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Supplemental Materials:

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Translational Diffusion, Molecular Brightness, and Energy Transfer Analysis of mEGFP–Linker–mScarlet–I Crowding Biosensor using Fluorescence Correlation Spectroscopy

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2. Materials and Methods:

2.3 FCS Data Analysis: In single-detector FCS, time-dependent fluorescence fluctuations, $\delta F(t)$ as individual molecules diffuse through the observation volume are described by (1):

$$\delta F(t) = F(t) - \langle F(t) \rangle \tag{S.1}$$

The observed fluorescence fluctuation is caused by the changing number of molecules in the observation volume because of translational diffusion, chemical reactions, and fast photophysical processes, including fluorescence blinking, intersystem crossing, or conformational changes (2-4). The corresponding autocorrelation curve, $G(\tau)$, is calculated using the measured time-dependent fluorescence fluctuation, $\delta F(t)$, with itself as a function of a lag time (τ), *i.e.,* $\delta F(t+\tau)$, such that (5, 6):

$$G(\tau) = \frac{\langle \delta F(t) \otimes \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \tag{S.2}$$

Assuming a three-dimensional (3D) Gaussian profile of the observation volume, the fluorescence fluctuation autocorrelation curve, $G(\tau)$, due to translational diffusion plus photophysical processes such as fluorescence blinking (with a population fraction f_B and time constant τ_B), can be written as follows: (7)

$$G(\tau) = \frac{1}{N}\left(1 - \frac{\tau}{\tau_D}\right)^{-1}\left(1 - \frac{\tau}{s^2 \tau_D}\right)^{-1/2} \times \left(1 - f_B + f_B \cdot e^{-\tau/\tau_B}\right) \tag{S.3}$$

where N is the average number of molecules residing in the observation volume, whose structure parameter (s) is the ratio of the axial (z) to the lateral (r) extension of that volume such that $s = z/r$. It is worth noting that under 488-nm excitation of the donor, the autocorrelation curve of cleaved and intact GE2.3 does not exhibit a fluorescence blinking component (i.e., $f_B = 0$) in contrast to the autocorrelation of the acceptor under 561-nm excitation. By selecting a low laser intensity region and the linear response regime of the excited molecule, it is possible to minimize the photobleaching, additional laser-induced

photophysical processes, and potential direct excitation of the acceptor. The measured diffusion time (τ_D) and the translational diffusion coefficient (D_T) of a given molecule are related, where (8):

$$\tau_D = \frac{r^2}{4D_T} \tag{S.4}$$

To calibrate the FCS setup under 488-nm excitation, the diffusion time of Rh110 with a known translational diffusion coefficient (4.3×10^{-6} cm^2/s) is used to determine the radius of the observation volume (9-11).

According to equation (S.3), the initial amplitude of the autocorrelation function, $G_i(\tau = 0)$, of the ith species ($i = D$ for cleaved, $i = DA$ for intact GE2.3) equals the inverse of the average number of molecules (N_i) residing in the open observation volume such that:

$$G_i(\tau = 0) = \frac{1}{N_i} \tag{S.5}$$

Using the time-averaged fluorescence signal (1000 photons per second or kHz), $\langle F_i(t) \rangle$, of the ith species, the corresponding molecular brightness (ψ_i) under a given laser intensity was calculated experimentally using FCS such that:

$$\psi_i = \frac{\langle F_i(t) \rangle}{N_i} \tag{S.6}$$

The molecular brightness is defined as the average number of fluorescence photons detected per fluorophore during their random walk in the open observation volume. In this approach for FRET analysis, the molecular brightness (ψ, kHz/molecule) of the donor in the presence and absence of the acceptor were measured under the same experimental conditions. In these FCS measurements, the concentrations of GE2.3 (both cleaved and intact) and Rh110 were adjusted to yield on average 20-60 molecules residing in the observation volume such that the detected fluorescence signal was higher (>95%) of any background signal from the blank samples (i.e., buffer enriched with Ficoll only).

