, single molecule fluorescence (SMF) microscopy collects data from individual molecules allowing direct measurement of conformational distributions (Dahan *et al.*, 1999; Li *et al.*, 2003; Orte *et al.*, 2008a). SMF can also reveal how conformational distributions change over time during processes such as polymer formation (Chiou *et al.*, 2009; Purkayastha *et al.*, 2005) and protein folding (Chattopadhyay *et al.*, 2005; Chen *et al.*, 2007; Schuler and Eaton, 2008; Sherman *et al.*, 2008). Thus, along with other methods such as mass spectrometry and NMR that can also report on conformational heterogeneity, SMF and related fluorescence techniques provide a window unto the complicated structural landscape populated by serpins.

In this review, we begin with general technical aspects of SMF experiments from how to label proteins to the necessary instrumentation. Three different SMF methods have been applied to serpins. Gai and colleagues have used fluorescence correlation spectroscopy (FCS) to monitor α_1 -anti-trypsin (α_1 AT) polymerization (Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005) while Klenerman and coworkers studied neuroserpin polymerization using two-color coincidence detection (TCCD) (Chiou *et al.*, 2009). We have used single pair Förster resonance energy transfer (spFRET) to determine conformational distributions of trypsin- α_1 AT covalent complexes (Liu *et al.*, 2006, 2007). For all of these studies, we introduce the relevant SMF technique, provide protocols, and review the experimental findings. SMF is a growing field in which technical advances, particularly for imaging proteins in living cells (Patterson *et al.*, 2010), are continuing at a fast pace and we hope to inspire scientists working on serpins to interrogate their systems using SMF.

2. Labeling Serpins and Proteases with Fluorophores

SMF experiments require bright, photostable fluorophores that emit in the visible or near-IR. Commonly used fluorophores include the Alexa Fluor dyes (Invitrogen), Atto dyes (Atto-Tec, Germany), cyanine dyes such as Cy3 and Cy5 (GE Healthcare), and HiLyte dyes (Anaspec). These are photostable fluorophores with high-absorption cross-sections and high fluorescence quantum yields (Buschmann *et al.*, 2003; Roy *et al.*, 2008). Serpins have been covalently labeled with such fluorophores using Cys-maleimide linkages because of the stability and specificity this reaction affords. Native or unfolded proteins may be labeled in solution, and efficient labeling for ammonium sulfate precipitated proteins was recently reported (Kim *et al.*, 2008).

If a single, solvent-accessible Cys is present in wildtype protein it can be specifically labeled for FCS and/or TCCD experiments. For proteins that lack solvent-accessible Cys residues and for spFRET experiments where two fluorescent labels are required, solvent-accessible Cys residues may be introduced at various positions and, when necessary, native Cys residues may be mutated, usually to Ser or Ala. For proteins such as trypsin and related serine proteases in which all of the Cys residues are disulfide bonded, an additional solvent-accessible Cys may be introduced without mutating existing Cys residues (Mellet *et al.*, 2002). The resulting Cys variants, both unlabeled and labeled, should be checked for function, stability and, for serpins, polymerization propensity.

2.1. A general protocol for labeling proteins with maleimide derivatized fluorophores

1. Maleimide reactions are sulfhydryl specific between pH 6.5 and 7.5 (Hermanson, 1996), and labeling reactions are generally performed between pH 7.0 and 7.5 in a variety of buffers.

2. Protein concentrations should be at least 10 μM for effective labeling and higher concentrations, 25–100 μM , are generally recommended by the dye manufacturers.
3. Prior to labeling, the protein should be reduced using at least a 10-fold molar excess of DTT or TCEP usually for 10–15 m at room temperature. Note that TCEP can irreversibly inhibit Ser proteases. DTT must be removed prior to labeling by gel filtration. PD-10 or NAP-10 columns (GE Healthcare) containing Sephadex G-25, as well as Toyo-pearl HW-40C or HW-50F (Tosoh Biosciences) resins are all commonly used. TCEP need not be removed prior to labeling.
4. Prepare a 1–10 mM fluorophore stock solution immediately before use and protect this solution from light. To attain millimolar concentrations, hydrophobic fluorophores may need to be dissolved in an organic solvent such as dimethyl sulfoxide. The fluorophore is added to the protein solution with final fluorophore to protein molar ratios usually between 10:1 and 20:1. The final protein solution should contain 5% or less organic solvent to avoid protein denaturation. Samples may be layered with argon gas to avoid oxidation, and tubes should be sealed with parafilm and protected from light. Reactions are generally allowed to proceed for at least 2 h at room temperature and/or at 4 °C overnight.
5. The reaction is stopped by adding β -mercaptoethanol or glutathione, and free dye is removed by gel filtration (see Section 2 for a list of commonly used resins). Assuming that the protein environment does not alter the spectral properties of the fluorophore, labeling efficiency may be determined from an absorption spectrum using the extinction coefficient of the free fluorophore at or near its peak absorption (where the protein does not absorb), $\epsilon_{\max,fl}$, the fluorophore's relative extinction coefficient at 280 nm, $\epsilon_{280,fl}/\epsilon_{\max,fl}$, and the protein's extinction coefficient at 280 nm, $\epsilon_{280,prot}$:

$$\text{DOL} = \frac{[\text{fluorophore}]}{[\text{protein}]} = \frac{A_{\max,fl}/(\epsilon_{\max,fl}l)}{A_{280,prot}/(\epsilon_{280,prot}l)} \quad (16.1)$$

$$= \frac{A_{\max,fl}\epsilon_{280,prot}}{(A_{280} - A_{\max,fl}(\epsilon_{280,fl}/\epsilon_{\max,fl}))\epsilon_{\max,fl}}$$

where A is the measured absorbance, l is the pathlength of the cuvette, and DOL is the degree of labeling or the labeling efficiency. The DOL may also be measured using mass spectrometry, and this method is often more accurate.

