Dataset S1: Appendix, Single-molecule data analysis summaries

A Kinetic and Thermodynamic Framework for P4-P6 RNA Reveals Tertiary Motif Modularity and Modulation of the Folding Preferred Pathway

Namita Bisaria[a*], Max Greenfeld[a,b*], Charles Limouse[c], Dmitri Pavlichin[c], Hideo Mabuchi[c], Daniel Herschlag[a,b,d,e]

[a] Dept. of Biochemistry, Stanford University, Stanford, CA, USA
[b] Dept. of Chemical Engineering, Stanford University, Stanford, CA, USA
[c] Dept. of Applied Physics, Stanford University, Stanford, CA, USA
[d] Dept. of Chemistry, Stanford University, Stanford, CA, USA
[e] Email: herschla@stanford.edu
[*] These authors contributed equally to this work

*

Contents

SI Appendix:Data Reports

Table S6-1.Variant and Conditions

Variant:	WT
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	92
SNR Threshold[2]	0.75
Number of Traces	368

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S6-2. Folding parameters of smFRET the variant WT inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	6.3	5.8 - 6.8	2.2
	$k_u(s^{-1})$	0.2	0.2 - 0.3	2.8
	K_{eq}	26.8	23.0 - 30.4	4.1
	SNR green	2.4	2.3 - 2.5	0.5
	SNR red	1.8	1.8 - 1.9	0.6
	ΔG(kcal/mol)	-2.2	-2.0 - -1.8	0.8
Fits from Cumulative Data[2]	Lifetime (s)	130.6	118.2 - 145.0	130.6
	$k_{f,\ bulk}(s^{-1})$	4.4	4.4 - 4.3	3.1
	$k_{u,\ bulk}(s^{-1})$	0.3	0.3 - 0.3	0.3
	$K_{eq,bulk}$	12.6	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.5	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

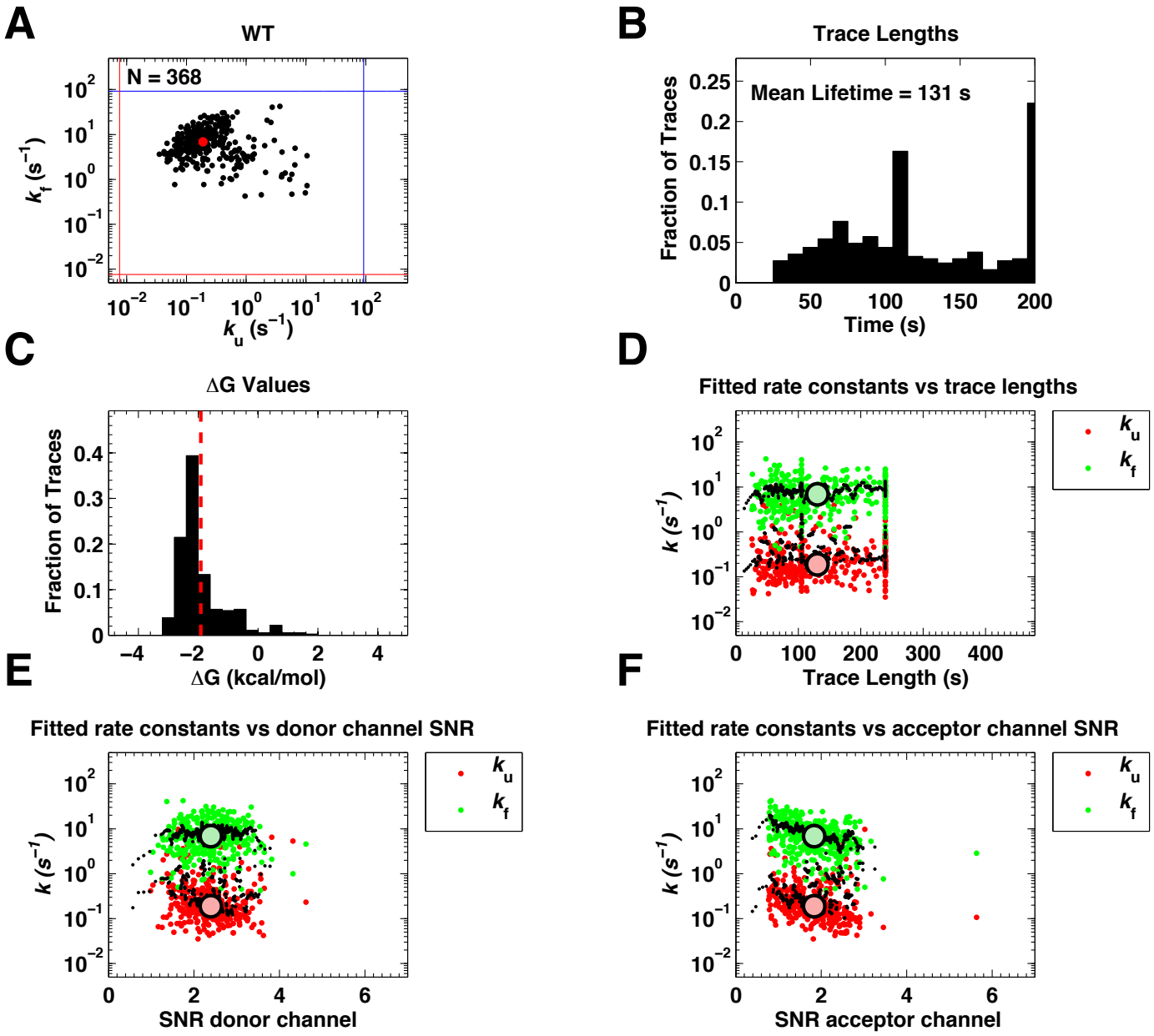


Figure S6-1. smFRET data assessment for WT. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

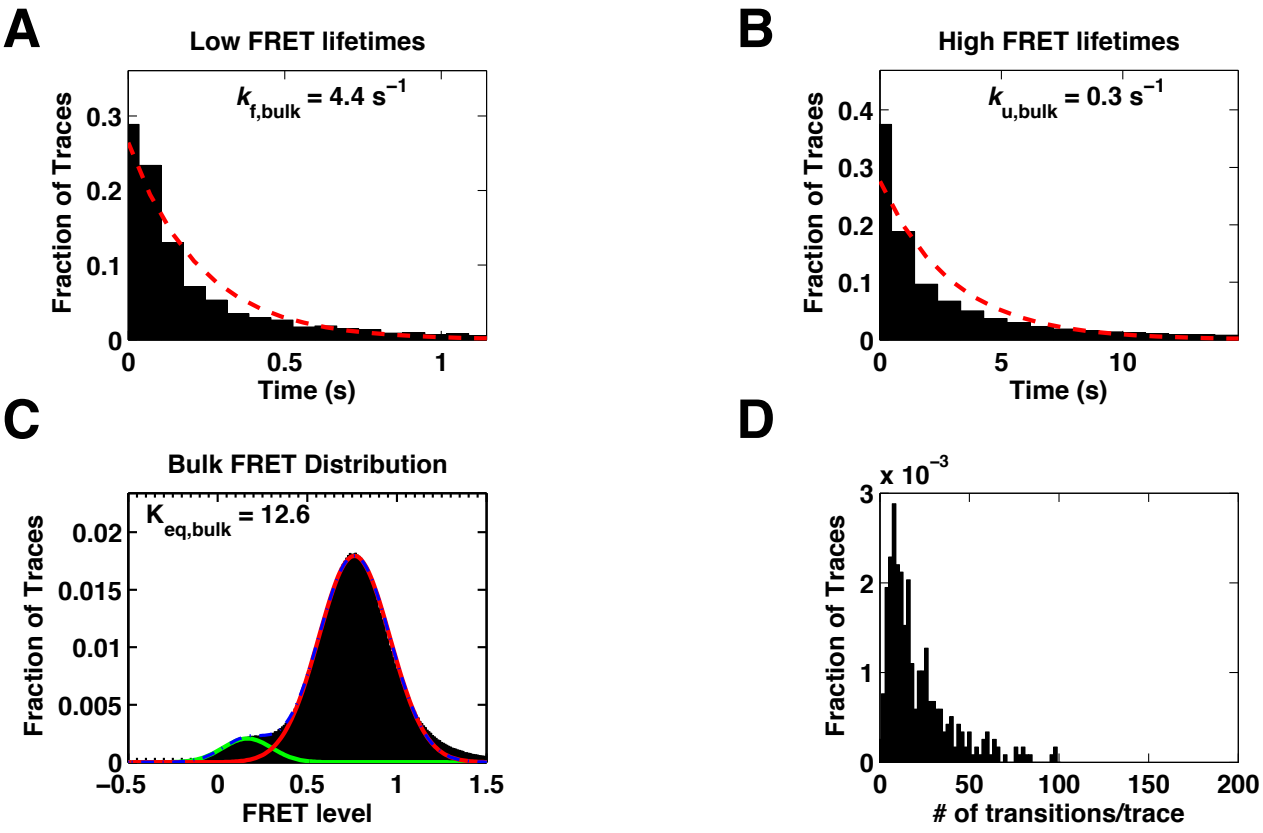


Figure S6-2. smFRET data assesment of aggregate data for WT. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

