Supplementary Information for *Detecting Intramolecular Dynamics and Multiple FRET States by Fluorescence Correlation Spectroscopy*

E. Shane Price, Matthew S. DeVore, and Carey K. Johnson

Burst measurements for Gly-(Pro)$_{15}$-Cys

For burst measurements, the concentration of polyproline was lowered to 200 pM and the laser power increased to 20 μW so that bursts could be detected as single-molecules diffused through the observation volume. The data were binned with 300 μs time resolution. All bins where the sum of the green and red channels was greater than 14 were collected into an array of green and red counts and the FRET efficiencies were calculated. Often in burst measurements a minimum count threshold is set for the red channel to ensure that both green and red fluorophores were photoactive. However, because such selection is not possible in FCS measurements, to characterize the FRET states detected in FCS, bursts with low acceptor counts were not rejected. The FRET efficiency was calculated by

$$E = \frac{c(I_A - bI_D)}{c(I_A - bI_D) + I_D}$$

were b=0.11 is the correction for cross-talk of 0.11 of the donor in the red channel and c=1.1 corrects for the detection efficiency of the red channel relative to the green channel. The resulting histogram of the apparent FRET efficiencies is shown in Figure S1. A dividing line was chosen at a FRET efficiency of 0.4 and the average E and fraction above and below the threshold was calculated. The average efficiency E_1 above the boundary was 0.82 with a fraction of 0.74, and the average efficiency E_2 below the boundary was 0.08 with a fraction of 0.26.

These values are consistent with the initial FCS amplitudes (see manuscript text).

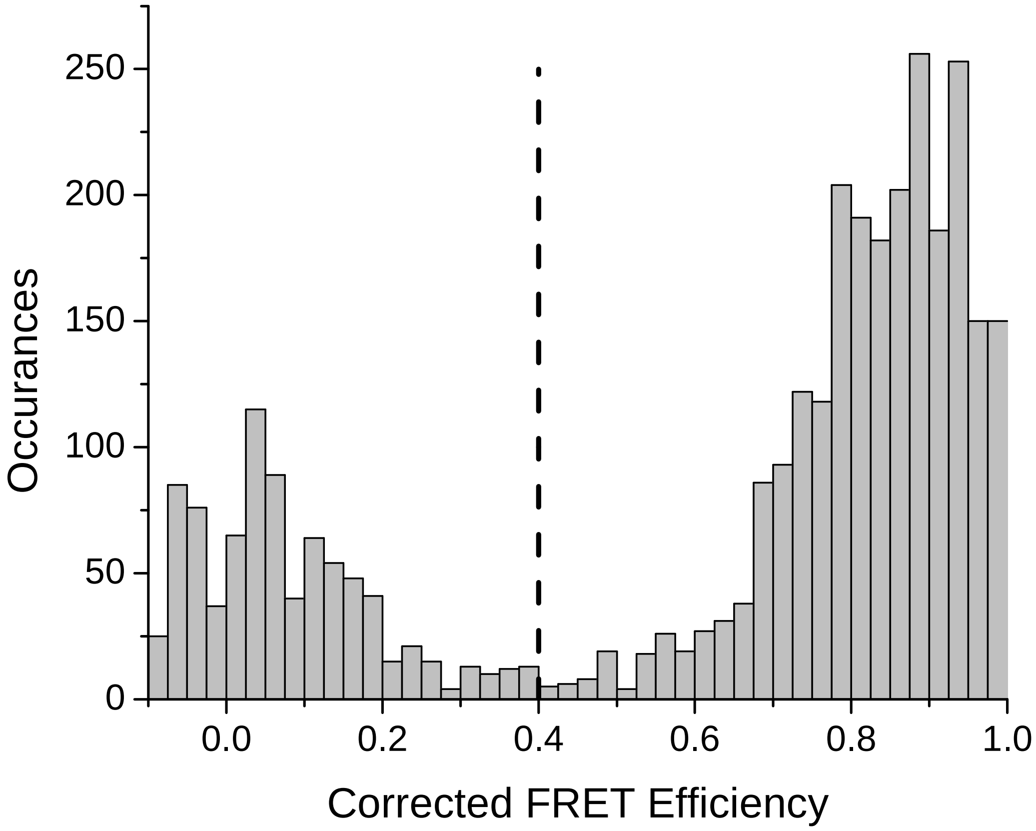

Figure S1: FRET efficiency distribution for polyproline-AF 488-TR. The average E and fraction above (E_1, f_1) and below (E_2, f_2) the threshold level (dashed line) were calculated: E_1 =0.82, f_1 = 0.74, E_2 = 0.08, and f_2 =0.26.