3. Overview of Single Molecule Fluorescence Techniques

To our knowledge, all serpin SMF experiments have been conducted on serpin monomers, polymers, and molecular complexes *freely diffusing* in solution (Chiou *et al.*, 2009; Chowdhury *et al.*, 2007; Liu *et al.*, 2006; Purkayastha *et al.*, 2005) or, in one case, when microinjected into cells (Speil and Kubitschek, 2010). While many of the most familiar SMF experiments on other biomolecules have been performed using total internal reflection microscopy and surface immobilized biomolecules (for reviews see Moerner and Fromm, 2003; Myong and Ha, 2010; Peterman *et al.*, 2004), our efforts to immobilize serpins have resulted in dysfunctional molecules. We therefore focus on SMF techniques that interrogate freely diffusing molecules (Table 16.1) using microscope-based instrumentation as depicted in Fig. 16.1.

In order to interrogate an individual molecule, there must be a very low probability that two or more molecules are in the observation volume at the same time. This places two restrictions on the experimental conditions: (i) low (10–100 pM) concentrations of labeled molecules must be used and (ii) observation volumes must be small, sub-femtoliter. To put the concentration and volume limitations in context, for a 1 nM solution a 1 fl volume will,

on average, contain 1 molecule. Thus, 1 nM is too high a concentration for true single molecule experiments because the probability that 2 molecules will be detected is not negligible. The concentration limitations apply only to the fluorescently labeled protein of interest allowing unlabeled molecules to be in solution at arbitrary concentrations. Concentration restrictions are also relaxed for experiments on oligomers where the relevant concentration is that of the oligomer and not of the monomer as well as for FCS experiments that rely on small numbers of molecules rather than single molecules allowing a broader concentration range, from pM to 100 s of nM. The size of the observation volume is determined by the microscope optics and is diffraction limited so that shorter excitation wavelengths, λ_{ex} , and higher microscope objective numerical apertures, NA, result in smaller observation volumes.

3.1. SMF instrumentation

SMF instrumentation is often based on an inverted microscope where a dichroic mirror both reflects the laser light into the microscope objective and transmits the lower energy, higher wavelength fluorescence emitted by the sample (Fig. 16.1; Dahan *et al.*, 1999; Petersen and Elson, 1986; Sisamakis *et al.*, 2010). For conventional one-photon excitation, fluorescence arises from the entire illuminated volume, and a confocal pinhole is used to restrict the observation volume to a small volume centered at the focus (Dahan *et al.*, 1999; Magde *et al.*, 1974; Petersen and Elson, 1986). The pinhole is placed in the conjugate image plane and acts as a spatial filter, rejecting fluorescence from out-of-focus molecules thereby restricting the observation volume.

For spFRET, TCCD, and other experiments involving fluorophores with different colors, a dichroic mirror in the emission path is used to split the fluorescence by color and the fluorescence is collected by high-efficiency detectors such as single photon counting avalanche photodiodes (APDs). Some common alternative configurations include anisotropy experiments where the fluorescence may be split by polarization using a polarizing beamsplitter in place of or in addition to the dichroic mirror (Liu *et al.*, 2006; Sisamakis *et al.*, 2010) and single color experiments requiring high time resolution where the dichroic mirror may be replaced by a nonpolarizing 50–50 beamsplitter. Bandpass filters are often positioned in front of the detectors to restrict cross talk between detectors for multicolor experiments and to filter out the Raman scattering from water (Fig. 16.1).

In many two-color experiments, such as the TCCD work by Klennerman and coworkers (Chiou *et al.*, 2009; Orte *et al.*, 2008a), fluorophores with very different excitation spectra must be simultaneously excited. This requires colinear alignment of two different wavelength laser lines that are then focused in the sample (Fig. 16.1). For spFRET experiments, the acceptor fluorophore absorbs at redder wavelengths than does the donor fluorophore. While spFRET experiments may be performed by exciting only the donor fluorophore, alternately exciting the donor and the acceptor with two different wavelengths allows discrimination between molecules with low FRET efficiencies and those in which the acceptor is missing or has photobleached (Kapanidis *et al.*, 2005; Müller *et al.*, 2005). This again requires proper alignment of two different wavelength laser lines.

More comprehensive discussions of FCS instrumentation may be found in a review by Krichinsky and Bonnet (2002). TCCD experimental setups have been thoroughly described by Klennerman and coworkers (Li *et al.*, 2003; Orte *et al.*, 2008b). More detailed descriptions of SMF instrumentation for FRET may be found in recent reviews by Seidel and coworkers (Sisamakis *et al.*, 2010) as well as by Ha and coworkers (Roy *et al.*, 2008).

4. Serpin Polymerization

Mutations in metastable serpins can lead to polymerization resulting in disease (Davis *et al.*, 1999; Lomas *et al.*, 1992). While it is relatively easy to monitor polymers once they are formed, interrogating intermediates in polymer formation can be quite difficult. FCS and TCCD studies of serpin polymers allow researchers to probe early events in polymer formation including both changes in monomer structure that significantly alter translational diffusion and the formation of small oligomers (Chiou *et al.*, 2009; Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005).

4.1. α_1 AT polymerization and depolymerization monitored by fluorescence correlation spectroscopy

Increases in molecular size due to oligomerization slow translational diffusion and increase the diffusion time, τ_D , of fluorescent molecules through the microscopic observation volume as measured by FCS (Fig. 16.2) (Krichevsky and Bonnet, 2002; Thompson, 1991). Protein conformational changes that significantly alter the overall protein shape can also change τ_D (Sherman *et al.*, 2008), making FCS sensitive to both the monomer conformation and oligomerization. Feng Gai, Barry Cooperman, and coworkers have used FCS to study both the formation of α_1 AT polymers by heating (Purkayastha *et al.*, 2005) and the effects of peptide inhibitors on polymerization and depolymerization (Chowdhury *et al.*, 2007). FCS revealed that for α_1 AT polymerization at 45 °C, τ_D increases in less than 30 m from ~2 ms for monomeric α_1 AT to 4–6 ms indicating the formation of small oligomers and/or monomer conformational changes (Purkayastha *et al.*, 2005). After a lag of approximately 200 m, τ_D increases to 8–16 ms, but long polymers appear only after 400 m. As expected, the lag times in the production of oligomers and long polymers is concentration and temperature dependent, and higher concentrations result in longer polymers. The FCS data also suggest that large polymers form not by monomer addition but rather through the association of smaller polymers. Peptide driven depolymerization occurs in the opposite manner, large polymers dissociate into smaller polymers that then further dissociate (Chowdhury *et al.*, 2007). Below we describe how to analyze FCS data, further describe the findings of Gai and colleagues, and provide a protocol for FCS polymerization and depolymerization experiments based on their work (Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005). It should be noted that the choice of fluorescent dye, excitation wavelength, and excitation power can easily be optimized for the system of interest.