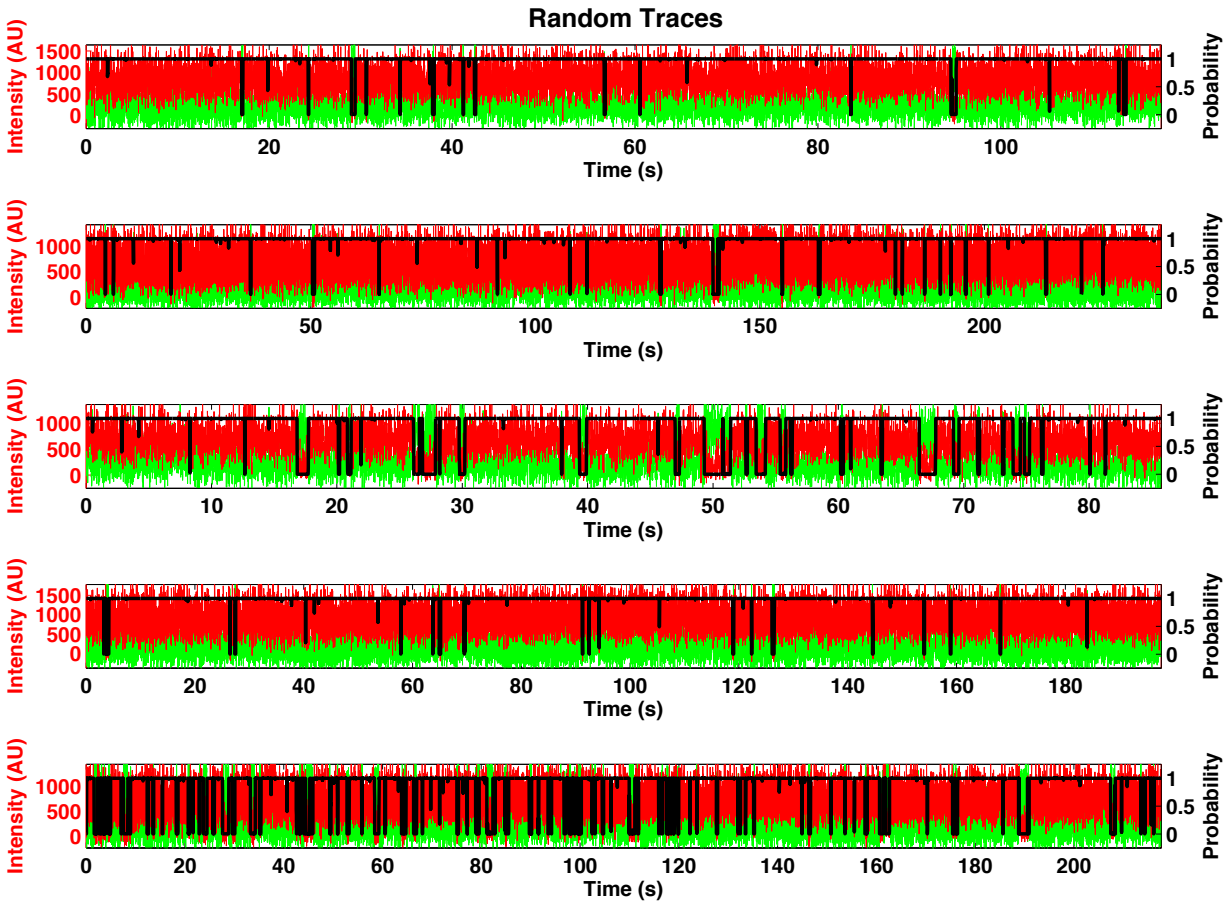


Figure S6-3. Randomly selected FRET traces of WT. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S7-1. Variant and Conditions

Variant:	Nat+2
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	92
SNR Threshold[2]	0.75
Number of Traces	117

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S7-2. Folding parameters of smFRET the variant Nat+2 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	6.5	5.1 - 8.0	3.4
	$k_u(s^{-1})$	0.2	0.2 - 0.2	2.7
	K_{eq}	34.2	26.4 - 41.8	3.4
	SNR green	2.8	2.6 - 2.9	0.8
	SNR red	1.8	1.9 - 2.2	1.0
	ΔG(kcal/mol)	-2.3	-2.2 - -1.9	0.7
Fits from Cumulative Data[2]	Lifetime (s)	111.9	94.1 - 135.3	111.9
	$k_{f,\ bulk}(s^{-1})$	6.5	6.8 - 6.3	3.1
	$k_{u,\ bulk}(s^{-1})$	0.3	0.4 - 0.3	0.3
	$K_{eq,bulk}$	14.1	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.5	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