FCS with three interchanging FRET states

We describe here the correlation response expected for interchange among three FRET states according to the chemical reactions:

$$E_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} E_3$$

Following methods described in references {Berne, 1976 #379;Widengren, 2002 #380} the fluctuations in the concentration of species E_1, E_2, and E_3 can be found. The resulting contributions to the FCS correlation functions in eqs (1) and (3) from intramolecular dynamics are then given by:

$$E_{DD}(\tau) = \left[1 + a\exp(-\tau/\tau_1) + b\exp(-(\tau/\tau_2)\right] \tag{S1}$$

$$E_{AA}(\tau) = \left[1 + c\exp(-\tau/\tau_1) + d\exp(-\tau/\tau_2)\right] \tag{S2}$$

$$E_{DA}(\tau) = E_{AD}(\tau) = \left[1 + \sqrt{ac}\exp(-\tau/\tau_1) + \sqrt{bd}\exp(-\tau/\tau_2)\right] \tag{S3}$$

where $E_{DD}(\tau)$ is the time dependence for intramolecular dynamics in the donor-donor autocorrelation function, $E_{AA}(\tau)$ is the time dependence for intramolecular dynamics in the acceptor-acceptor autocorrelation function, and $E_{DA}(\tau) = E_{AD}(\tau)$ is the time dependence for intramolecular dynamics in the donor-acceptor or acceptor-donor cross-correlation function. In eqs S1 to S3:

$$a = \frac{\left(Q_1^D - Q_2^D\right)^2 f_1 k_{12}(A-J)^2 + \left(Q_3^D - Q_2^D\right)^2 f_3 k_{32}(B-J)^2 - 2 f_1 f_3 L\left(Q_1^D - Q_3^D\right)^2}{J(J+K)\left(Q_1^D f_1 + Q_2^D f_2 + Q_3^D f_3\right)^2}$$

$$b = \frac{\left(Q_1^D - Q_2^D\right)^2 f_1 k_{12}(A-J)^2 + \left(Q_3^D - Q_2^D\right)^2 f_3 k_{32}(B-J)^2 - 2 f_1 f_3 L\left(Q_1^D - Q_3^D\right)^2}{J(J-K)\left(Q_1^D f_1 + Q_2^D f_2 + Q_3^D f_3\right)^2}$$

$$c = \frac{\left(Q_1^A - Q_2^A\right)^2 f_1 k_{12}(A-J)^2 + \left(Q_3^A - Q_2^A\right)^2 f_3 k_{32}(B-J)^2 - 2 f_1 f_3 L\left(Q_1^A - Q_3^A\right)^2}{J(J+K)\left(Q_1^A f_1 + Q_2^A f_2 + Q_3^A f_3\right)^2}$$

$$d=\frac{\left(Q_1^A-Q_2^A\right)^2 f_1 k_{12}\left(A-J\right)^2+\left(Q_3^A-Q_2^A\right)^2 f_3 k_{32}\left(B-J\right)^2-2 f_1 f_3 L\left(Q_1^A-Q_3^A\right)^2}{J\left(J-K\right)\left(Q_1^A f_1+Q_2^A f_2+Q_3^A f_3\right)^2}$$

$$\tau_1=2\left(K+J\right)^{-1} \qquad \tau_2=2\left(K-J\right)^{-1}$$

$$A=k_{12}+k_{21}+k_{23}-k_{32} \qquad B=-k_{12}+k_{21}+k_{23}+k_{32}$$

$$K=k_{12}+k_{21}+k_{32}+k_{23} \qquad L=k_{12}k_{23}+k_{32}k_{21}+k_{12}k_{32}$$

$$J=\left[\left(k_{12}+k_{21}-k_{23}-k_{32}\right)^2+4k_{23}k_{21}\right]^{1/2}$$

$$Q_i^D=q_D\sigma\phi^D\left(1-E_i\right) \qquad Q_i^A=q_A\sigma\phi^A E_i$$

where $q_{D(A)}$ is the detection efficiency in the donor (acceptor) channel, σ is the excitation cross section, $\phi^{D(A)}$ is the quantum efficiency of the donor (acceptor), and f_i is the fractional population in state i, given by $f_1=\frac{k_{32}k_{21}}{L}$; $f_2=\frac{k_{12}k_{32}}{L}$; $f_3=\frac{k_{12}k_{23}}{L}$. We note that for τ=0, eqs S1-S3 reduce to eqs 5-7 (extended to 3 FRET states).