4.1.1. Introduction to FCS—Diffusion of molecules in and out of the observation volume results in fluorescence fluctuations, and the time scale of these fluctuations is related to the diffusion time, τ_D , which can be determined using FCS (Elson and Magde, 1974; Magde *et al.*, 1974; Thompson, 1991). The concentrations of fluorescently labeled molecules needed for FCS experiments range from < 100 nM to pM. If the concentrations are too high, ≥ 1000 molecules in the observation volume (Thompson, 1991), for every molecule that leaves the observation volume another one enters damping the fluctuations and degrading the FCS signal. At lower concentrations, diffusion of molecules in and out of the observation volume results in large changes in the number of molecules in the observation volume at a given time, large fluorescence fluctuations, and larger amplitude FCS signals (Fig. 16.2). The diffusion time, τ_D , may be determined by correlating the time-dependent fluorescence intensity, $I(t)$, and finding the resulting autocorrelation, $G(\tau)$ (Elson and Magde, 1974; Krichevsky and Bonnet, 2002; Magde *et al.*, 1974; Thompson, 1991) (Fig. 16.2):

$$G(\tau) = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2} \quad (16.2)$$

$$\delta I(t) = I(t) - \langle I(t) \rangle \quad (16.3)$$

where $\langle \rangle$ indicates a time average, τ is the time lag between the time-resolved fluorescence intensities, and $\delta I(t)$ are the fluorescence fluctuations. Correlation functions may be calculated using a hardware correlator (e.g., Correlator.com) or by software correlators using either commercial software (e.g., ISS, Zeiss, or Picoquant) or by programs written in various laboratories (e.g., Purkayastha *et al.*, 2005). Assuming that the excitation intensity in the observation volume is essentially a Gaussian ellipsoid that is brightest at the center and less bright toward the edges (Fig. 16.2A), the radius and axial extent of the observation volume are given by ω_0 and z_0 , respectively. For a single fluorescent species, the calculated correlations may then be fit to

$$G(\tau) = \frac{1}{\langle N \rangle} \left(1 + \frac{\tau}{\tau_D} \right)^{-1} \left(1 + \frac{\tau}{S^2 \tau_D} \right)^{-1/2} \quad (16.4)$$

$$\tau_D = \frac{\omega_0^2}{4D} \quad (16.5)$$

where $\langle N \rangle$ is the average number of molecules in the observation volume, τ_D is related to the translational diffusion coefficient, D , by Eq. (16.5), and $S = z_0/\omega_0$ is the axial to radial ratio of the observation volume. Oligomerization (Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005) or protein unfolding (Sherman *et al.*, 2008) will slow translational diffusion, increasing the time it takes for the molecule to diffuse through the observation volume and shifting the correlation function to longer times (Fig. 16.2C).

The number of fitting parameters for FCS is often large compared to the number of data points making the fits underdetermined and much care must be taken to ensure that the results from the fits are experimentally meaningful. To avoid this problem, the radius and extent, ω_0 and z_0 , of the observation volume are calibrated using a fluorescent dye with a known diffusion coefficient (Sherman *et al.*, 2008), and these values may be fixed when fitting protein data. The fit given in Eq. (16.4) is for a single molecular species. Many FCS experiments are performed on mixtures of species. If the fluorescence intensity, also called the molecular brightness, of all of the species is the same then $G(\tau)$ may be fit to (Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005)

$$G(\tau) = \frac{1}{\langle N \rangle} \sum_k f_k \left(1 + \frac{\tau}{\tau_D^k} \right)^{-1} \left(1 + \frac{\tau}{S^2 \tau_D^k} \right)^{-1/2} = \frac{1}{\langle N \rangle} \sum_k f_k g_k(\tau) \quad (16.6)$$

where $\langle N \rangle$ is the time-averaged total number of molecules in the observation volume, f_k is the fraction of species k in solution with diffusion time τ_D^k , and $g_k(\tau)$ is the autocorrelation for species k .

When fluorescently labeled proteins oligomerize the brightness of the oligomers increases with size, so a dimer is twice as bright as a monomer, a trimer is three times as bright as a monomer, etc. Note that this simple description assumes that changes in the fluorophore

environment due to oligomerization do not affect the brightness. FCS is sensitive to brightness and the autocorrelations dependent on both the number of each species in the observation volume, N_k , and the brightness of each species, B_k (Krichevsky and Bonnet, 2002; Thompson, 1991):

$$G(\tau) = \frac{\sum_k N_k B_k^2 g_k(\tau)}{(\sum_j N_j B_j)^2} = \frac{\sum_k N_k \alpha_k^2 g_k(\tau)}{(\sum_j N_j \alpha_j)^2} \quad (16.7)$$

where $\alpha_k = B_k/B_1$ is the brightness of species k relative to species 1, the monomer for oligomerization experiments. Thus, $\alpha = 1$ for monomers, 2 for dimers, 3 for trimers, etc., in an ideal polymerization experiment. For polymerization experiments, Eq. (16.7) effectively means that $G(\tau)$ is biased toward higher-order oligomers.