Statistical analysis of the molecular brightness of the cleaved and intact GE2.3 was carried out using a students *t*-test (ANOVA, OriginPro software).

Under the same experimental conditions of laser intensity, excitation wavelength of the donor, observation volume, and the detection efficiency, the FRET efficiency can be written in terms of the molecular brightness of the donor in the presence (ψ_{DA}) and absence (ψ_{D}) of the acceptor such that (12):

$$E(\%) = \left(1 - \frac{\psi_{DA}}{\psi_{D}}\right) \times 100 \qquad \text{(S.7)}$$

As a result, FRET analysis can be carried out on freely diffusing donor–linker–acceptor constructs at the single molecule level in terms of the molecular brightness of the excited donor, in the presence and absence of an acceptor, using a traditional single-detector FCS setup. This approach eliminates any error due to the difference in concentrations of the prepared sample used in steady-state fluorescence intensity of the donor-acceptor constructs and their cleaved counterpart for FRET analysis. Importantly, this experimental design accounts for the effects of the refractive index of Ficoll-70 solutions on the measured molecular brightness of cleaved and intact GE2.3.

In these measurements, we recorded the fluorescence fluctuations (20 scans, 10 seconds each) and the corresponding autocorrelation curve for both the cleaved and intact sensor under the same experimental conditions. Each scan was used to estimate the average number of molecules, the average fluorescence fluctuation signal, and therefore the corresponding molecular brightness for box and whisker plots. Under 488-nm excitation, the FCS observation volume was calibrated using Rh110 with known diffusion coefficient (4.3×10^{-6} cm^2/s) (13). As the Ficoll concentration increases, the background signal of blank samples starts to increase slightly above the dark noise of the detector or the blank buffer. As a result, we ensured that any background signal from the blank Ficoll solutions was less that 5% of the fluorescence signal from cleaved and intact GE2.3. This was accomplished by measuring the blank samples under the same experimental condition at different concentration of Ficoll and any observed background signal was subtracted from the corresponding protein signal under the same conditions. We also adjusted the

concentration and therefore the average number of molecules residing in the observation volume to ensure large fluorescence signal (>95%) from GE2.3 samples.

For translational diffusion analysis under 488 nm (exciting the donor) and 561 nm (exciting the acceptor), the measured diffusion time in our calibrated FCS setup was used to calculate the translational diffusion coefficient (D_T) and therefore the hydrodynamic radius (R_h) of cleaved and intact GE2.3 using Stokes-Einstein model using (11):

$$D_T = \frac{k_B T}{6\pi\eta R_h} \tag{S.8}$$

Where k_B and T are the Boltzmann constant (1.38 x 10^{-16} g cm^2 s^{-2} K^{-1}) and temperature (295 K). The molecular weight of a given protein can also be used to approximately calculate the corresponding hydrodynamic volume (V_h) (11, 14):

$$V_h\left(nm^3\right) = \frac{M}{N_A}\left(\upsilon + \vartheta h\right) \times 10^{21} nm^3 / cm^3 \tag{S.9}$$

Where N_A is Avogadro's number (6.023 x 10^{23} molecule/mole). The partial specific volume (υ) of a protein molecule ($\upsilon = 0.73\, cm^3/g$) and for a water molecule ($\vartheta = 1.0018\, cm^3/g$). The hydration ($h$) is assumed to be 0.3 g H_2O/g Protein [These two sentences need to be rewritten for clarity]. The hydration of the protein was accounted for using the partial specific volume (υ) of a protein molecule ($\upsilon = 0.73\, cm^3/g$) and for a water molecule ($\vartheta = 1.0018\, cm^3/g$). The hydration ($h$) is assumed to be 0.3 g H_2O/g Protein. Assuming a spherical shape for the protein, the corresponding hydrodynamic radius (R_h) can also be approximated, where:

$$R_h\left(nm\right) = \left(\frac{3V_h(nm^3)}{4\pi}\right)^{1/3} \tag{S.10}$$

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