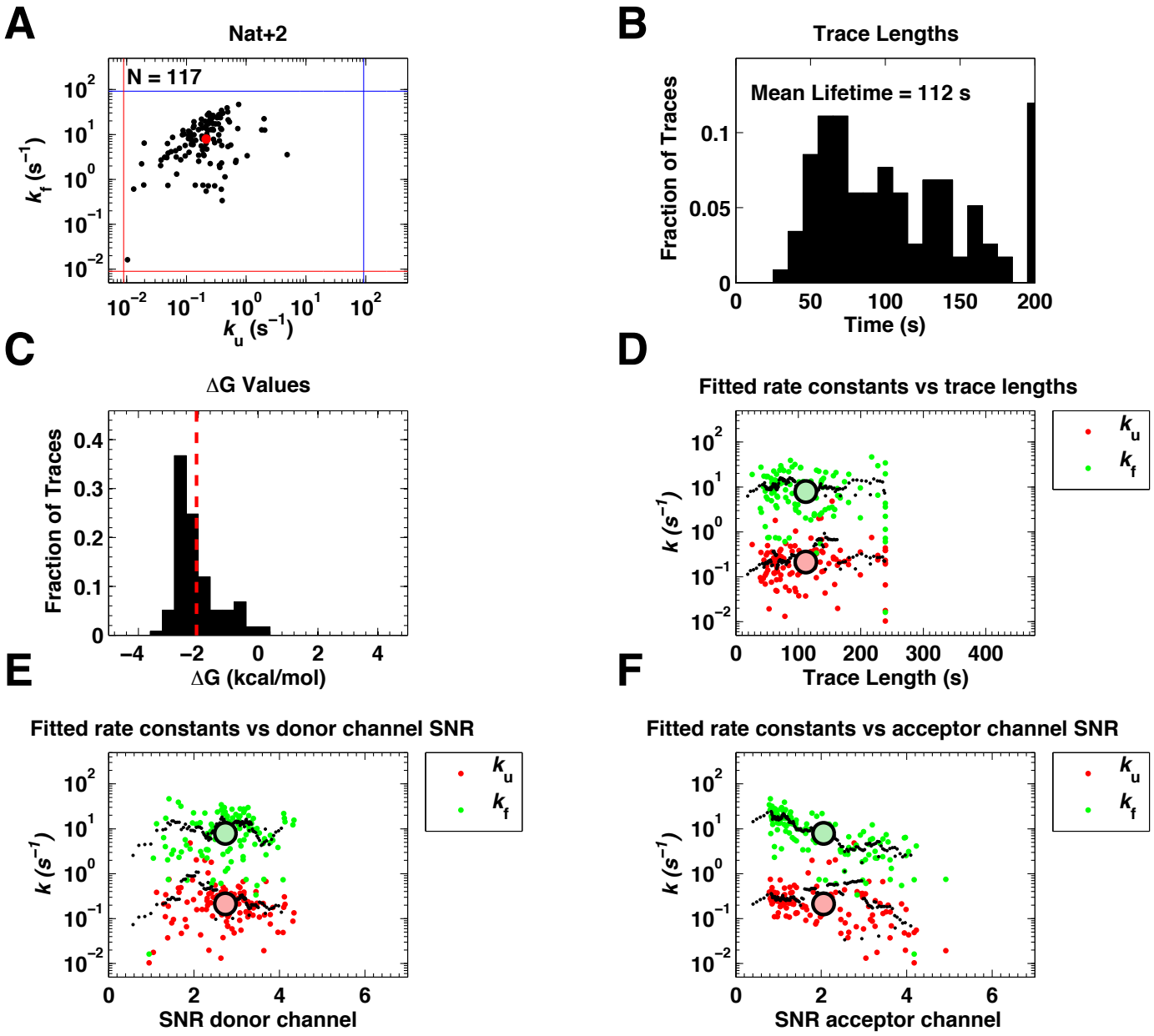


Figure S7-1. smFRET data assessment for Nat+2. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

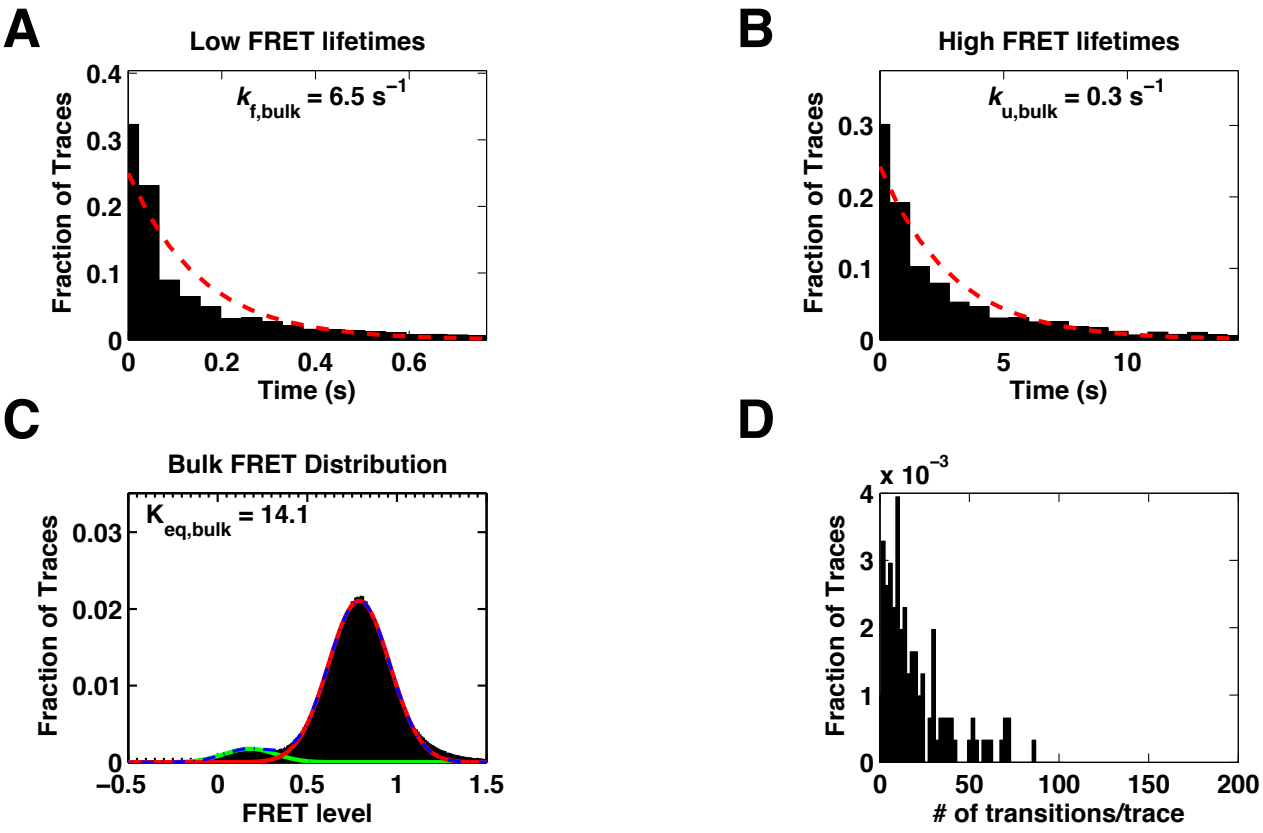


Figure S7-2. smFRET data assesment of aggregate data for Nat+2. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

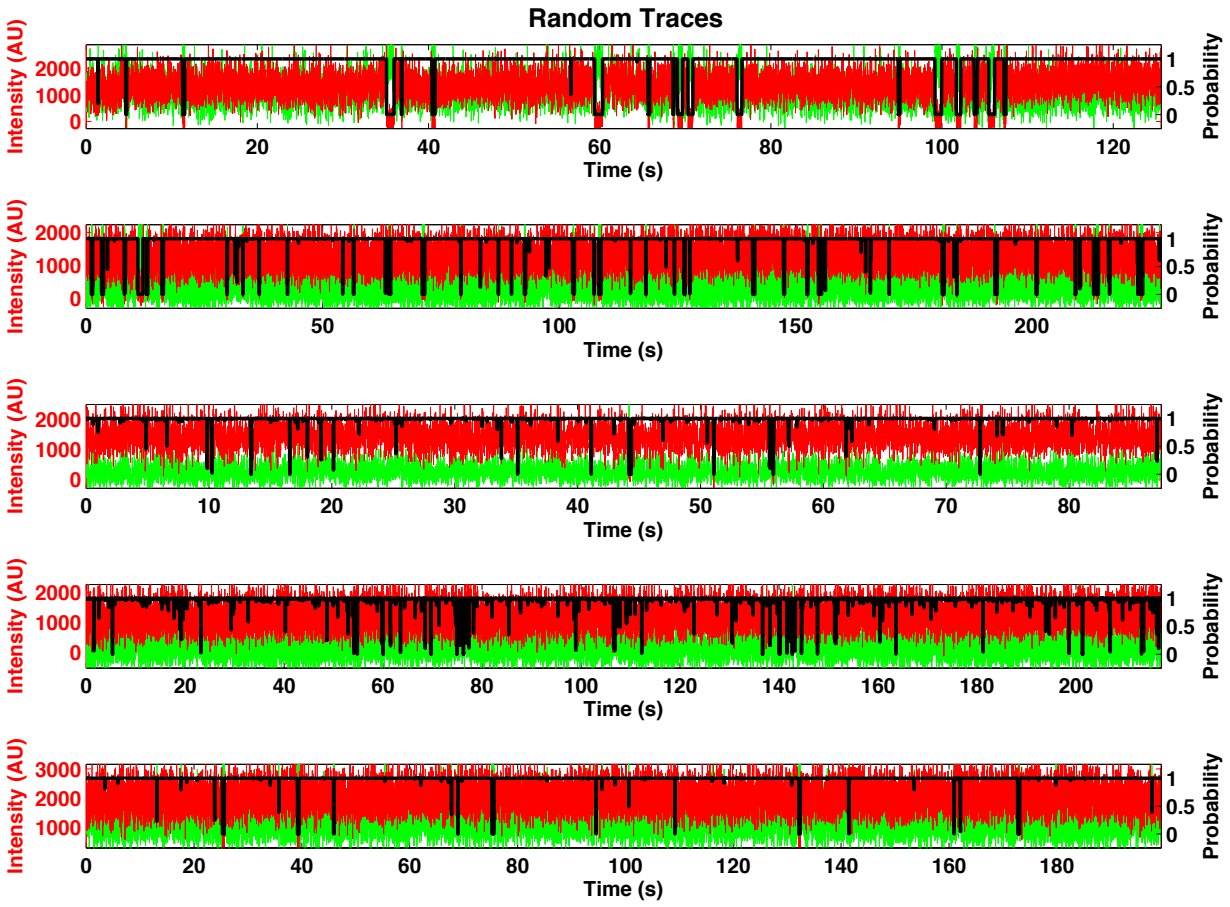


Figure S7-3. Randomly selected FRET traces of Nat+2. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S8-1. Variant and Conditions