4.1.2. Applying FCS to α_1 AT polymerization—Heat-induced polymerization results in heterogeneous samples containing monomers, dimers, and higher-order oligomers. As shown in Eq. (16.7), brighter species such as long polymers containing many labeled monomers will make a larger contribution to the autocorrelation than will less bright species such as monomers. To account for this heterogeneity, multiple short datasets were collected for each sample and each individual dataset was analyzed using Eq. (16.6) (Purkayastha *et al.*, 2005). This allowed the distribution of diffusion times to be determined at the various time points providing a time-resolved profile of polymerization or depolymerization (Fig. 16.2D) (Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005). While the diffusion times determined from Eq. (16.6) are correct, because the molecular brightness terms are omitted from this equation (see Eq. (16.7)) the fraction of the population corresponding to the brightest species, that is, the largest oligomers at each time point, may have been overestimated by Gai and colleagues. Nonetheless, the FCS results show that heat-induced polymerization of α_1 AT has two, concentration- and temperature-dependent lag phases (Purkayastha *et al.*, 2005), that the average polymer length increases with increasing temperature (Purkayastha *et al.*, 2005), and that small peptides such as WMDF can both prevent polymerization at 39 °C and lead to depolymerization of polymers formed at 45 °C. FCS can easily be applied to other polymer-prone serpins.

4.1.3. A protocol for monitoring α_1 AT polymerization with FCS (based on the work by Gai, Cooperman, and collaborators; Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005)

1. Monomers of the α_1 AT variant Cys232Ser/Ser359Cys (P1'C α_1 AT) were labeled with the Cys reactive dye tetramethyl-rhodamine at a fluorophore to protein ratio of 20:1 in 50 mM Tris, 50 mM KCl, pH 8.3.
2. FCS experiments were performed using ~270 μ W of 514.5 nm excitation from an argon ion laser and a 50 μ m confocal pinhole. The setup was calibrated with the dye rhodamine 6G ($D = 280 \mu\text{m}^2/\text{s}$ at 22 °C (Magde *et al.*, 1974)). All FCS experiments were performed at 23 °C.
3. Monomer experiments: P1'C α_1 AT was diluted to 1 nM in 50 mM Tris, 50 mM KCl, pH 8.3, and FCS data were collected. Due to the presence of free dye, the resulting correlations were fit to two species using Eq. (16.6) where the first species corresponds to <10% free, tetra-methyl-rhodamine and the second species, with a much longer diffusion time, to P1'C α_1 AT.
4. Polymer formation: 5 μ M labeled P1'C α_1 AT in 50 mM Tris, 50 mM KCl, pH 8.3, was incubated at 45, 50, or 55 °C.

5. FCS measurements: At various times, aliquots were removed from the heated samples and quickly diluted with 50 mM Tris, 50 mM KCl, pH 8.3, at 23 °C to 1 nM P1'C α_1 AT (where 1 nM is the monomer concentration). FCS data were collected for a total of 100 s with an integration of 100 μ s per time point. 10–100 datasets were collected for each diluted sample.
6. Polymerization blocking experiments (Chowdhury *et al.*, 2007): Polymers were formed by incubating 7 μ M of labeled P1'C α_1 AT in 50 mM Tris, 50 mM KCl, pH 8.3, at 39 or 52 °C in the presence or absence of 1.4 μ M peptide, and FCS experiments were performed as described above.
7. Depolymerization experiments (Chowdhury *et al.*, 2007): Polymers were preformed at 45 °C for ~36 h using 7 μ M of labeled P1'C α_1 AT in 50 mM Tris, 50 mM KCl, pH 8.3, the temperature was decreased to 39 °C and a 200-fold molar excess of peptide was added. FCS experiments were performed as described above.

4.2. Early events in neuroserpin polymerization measured by two-color coincidence detection

Amyloid formation by A β peptides is associated with Alzheimer's disease and interactions between neuroserpin and A β peptides have been shown to reduce neuroserpin polymerization both *in vitro* and in model systems (Kingham *et al.*, 2006). Conversely, neuroserpin reduces the toxicity of A β_{1-42} oligomers in cells by accelerating aggregation (Kingham *et al.*, 2006). David Klenerman, David A. Lomas, and coworkers have taken advantage of SMF-based coincidence methods to monitor polymerization of neuroserpin and how interactions between neuroserpin and A β_{1-40} peptides affect polymer formation (Chiou *et al.*, 2009).

Similarly to the α_1 AT FCS experiments, polymers for TCCD experiments were formed at μ M neuroserpin concentrations and then diluted to ~50 pM (monomer concentration) for SMF microscopy (Chiou *et al.*, 2009). Dilutions of this magnitude can lead to dissociation of loosely bound species, and while serpin polymers are still intact at low concentrations (Chiou *et al.*, 2009; Purkayastha *et al.*, 2005), very few neuroserpin/A β_{1-40} complexes were detected (Chiou *et al.*, 2009). Nonetheless, a dissociation constant of 10 ± 5 nM for neuroserpin/A β_{1-40} complexes could be estimated from the TCCD data. While overnight incubation with A β_{1-40} inhibited neuroserpin polymerization, addition of A β_{1-40} immediately before or during polymerization accelerated neuroserpin polymerization (Chiou *et al.*, 2009). TCCD experiments on neuroserpin polymerization in the absence of A β_{1-40} show that a conformational change in monomeric neuroserpin is the rate-limiting step for polymerization. In the following sections, the principles behind TCCD are introduced, the TCCD results are further described and a protocol for TCCD serpin experiments is given based on the work of Klenerman, Lomas, and colleagues (Chiou *et al.*, 2009).

