Appendix

Real-time Observation of Flexible Domain Movements in Cas9

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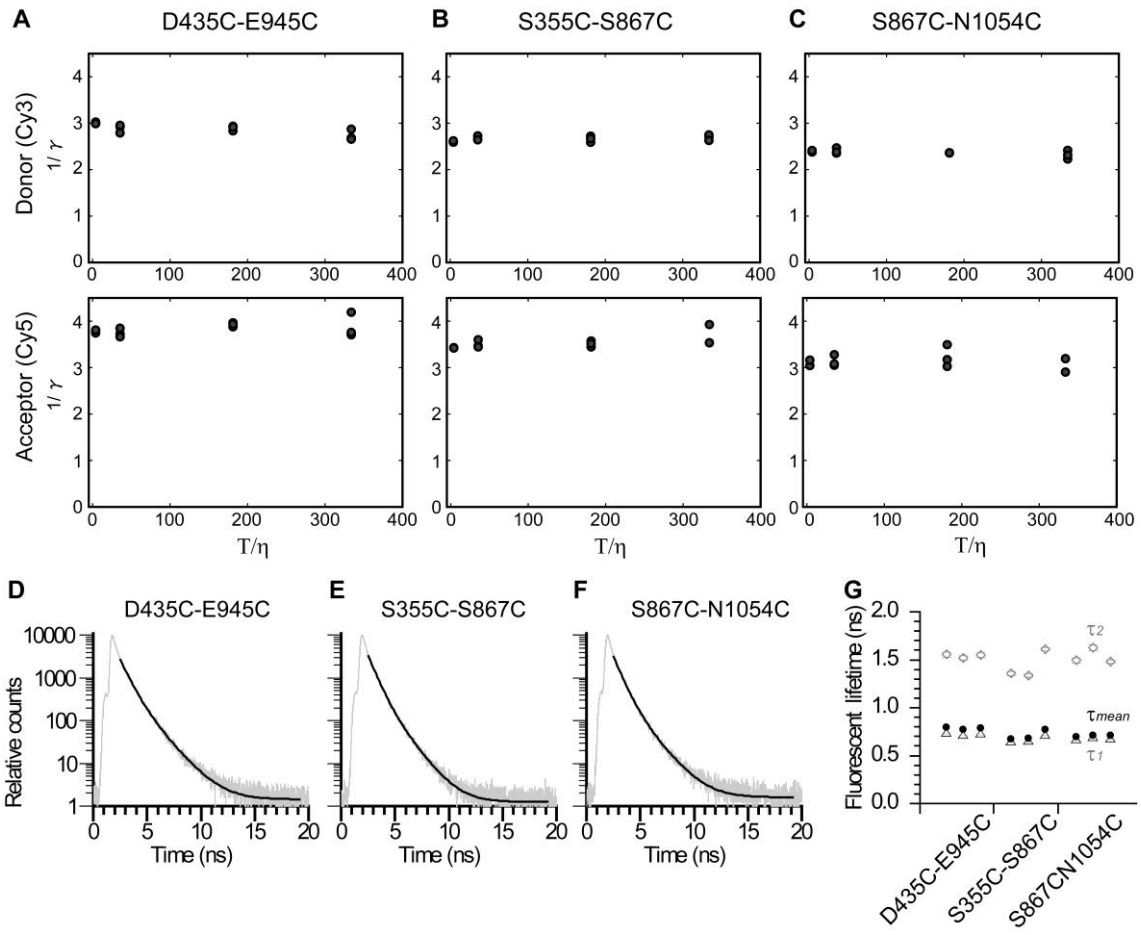
Contents:



Appendix Figure S1 - Fluorescence anisotropy and lifetime of dyes on Cas9 constructs.