Variant:	Nat+3
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	92
SNR Threshold[2]	0.75
Number of Traces	205

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S8-2.Folding parameters of smFRET the variant Nat+3 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	6.7	5.9 - 7.4	2.4
	$k_u(s^{-1})$	0.2	0.1 - 0.2	3.3
	K_{eq}	38.6	31.5 - 45.8	4.0
	SNR green	2.7	2.5 - 2.7	0.6
	SNR red	2.1	2.0 - 2.2	0.8
	ΔG(kcal/mol)	-2.3	-2.2 - -2.0	0.8
Fits from Cumulative Data[2]	Lifetime (s)	99.1	86.8 - 114.2	99.1
	$k_{f,\ bulk}(s^{-1})$	6.1	6.3 - 5.9	4.0
	$k_{u,\ bulk}(s^{-1})$	0.4	0.4 - 0.4	0.3
	$K_{eq,bulk}$	15.9	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.6	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

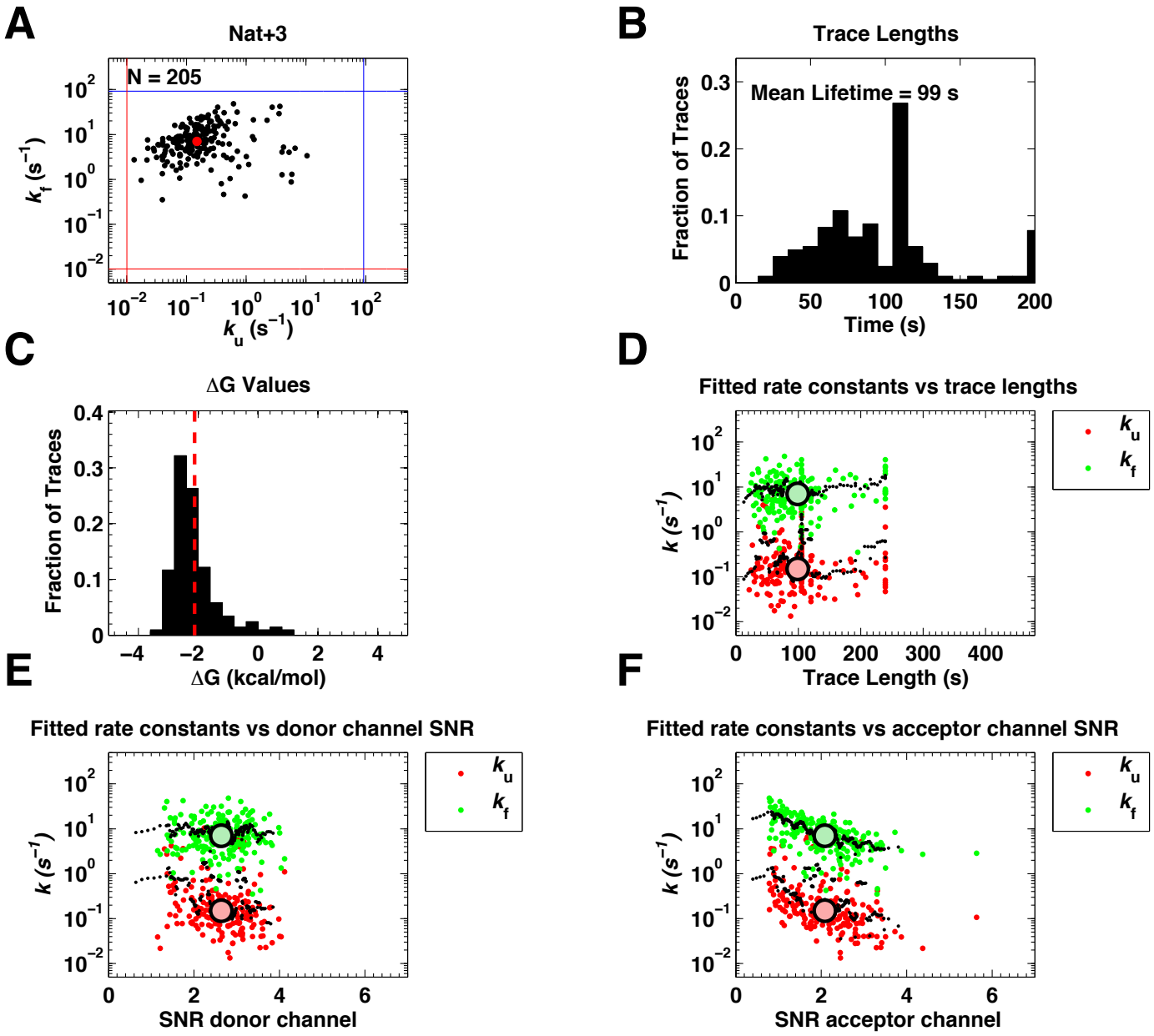


Figure S8-1. smFRET data assessment for Nat+3. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

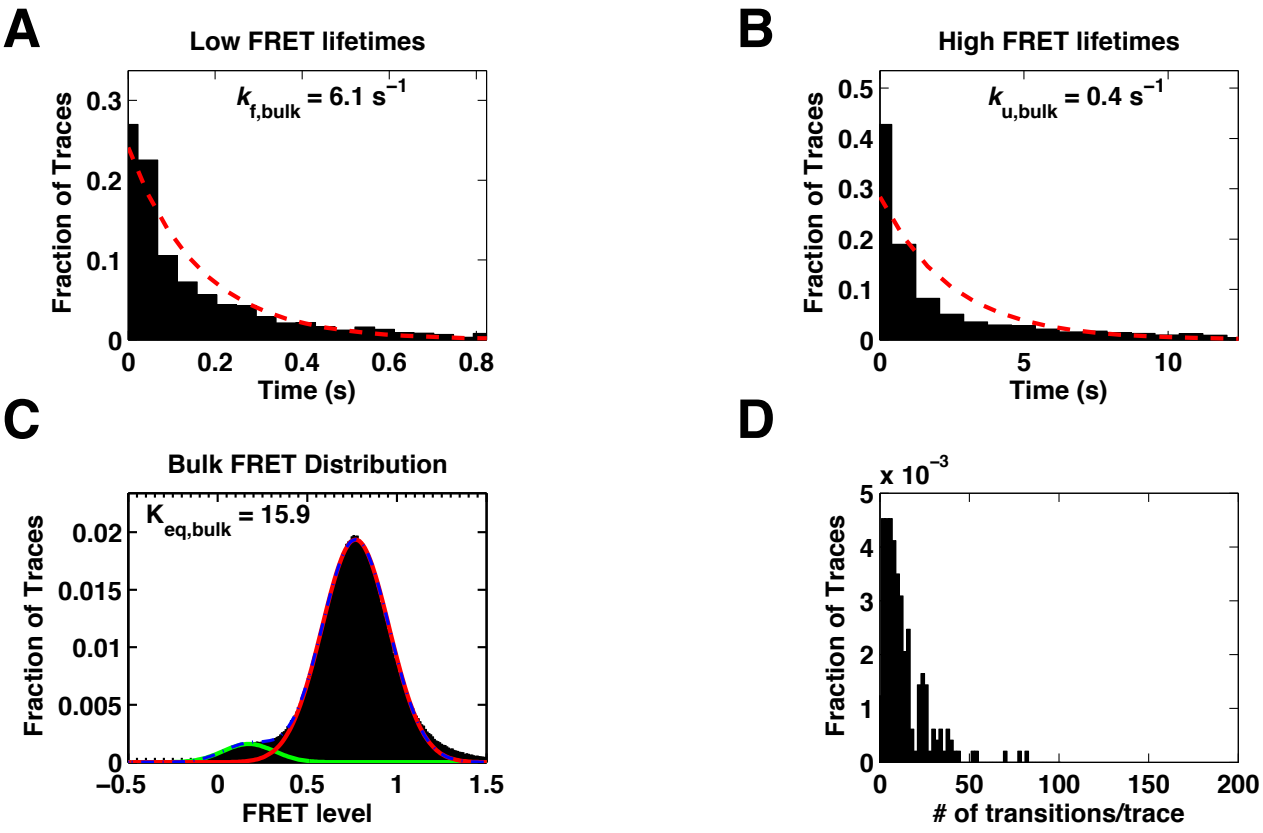


Figure S8-2. smFRET data assesment of aggregate data for Nat+3. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