4.2.1. Introduction to TCCD—Oligomerization, particularly early events in polymerization, may be monitored using two pools of protein labeled with spectrally distinct fluorophores, for example, a blue and a red fluorophore (Li *et al.*, 2003; Orte *et al.*, 2008a). At single molecule concentrations, association of blue and red labeled monomers results in diffusion of a blue and red labeled oligomer through the observation volume and coincident signals will be observed on the two detectors (Figs. 16.1 and 16.3). The intensity of the signal from the blue (red) detector is proportional to the number of monomers in the oligomer that are labeled with the blue (red) fluorophore. To discriminate signal from noise, the number of photons in a burst must be above a minimum threshold (number of photons/unit time) and the thresholds are individually optimized for the red and blue channels,

respectively (Clarke *et al.*, 2007). The association quotient, Q , which increases as oligomers/polymers get larger, may then be determined as (Chiou *et al.*, 2009; Orte *et al.*, 2006)

$$Q = \frac{r_s}{r_B + r_R - r_s} \quad (16.8)$$

$$r_s = r_C - r_E \quad (16.9)$$

where the photon burst rates in the blue and red detection channels are given by r_B and r_R , respectively. The rate of bursts that are coincident on both channels is given by r_C which must be corrected for the rate of random coincidences, r_E , to reveal the rate of coincident events, r_s , arising from association (Eq. (16.9)). As polymers form and grow, Q increases with the time of incubation, t , according to Chiou *et al.* (2009):

$$Q(t) = y_0 + A \exp(-t/\tau) \quad (16.10)$$

where y_0 is the value of Q when polymerization has reached a steady state, A ($A < 0$) is the normalization constant, and τ is the rise time for polymerization.

The simplest application of TCCD requires two spectrally distinct fluorophores so that the signal in the blue (red) channel comes almost exclusively from the blue (red) fluorophore and FRET between the fluorophores is unlikely (although a more recent application combines TCCD and spFRET; Orte *et al.*, 2008b). The most important controls include experiments on monomers labeled with only the red or blue fluorophore to determine the signal intensity expected for each fluorophore and to make sure that the two detection channels are specific for each color as well as experiments in which red and blue monomers are mixed in order to determine the probability that simultaneous signals on both detectors occur by chance, r_E . TCCD was developed by Klennerman and colleagues, and more detailed descriptions of experimental considerations and data analysis are described in their work (Chiou *et al.*, 2009; Clarke *et al.*, 2007; Orte *et al.*, 2006, 2008a,b).

4.2.2. Applying TCCD to neuroserpin polymerization—At the earliest stages of polymerization TCCD reveals that the percentage of polymers is less than 1%. The initial change in Q , as determined from $(dQ/dt)_{t=0}$, is approximately $2.2 \pm 0.8 \times 10^{-6} \text{ s}^{-1}$ at all neuroserpin concentrations indicating that the initial rate-limiting step in polymerization is unimolecular, presumably a conformational change in the neuroserpin monomer. As observed in the FCS α_1 AT polymerization experiments (Purkayastha *et al.*, 2005), the final neuroserpin polymer length is concentration dependent with higher concentrations resulting in larger polymers (Chiou *et al.*, 2009). In addition, as shown by a combination of modeling and fits to the TCCD data, TCCD may be used to test kinetic models of polymerization (Fig. 16.3C) (Chiou *et al.*, 2009).

4.2.3. A protocol for monitoring neuroserpin polymerization with TCCD (based on the work of Klennerman, Lomas, and collaborators; Chiou *et al.*, 2009)

1. **Protein labeling:** The Ser229Cys (S229C) mutation was introduced into neuroserpin (Chiou *et al.*, 2009) and the S229C variant with an N-terminal 6× His tag was purified (Belorgey *et al.*, 2002; Chiou *et al.*, 2009). S229C neuroserpin was labeled with 10- to 20-fold molar excess of Alexa Fluor 488 (AF488, blue) or Alexa Fluor 647 (AF647, red).
2. **Polymer formation:** Equimolar concentrations of blue and red labeled neuroserpin were mixed in phosphate buffered saline at total neuroserpin concentrations

ranging from 2.2 to 6.6 μM and incubated at 45 °C for up to 480 m. Aliquots were removed at various times and snap-frozen in liquid nitrogen. More detailed protocols for neuroserpin polymerization may be found in a recent paper by Lomas and colleagues (Belorgey *et al.*, 2011).

3. Brief description of the TCCD setup: AF488 was excited with 220 μW of 488 nm laser light while AF647 was excited with 60 μW of 633 nm laser light from two overlapping Gaussian laser beams in a confocal microscope with a 60 $\times$ NA 1.40 oil objective and a 50 μm pinhole (Fig. 16.1). The fluorescence was separated using a dichroic mirror (585DRLP, Omega Optical) and sent to two APDs, one for each color (Chiou *et al.*, 2009).
4. Glass coverslips used for TCCD experiments were incubated with 1 mg/ml bovine serum albumin (BSA) for at least 1 h at room temperature to prevent nonspecific protein adsorption. The BSA solutions were removed, snap-frozen neuroserpin samples were thawed, quickly diluted to 50 pM for TCCD experiments and applied to the coverslips.
5. The association quotient, Q , was calculated from the time-resolved photon counts from the blue and red detectors according to Eq. (16.8) (Chiou *et al.*, 2009; Orte *et al.*, 2006, 2008a) and the time dependence of Q was fit to Eq. (16.10). These experiments were performed for neuroserpin alone or in the presence of A β ₁₋₄₀ (1:1 neuroserpin: A β ₁₋₄₀ ratio).
6. Neuroserpin-A β interactions: A β ₁₋₄₀ labeled at the N-terminus with HiLyte Fluor 488 was purchased from Anaspec. A 1:1 ratio of AF647 labeled neuroserpin (red) and HiLyte Fluor 488 labeled A β ₁₋₄₀ (red) were incubated at μM concentrations and 45 °C to promote neuroserpin polymerization, and TCCD experiments were performed as described above.

4.3. The utility of FCS and TCCD for studying polymerization

Both FCS and TCCD can easily be applied to any serpin that can be fluorescently labeled. Because both methods can measure the size of oligomers, they can be used to obtain detailed information on early events in polymerization and how protein-protein as well as protein-peptide interactions affect early and late events in polymerization. Finally, both methods may be combined with FRET to provide even more information on polymer structures (Li *et al.*, 2005; Nath *et al.*, 2010; Orte *et al.*, 2008b). Unlike FCS, which is often used to interrogate small numbers of molecules, TCCD is a real single molecule technique collecting data from one molecule at a time. However, TCCD is still being developed, and for the moment FCS is easier to apply with commercially available hardware and software.