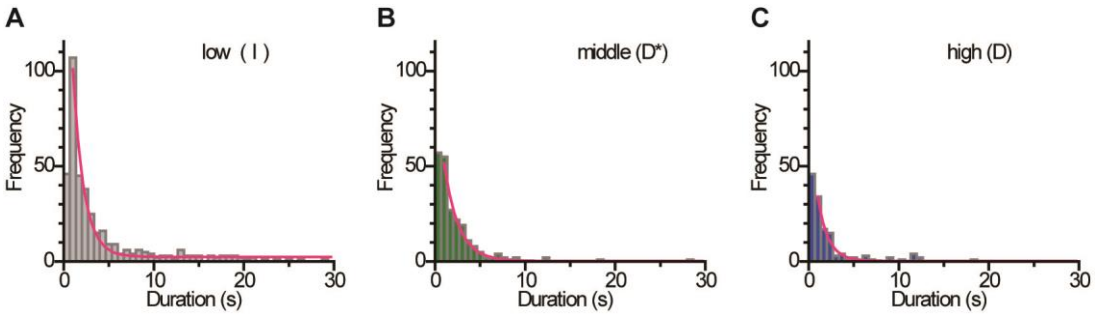
A-C Plots of the inverse fluorescence anisotropies (γ) of Cy3 and Cy5 on Cas9 constructs against T/η. Here, absolute temperature (T) = 298 K, and the viscosities of the sample (η) corresponding to 0, 0.001, 0.01 and 0.1% methyl cellulose solutions were 0.89, 1.64, 8.39 and 75.89, respectively. The plots are summaries of three individual experiments for each construct (A: D435C-E945C, B: S355C-S867C, C: S867C-N1054C). The y-intercepts were calculated by extrapolating the plots to a linear function, yielding the estimated anisotropy values. The values for Cy3 anisotropy (upper panels) were 0.34 ± 0.006 in D435C-E945C, 0.38 ± 0.004 in S355C-S867C and 0.41 ± 0.004 in S867C-N1054C (mean ± SEM, n = 3), and the

values for Cy5 (lower panels) were γ = 0.27 ± 0.005 in D435C-E945C, 0.29 ± 0.006 in S355C-S867C and 0.32 ± 0.009 in S867C-N1054C (mean ± SEM, n = 3).

In the case of low anisotropy, the orientation factor, κ^2, is close to the dynamic isotropic limit of $\kappa^2 = 2/3$. Otherwise, κ^2 is widely distributed in the range of $0 \leq \kappa^2 \leq 4$. Thus, the high anisotropies of Cy3 and Cy5 obtained, which are close to the theoretical maximum value of 0.4, obscured the value of κ^2 so that we were unable to estimate accurate distances between the two fluorochromes on Cas9 molecules from the FRET efficiency.

D-F Representative time traces of the fluorescence lifetime of Cy3 on Cas9 constructs (D: D435C-E945C, E: S355C-S867C, F: S867C-N1054C). The time trace data (gray) were fitted to double-exponential decay curves (black) to calculate the fluorescence lifetimes.

G Summary of Cy3 lifetimes calculated from three individual experiments for each construct. The grey triangles and diamonds represent the fast (τ_1) and slow (τ_2) components of the lifetime. The mean lifetimes (τ_{mean}, black circle) were 0.78 ± 0.01 for D435C-E945C, 0.71 ± 0.05 for S355C-S867C and 0.71 ± 0.01 for S867C-N1054C (mean ± SEM, n = 3). Bars are SEM. The lifetimes obtained were shorter than normal dye tumbling time (1-10 ns). Therefore, the high anisotropy of the dye on Cas9 constructs (A-C) is most likely due to the short fluorescence lifetimes (D-G).



Appendix Figure S2 - Dwell time histograms of the HNH domain in three positions during flexible movements.

A-C Dwell time distributions of the HNH domain in the I (A), D* (B) and D (C) positions in fluctuating S355C-S867C molecules. Assays were performed in the presence of Mg^{2+}, sgRNA and target DNA. By fitting the distributions to a single exponential decay function (red curves), the mean dwell times were determined as 1.22 ± 0.07 s for the I position (n = 399), 1.61 ± 0.08 s for the D* position (n = 219) and 1.14 ± 0.08 s for the D position (n = 124). Data are shown as the mean ± SEM.

Appendix Figure S3 - DNA sequence used in this study.

The pUC119 plasmid containing the 20-nt target sequence (blue) and the NGG PAM (red) was linearized by *Eco*RI (green) and used as the target DNA. The longest off-target matching sequence to the sgRNA was 4-nt with a PAM sequence (grey highlight). Since Cas9 binding to such a short matching sequence is highly unstable (Singh *et al.*, 2016), we concluded that almost all Cas9 in the sgRNA/DNA-Cas9 ternary complex observed was bound to the target sequence in the DNA.