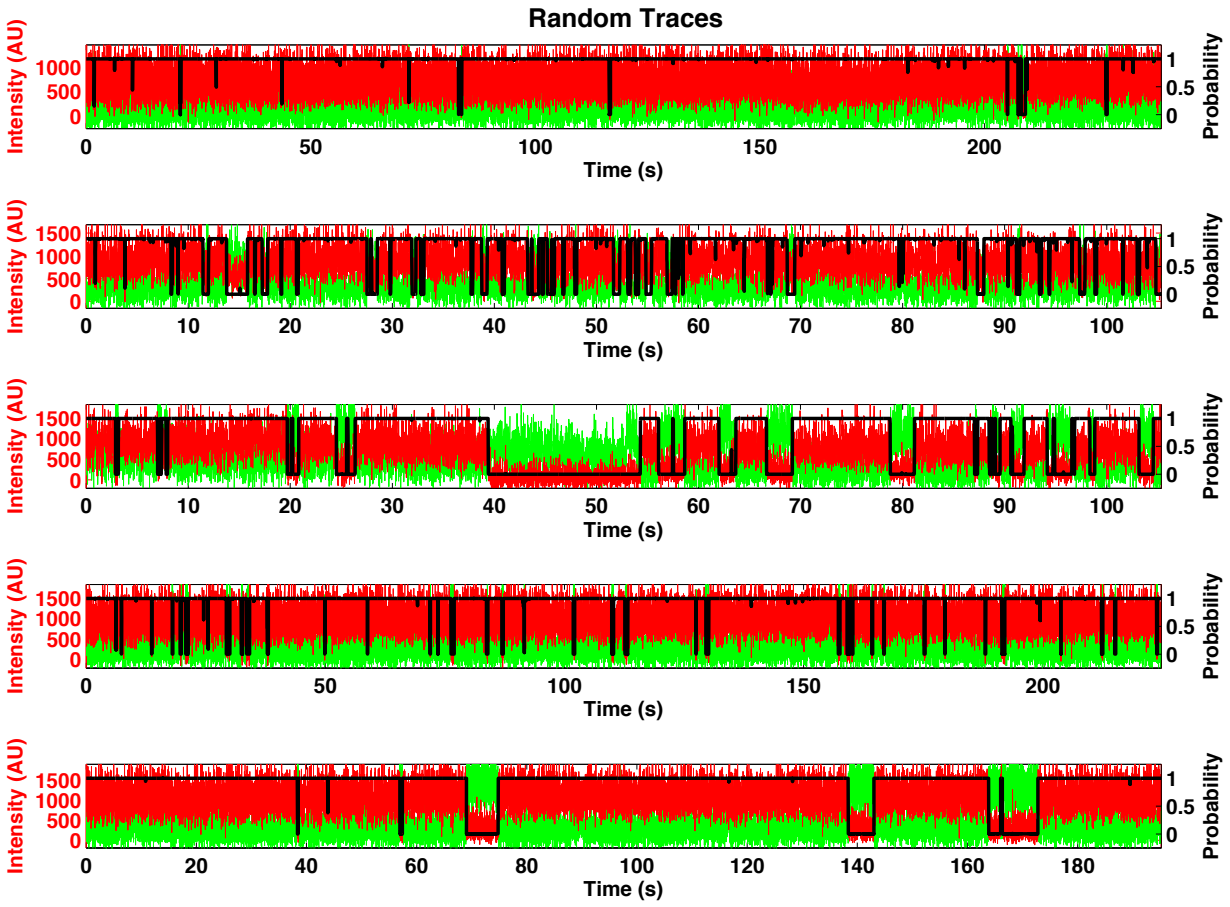


Figure S8-3. Randomly selected FRET traces of Nat+3. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S9-1. Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	154
SNR Threshold[2]	0.75
Number of Traces	167

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S9-2. Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	1.3	1.2 - 1.4	1.7
	$k_u(s^{-1})$	9.5	9.1 - 9.9	1.4
	K_{eq}	0.1	0.1 - 0.1	1.8
	SNR green	2.7	2.6 - 2.8	0.7
	SNR red	2.2	2.2 - 2.3	0.4
	ΔG(kcal/mol)	1.2	1.1 - 1.2	0.4
Fits from Cumulative Data[2]	Lifetime (s)	35.8	30.9 - 41.9	35.8
	$k_{f,\ bulk}(s^{-1})$	1.4	1.4 - 1.4	1.3
	$k_{u,\ bulk}(s^{-1})$	9.1	9.3 - 8.9	8.4
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.9	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