5. Conformational Distributions of Protease-Serpin Complexes

Serpins inhibit target proteases by translocating the covalently attached protease more than 70 Å from one pole of the serpin to the other, concomitantly mechanically deforming the protease active site (Dementiev *et al.*, 2006; Huntington *et al.*, 2000; Shin and Yu, 2006). Ensemble FRET and other fluorescence techniques have been used to monitor protease interactions with inhibitory serpins providing information on the average conformation of the population (Lawrence *et al.*, 1994; Mellet *et al.*, 2002; Shin and Yu, 2002, 2006; Shore *et al.*, 1995; Swanson *et al.*, 2007; Tew and Bottomley, 2001). SpFRET experiments provide additional information by revealing the conformational distributions of protease-serpin complexes.

SpFRET experiments have been conducted on covalent complexes between α_1 AT and bovine trypsin (BTryp) as well as its more stable cousin, rat trypsin (RTryp) (Liu *et al.*,

2006, 2007). SpFRET results suggest that BTryp is more disrupted in the covalent complex than is the more stable RTryp (Liu *et al.*, 2006). The orientation of the protease relative to α_1 AT is also different in BTryp and RTryp complexes. X-ray crystal structures of covalent complexes between α_1 AT and two noncognate serine proteases, BTryp (Huntington *et al.*, 2000) and porcine pancreatic elastase (Dementiev *et al.*, 2006), also revealed differences in protease structural disruption and orientation. While the extent of protease disruption may be affected by protease self-cleavage in crystals (Dementiev *et al.*, 2006), self-cleavage is unlikely in spFRET experiments due to the limited, 15 m, incubation time required for complex formation at μM concentrations and the pM concentrations used when collecting spFRET data. Thus, both the crystal structures and the spFRET results suggest that the protease conformation and orientation in protease–serpin covalent complexes may be affected by protease stability. In addition, spFRET has revealed a fluorophore-trapped covalent complex in which trypsin is only partially disrupted and partially translocated by α_1 AT. Below, we give a brief introduction to spFRET and describe how it has been applied to trypsin– α_1 AT complexes.

5.1. Introduction to single pair Förster resonance energy transfer

FRET takes advantage of distance dependent dipole–dipole interactions between a donor fluorophore in the excited state and a ground-state acceptor. These interactions can result in energy transfer, with the probability of energy transfer increasing as, r , the distance between the donor and acceptor dipole moments decreases (Lakowicz, 2006; Sisamakias *et al.*, 2010; Steinberg, 1971; Stryer, 1978):

$$E = \frac{R_0^6}{(r^6 + R_0^6)} \quad (16.11)$$

where E is the energy transfer efficiency ($0 \leq E \leq 1$) and R_0 is the inter-fluorophore distance at which the probability of energy transfer is 50%. The efficiency of energy transfer, and thus R_0 , depends on the fluorescence quantum yield of the donor, Q_D , the overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor calculated using the overlap integral, $J(\lambda)$, the relative orientation of the donor and acceptor dipole moments given by the orientation factor, κ^2 , and the refractive index, n , generally assumed to be that of water, 1.33 (Lakowicz, 2006; Sisamakias *et al.*, 2010; Steinberg, 1971; Stryer, 1978):

$$R_0(\text{\AA}) = 9.78 \times 10^3 (\kappa^2 n^{-4} Q_D J(\lambda))^{1/6} \quad (16.12)$$

If the donor and acceptor are freely rotating, $\kappa^2 = 2/3$, and the validity of this assumption may be tested by measuring the fluorescence anisotropy of the donor and acceptor. Detailed discussions of how to calculate R_0 may be found in work by Lakowicz (2006) and for single molecule measurements by Seidel and coworkers (Sisamakias *et al.*, 2010). For most donor–acceptor pairs R_0 is between 30 and 70 $\text{\AA}$, and typical R_0 values for a number of donor–acceptor pairs used for spFRET experiments are given in Table 16.2. FRET is most sensitive in the distance range between $\sim 0.5R_0$ and $\sim 1.5R_0$ and donor–acceptor pair should be carefully chosen so that expected separations fall within this range. In general, FRET is very good at determining relative changes in distance, for example, changes in the donor–acceptor separation upon protein unfolding or protein–protein interactions, but absolute distance determinations should only be made with great care.

For spFRET experiments, fluorescence emission is split by color and donor fluorescence is collected by one detector (Det1 in Fig. 16.1) while acceptor fluorescence is collected by a

second detector (Det2 in Fig. 16.1). The energy transfer efficiency, E , is then calculated from these fluorescence intensities (Dahan *et al.*, 1999):

$$E = I_A / (I_A + \gamma I_D) \quad (16.13)$$

where I_A and I_D are the intensities of a photon burst from the donor, D, and acceptor, A, respectively, corrected for background and cross talk between the donor and acceptor detectors. In Eq. (16.13), γ corrects for differences in quantum yield and detection efficiency between the donor and acceptor fluorophores. To discriminate between signal and noise, E is only calculated for photon bursts in which the total number of photons, $I_A + I_D$, are above a minimum threshold, and the resulting spFRET efficiencies from each photon burst (Fig. 16.4A) are compiled into a histogram where the number of peaks and the peak widths detail the conformational distribution (Fig. 16.4B and C) (Dahan *et al.*, 1999). SpFRET efficiency histograms are often fit to Gaussian distributions and detailed discussions of data analysis for spFRET may be found in work by Seidel and coworkers (Antonik *et al.*, 2006; Kalinin *et al.*, 2007; Sisamakis *et al.*, 2010) and by Gopich and Szabo (2007, 2010).