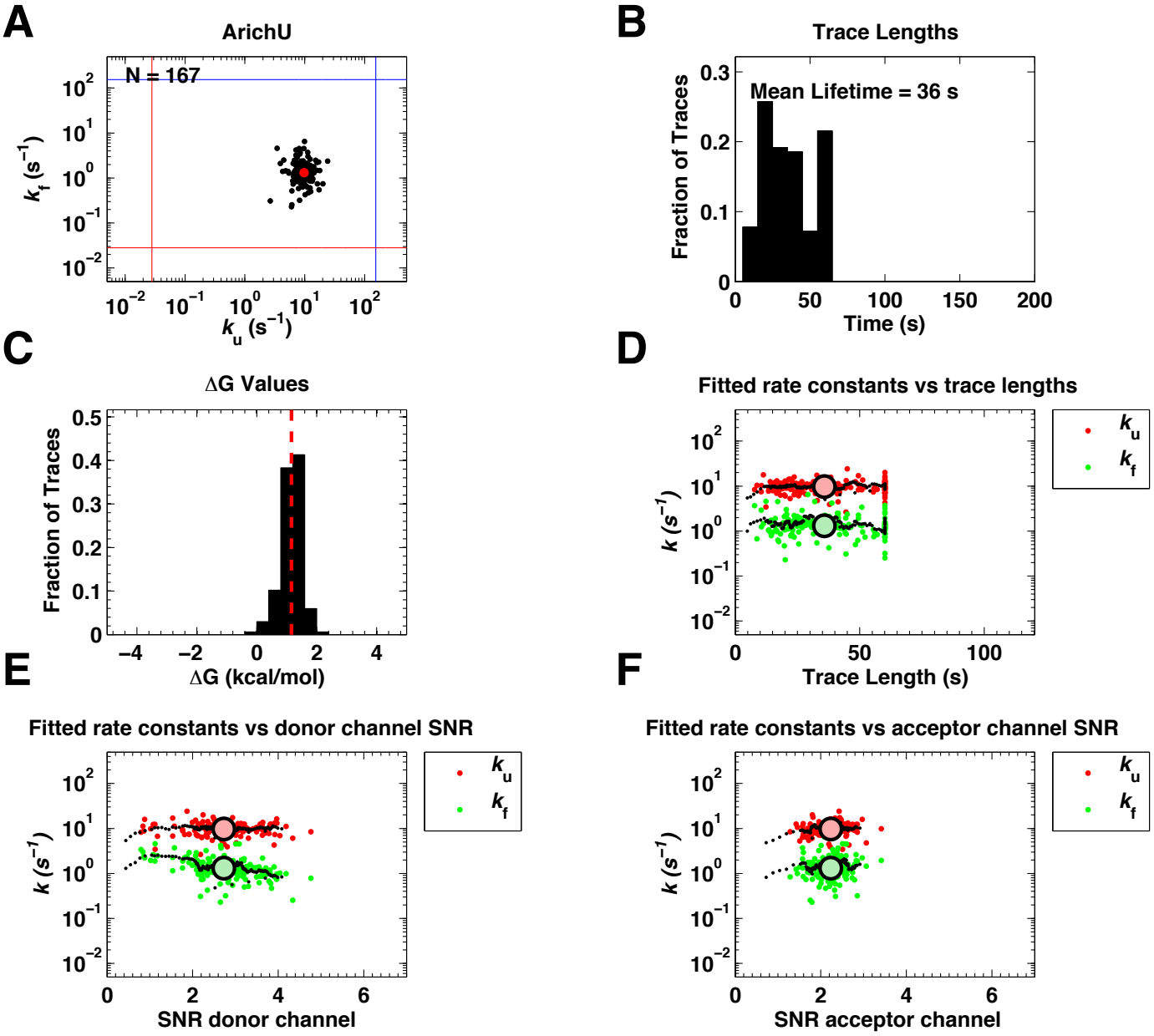


Figure S9-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

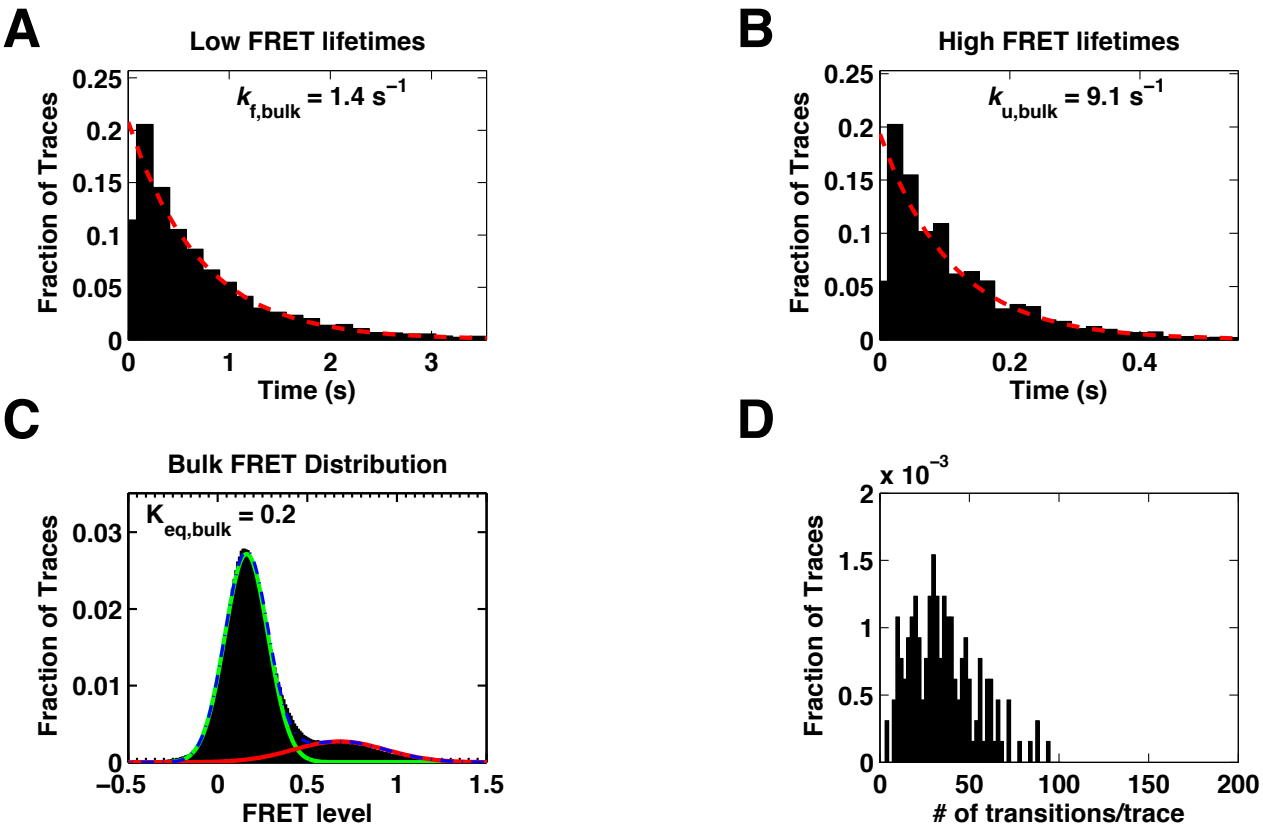


Figure S9-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

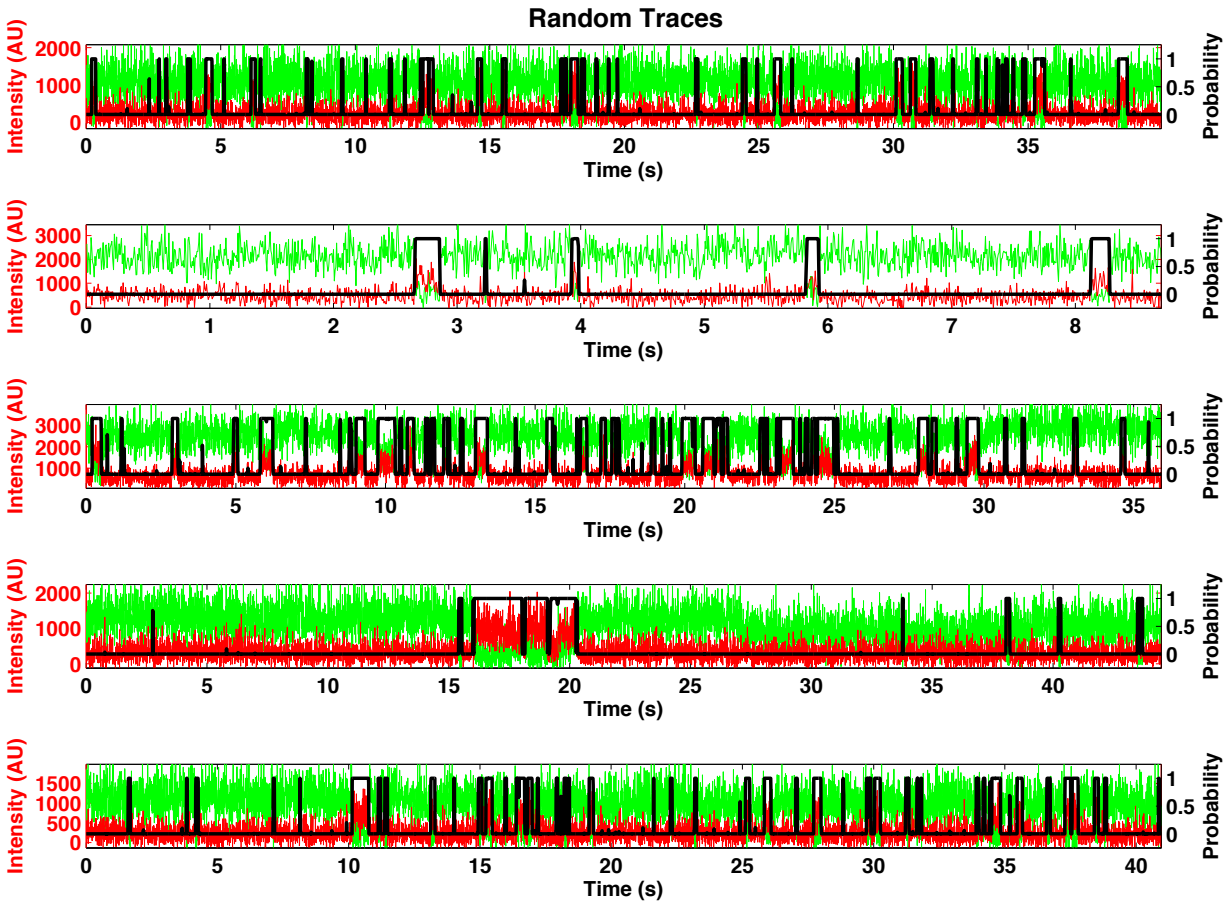


Figure S9-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S10-1. Variant and Conditions

Variant:	A186U
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	154
SNR Threshold[2]	0.75
Number of Traces	156

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S10-2. Folding parameters of smFRET the variant A186U inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	1.2	1.1 - 1.3	1.6
	$k_u(s^{-1})$	8.7	8.3 - 9.1	1.4
	K_{eq}	0.1	0.1 - 0.1	1.7
	SNR green	3.2	3.0 - 3.3	1.0
	SNR red	2.6	2.5 - 2.7	0.5
	ΔG(kcal/mol)	1.2	1.1 - 1.2	0.3
Fits from Cumulative Data[2]	Lifetime (s)	34.0	29.3 - 40.1	34.0
	$k_{f,\ bulk}(s^{-1})$	1.2	1.3 - 1.2	1.1
	$k_{u,\ bulk}(s^{-1})$	8.5	8.7 - 8.3	7.9
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.9	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

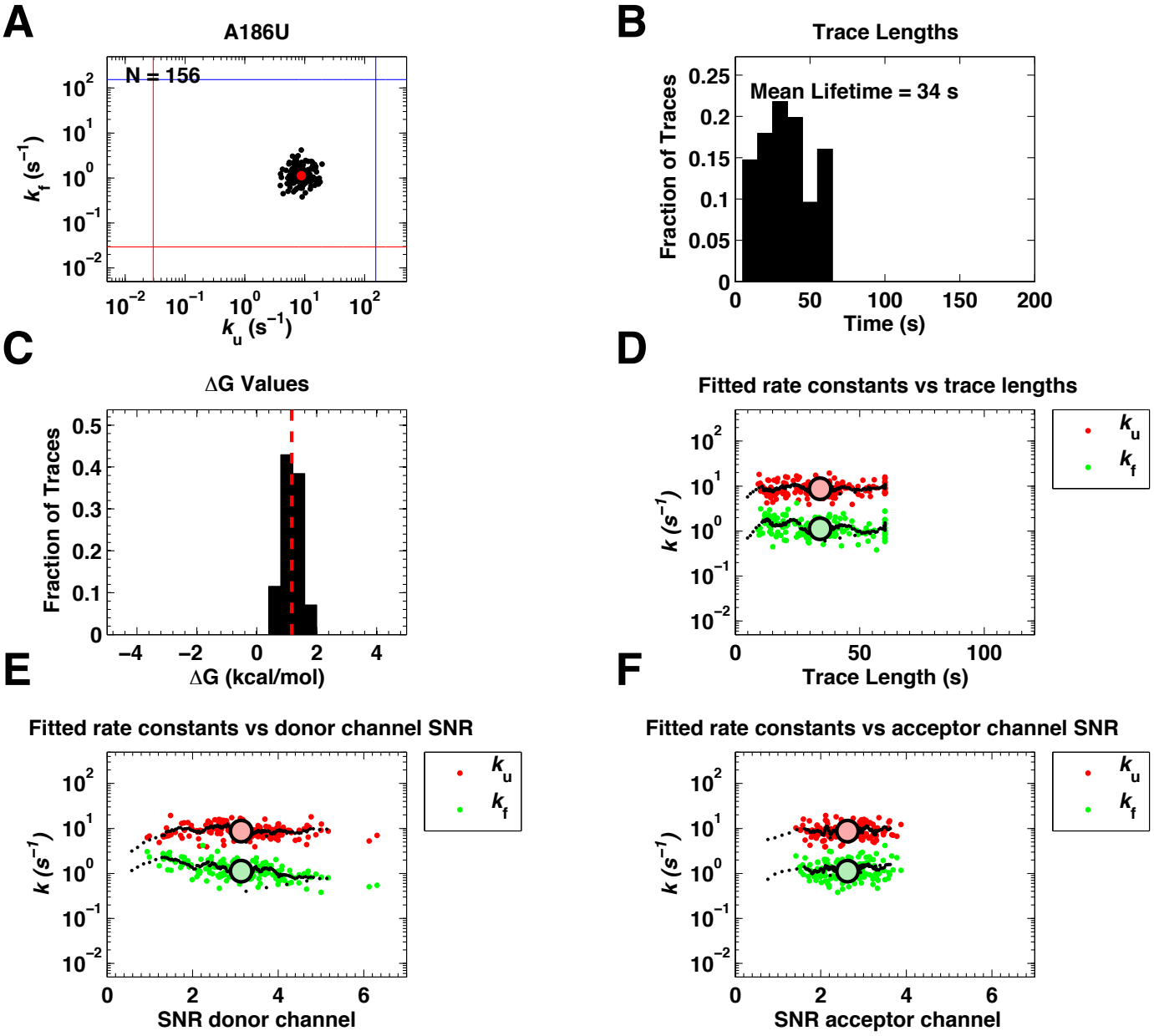


Figure S10-1. smFRET data assessment for A186U. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

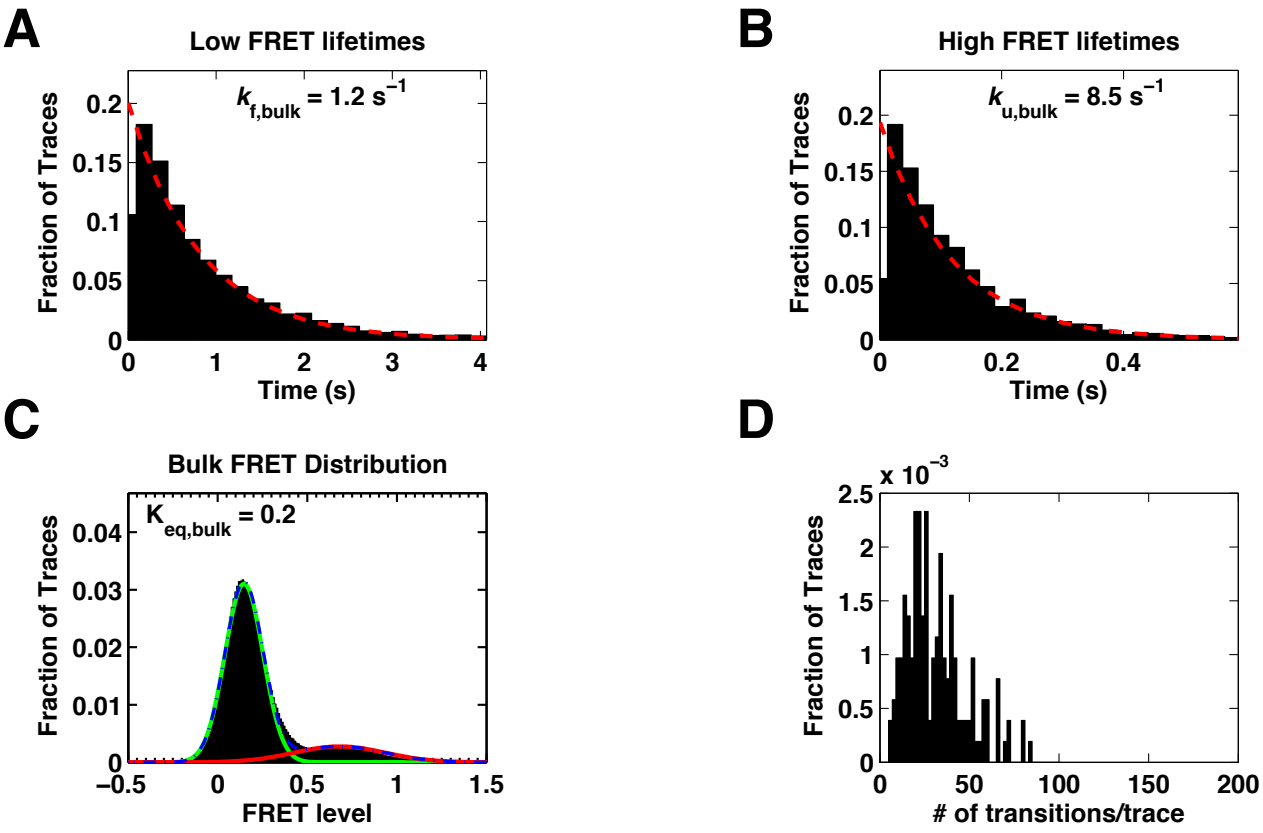


Figure S10-2. smFRET data assesment of aggregate data for A186U. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