5.2. Conformational distributions of trypsin- α_1 AT complexes

Peaks in spFRET efficiency histograms have an inherent width associated with noise, and conformational heterogeneity can further increase this width (Antonik *et al.*, 2006; Gopich and Szabo, 2007). In agreement with differences in protease structural disruption between X-ray crystal structures for BTryp- α_1 AT (Huntington *et al.*, 2000) and elastase- α_1 AT (Dementiev *et al.*, 2006), BTryp- α_1 AT covalent complexes have wider spFRET peaks than do corresponding RTryp- α_1 AT complexes indicating that the structure around trypsin residue 113 is more disrupted in BTryp complexes. Despite the conformational differences between the BTryp and RTryp covalent complexes, time-dependent spFRET experiments show that both complexes dissociate with similar rate constants ($6-7 \times 10^{-6} \text{ s}^{-1}$) at pH 7.4 when the protease is fully translocated. Interestingly, the fluorophores used in this study mediated trapping of covalent complexes in which RTryp is not fully translocated 70 Å across α_1 AT (Liu *et al.*, 2006, 2007). This species was identified from an additional peak in RTryp- α_1 AT spFRET histograms (Fig. 16.4C) and only occurs when α_1 AT is labeled with a cationic fluorophore at Cys232. Interactions between cationic fluorophores and anionic RTryp stabilize the partially translocated RTryp resulting in RTryp- α_1 AT complexes with a shorter lifetime, ~ 3 h at pH 7.4, and dissociation of the partially translocated complexes is accelerated at both low and high pH indicating that the RTryp active site is not fully disrupted in these complexes (Liu *et al.*, 2007).

These experiments demonstrate the utility of spFRET for monitoring protease-serpin covalent complexes both for its ability to delineate the conformational distribution and to monitor changes in this distribution over time. The serendipitous trapping of shorter-lived covalent complexes suggest that protease-serpin covalent complex stability may be tuned not only by altering the length of the reactive center loop (Plotnick *et al.*, 2002; Zhou *et al.*, 2001) but also by modulating electrostatic interactions between proteases and serpins.

5.3. A protocol for spFRET on protease-serpin complexes

1. Trypsin purification and labeling: Both trypsins have 12 native Cys residues all of which are disulfide bonded. For labeling, the surface accessible residue 113 was mutated to Cys (Liu *et al.*, 2006; Mellet *et al.*, 2002). Ser113Cys BTryp was expressed as inclusion bodies in *Escherichia coli* cells, purified and activated (Peterson *et al.*, 2001). Lys113Cys RTryp was expressed in yeast cells as a secreted protein, purified and activated (Hedstrom *et al.*, 1994). The trypsin variants were

generally labeled with the donor fluorophore Alexa Fluor 555 maleimide (Invitrogen) and a soybean trypsin inhibitor column (Sigma Chemical) was used to separate trypsin from unreacted fluorophores (Liu *et al.*, 2006).

2. α_1 AT purification and labeling: α_1 AT containing the single native Cys, Cys232, or single Cys variants in the Cys232Ser background, was expressed as inclusion bodies in *E. coli*, purified and checked for activity (Liu *et al.*, 2006). α_1 AT was generally labeled with the acceptor fluorophore Atto610 maleimide.
3. Covalent complex formation: Trypsin and α_1 AT (0.5–2 μ M) were incubated for 15 m at room temperature at a 1:2 molar ratio and complex formation was confirmed by SDS-PAGE. Samples were diluted to 100–50 pM in buffer containing 10 mM of the trypsin inhibitor benzamidine to inhibit free trypsin. SpFRET experiments were performed on 300 μ l samples on coverglass (Nalgene/Nunc) that had been incubated with 1 mg/ml BSA to prevent nonspecific protein adsorption.
4. Brief description of the spFRET setup: Samples were excited with the 488 nm laser light from an Ar–Kr laser in a confocal microscope based on an IX-70 inverted microscope (Olympus) with a 60 $\times$ NA 1.2 water objective and a 100 μ m pinhole (Fig. 16.1). The donor and acceptor fluorescence were separated using a dichroic mirror (Q605LP, Chroma Technologies) and sent to two APDs, one for each color (Liu *et al.*, 2006).
5. SpFRET analysis: The photon counts were collected using hardware and software from ISS, photon bursts were identified, and E was calculated according to Eq. (16.13) using programs written in Matlab (Math-works). Peaks in the spFRET histograms were fit using Origin (OriginLab).

6. Conclusions and Future Directions

The work by Gai and colleagues (Chowdhury *et al.*, 2007; Purkayastha *et al.*, 2005) and by Klenerman and coworkers (Chiou *et al.*, 2009) demonstrate the utility of SMF methods for monitoring early events in polymer formation. More detailed structural information on serpin conformational distributions during the lag phases in polymerization may arise from studies that combine FCS or TCCD with spFRET (Li *et al.*, 2005; Orte *et al.*, 2008b). SpFRET studies of serpin folding, which can help elucidate the structure of folding intermediates (Schuler and Eaton, 2008), could also contribute to an understanding of serpin polymerization by identifying conformational distributions associated with serpin folding intermediates.

Ideally, one would like to follow serpin folding, protease–serpin complex formation or serpin polymer formation by following the same molecule over time. While serpin immobilization has been difficult, a number of different approaches have been developed for monitoring one or more molecules encapsulated in a small volume (Huebner *et al.*, 2009; Reiner *et al.*, 2006; Srisa-Art *et al.*, 2010). Encapsulation of a labeled serpin and a labeled protease in the same droplet should allow protease–serpin complex formation to be followed from the initial encounter complex to the final covalent complex. Similarly, early events in polymer formation might be followed by encapsulating just a few serpins in a single droplet. Finally, newly developed super-resolution methods for imaging single molecules in cells (Patterson *et al.*, 2010) may allow for detailed studies of the motions and localization of intracellular serpins as was recently demonstrated for ovalbumin injected into cells (Speil and Kubitscheck, 2010).

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Abbreviations

α_1AT	α_1 -antitrypsin
AF488	Alexa Fluor 488
AF647	Alexa Fluor 647
APD	avalanche photodiode
BSA	bovine serum albumin
BTryp	bovine trypsin
FRET	Förster resonance energy transfer
FCS	fluorescence correlation spectroscopy
RTryp	rat trypsin
SMF	single molecule fluorescence
spFRET	single pair FRET
TCCD	two-color coincidence detection

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Figure 16.1.

Schematic of a one-photon SMF microscope designed to interrogate fluorescently labeled molecules freely diffusing in solution. The excitation wave-length(s) is reflected into the microscope objective, Obj, by a dichroic mirror, DM2, and is focused into the sample. The emitted fluorescence is transmitted by DM2, any residual laser light is blocked by a longpass or notch filter, F1, and the tube lens focuses the fluorescence onto the confocal pinhole which blocks fluorescence from out-of-focus molecules, spatially restricting the observation volume. The fluorescence is then collimated by lens L1. For spFRET or TCCD, the fluorescence is split by color using another dichroic mirror, DM3, and focused onto high quantum efficiency detectors, Det1 and Det2, by lenses L2 and L3, respectively. The bandpass filters in front of the detectors, F2 and F3, block Raman scattering from water and minimize cross talk between the detectors. DM3 may be replaced by a polarizing beamsplitter for anisotropy experiments or by a 50–50 beamsplitter. The first dichroic mirror, DM1, allows the use of two different wavelength excitation beams.

Figure 16.2.

Detecting oligomerization with FCS. (A) Schematic of a molecule diffusing through the observation volume, assumed to be a Gaussian volume with x - y radius ω_0 and axial extent z_0 . (B, C) FCS polymerization data for α_1 AT incubated at 45 °C for 0 h (black), 24 h (blue), and 48 h (red). (B) As polymerization proceeds, the number of independent proteins decreases, increasing the amplitude of the correlation curves. (C) Polymerization also increases the average size of the molecular assemblies, increasing the diffusion time and shifting the correlations to longer time as shown for the normalized correlation functions. (D) As the α_1 AT polymerization reaction proceeds, the distribution of diffusion times, τ_D , shifts to longer times. These data, adapted with permission from Purkayastha *et al.* (2005) copyright 2005 American Chemical Society, are for fits to Eq. (16.6) rather than Eq. (16.7) so the percentage of large polymers may be overestimated (see Section 4.1.1).

Figure 16.3.

Detecting oligomerization with TCCD. (A) For monomers (left), photons with a single color are detected on one of the detectors. Oligomers emit photons with two colors (right) resulting in coincident photon bursts. (B) Fluorescence intensity in the blue (bottom) and red (top) channels as a function of time with coincident photon bursts indicated by stars. The intensity of the signal in each channel is proportional to the number of labeled monomers in an oligomer. (A) and (B) were adapted from Orte and colleagues, copyright (2008) National Academy of Sciences, USA (Orte *et al.*, 2008a). (C) As neuroserpin polymerizes the probability of coincident photon bursts increases leading to a time-dependent increase in the association quotient, Q (Eq. (16.8)), that can be fit using a kinetic model for neuroserpin polymerization (Chiou *et al.*, 2009). Reprinted from Chiou *et al.* (2009) with permission from Elsevier.

Figure 16.4.

Detecting conformational distributions with spFRET (adapted from Liu *et al.* (2006)). (A) Detection of photons from single protease–serpin complexes, indicated by arrows, traversing the observation volume. These complexes are heterogeneous with some showing mostly donor fluorescence (low spFRET efficiency) and some showing mostly acceptor fluorescence (high spFRET efficiency). (B, C) SpFRET efficiencies from the individual complexes are compiled into a histogram to reveal the conformational distribution for protease–serpin complexes. The large “zero peak” centered around 0% efficiency arises from complexes containing only donor fluorophores or complexes in which the acceptor has photobleached. (B) Aside from the zero peak, BTryp– α_1 AT complexes show a single, broad conformational distribution with a Gaussian fit to the distribution given by the black line. (C) When cationic fluorophores are attached to Cys232 on α_1 AT, interactions between

anionic RTryp and the cationic fluorophore trap partially translocated RTryp resulting in a second peak at high FRET efficiency. The inset shows that this conformation is not observed for the RTryp- α_1 AT encounter complex where the serpin and protease are not covalently attached.

Table 16.1

Applications of SMF techniques to biological problems

Technique	Applications
FCS	<p>Serpin polymerization (Chowdhury <i>et al.</i>, 2007; Purkayastha <i>et al.</i>, 2005)</p> <p>Protein folding (Chattopadhyay <i>et al.</i>, 2005; Sherman <i>et al.</i>, 2008)</p> <p>Biomolecular interactions (Thompson, 1991)</p> <p>Protein conformational changes (Chen <i>et al.</i>, 2007; Neuweiler <i>et al.</i>, 2007)</p>
TCCD	<p>Serpin polymerization (Chiou <i>et al.</i>, 2009) and amyloid formation (Orte <i>et al.</i>, 2008a)</p> <p>Biomolecular interactions (Orte <i>et al.</i>, 2006)</p>
spFRET	<p>Conformational distributions during protein folding (Nienhaus, 2009; Schuler and Eaton, 2008)</p> <p>Protein conformational changes (Hoffmann <i>et al.</i>, 2007; Sharma <i>et al.</i>, 2008)</p> <p>Biomolecular interactions (Hoffman <i>et al.</i>, 2010; Liu <i>et al.</i>, 2006; Sharma <i>et al.</i>, 2008)</p>

Table 16.2Reported R_0 values for donor–acceptor pairs commonly used for spFRET experiments^a

Donor	Acceptor	R_0 (Å)
Alexa Fluor 488	Alexa Fluor 594	54 (Mukhopadhyay <i>et al.</i> , 2007; Müller-Späth <i>et al.</i> , 2010)
Alexa Fluor 488	Texas Red	48 ^b (Liu <i>et al.</i> , 2006)
Alexa Fluor 555	Atto 610	59 ^b (Liu <i>et al.</i> , 2006)
Alexa Fluor 555	Cy5	59 ^b (Liu <i>et al.</i> , 2006)
Cy3	Cy5	53 (Ishii <i>et al.</i> , 1999)
		~60 (Murphy <i>et al.</i> , 2004)

^a R_0 depends on the donor quantum yield and emission spectrum, the acceptor absorption spectrum, and the rotational freedom of the fluorophores (Eq. (16.12)) all of which may be influenced by the local fluorophore environment. R_0 should therefore be calculated based on experimental data for the biomolecule(s) of interest.

^bUsed in serpin spFRET experiments.