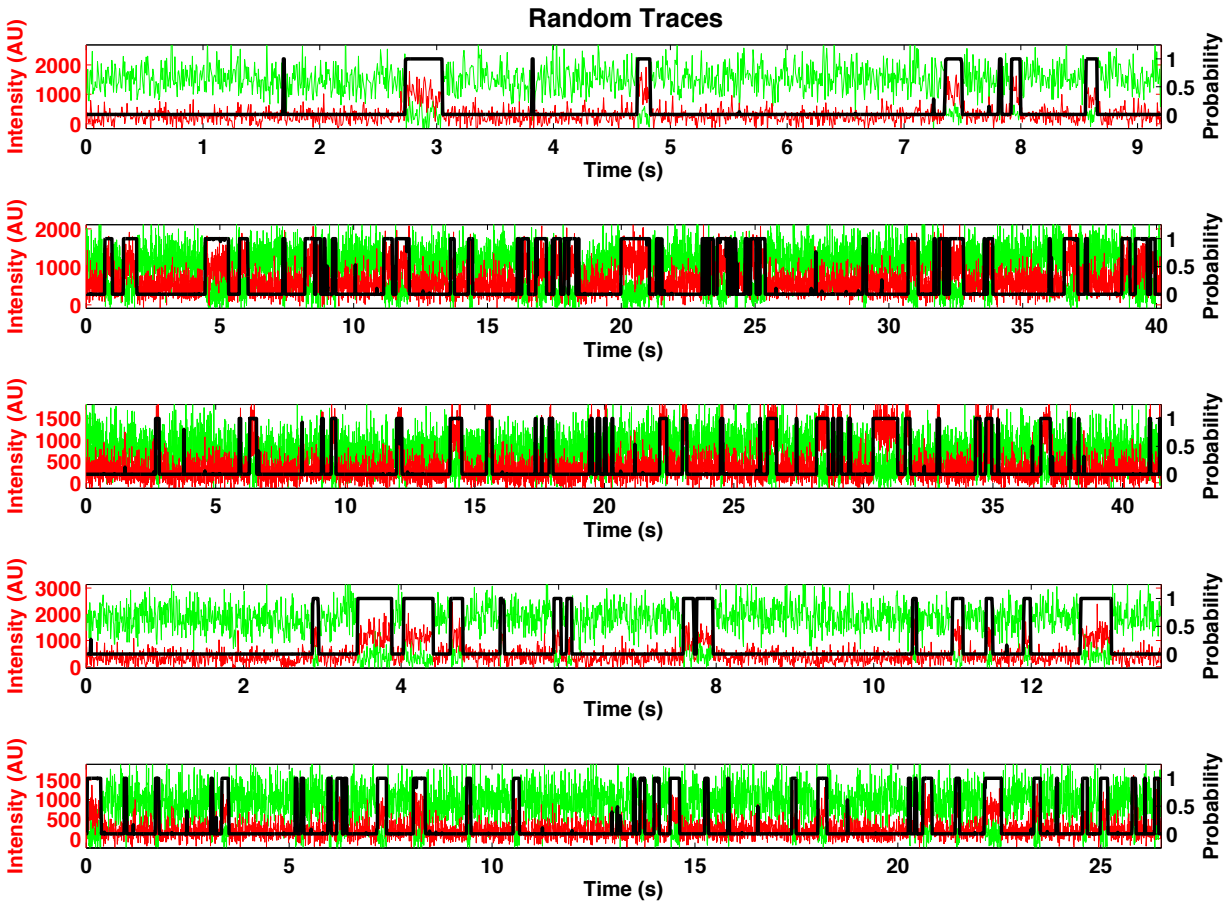


Figure S10-3. Randomly selected FRET traces of A186U. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S11-1. Variant and Conditions

Variant:	WT
$MgCl_2$ (mM)	0.0
$BaCl_2$ (mM)	5.0
KCl (mM)	100.0
pH	8.0
FPS[1]	50
SNR Threshold[2]	0.75
Number of Traces	166

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S11-2. Folding parameters of smFRET the variant WT inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	1.0	1.0 - 1.1	1.7
	$k_u(s^{-1})$	3.1	2.1 - 3.4	3.0
	K_{eq}	0.3	0.3 - 0.4	2.9
	SNR green	2.5	2.4 - 2.6	0.8
	SNR red	2.2	2.1 - 2.3	0.7
	ΔG(kcal/mol)	0.8	0.5 - 0.7	0.6
Fits from Cumulative Data[2]	Lifetime (s)	79.3	68.5 - 92.9	79.3
	$k_{f,\ bulk}(s^{-1})$	1.0	1.0 - 1.0	0.9
	$k_{u,\ bulk}(s^{-1})$	3.1	3.2 - 3.1	2.8
	$K_{eq,bulk}$	0.5	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.4	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

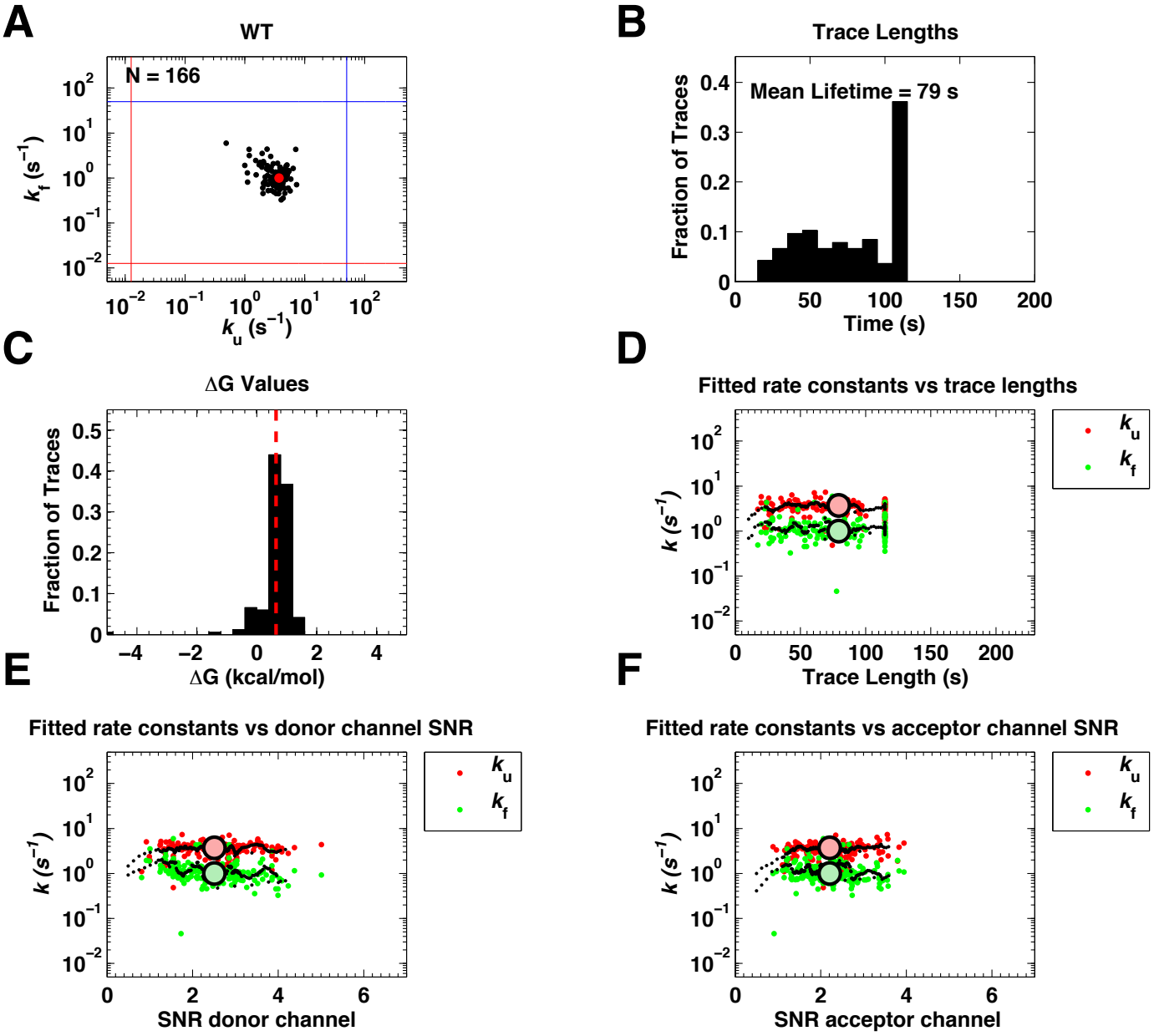


Figure S11-1. smFRET data assessment for WT. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

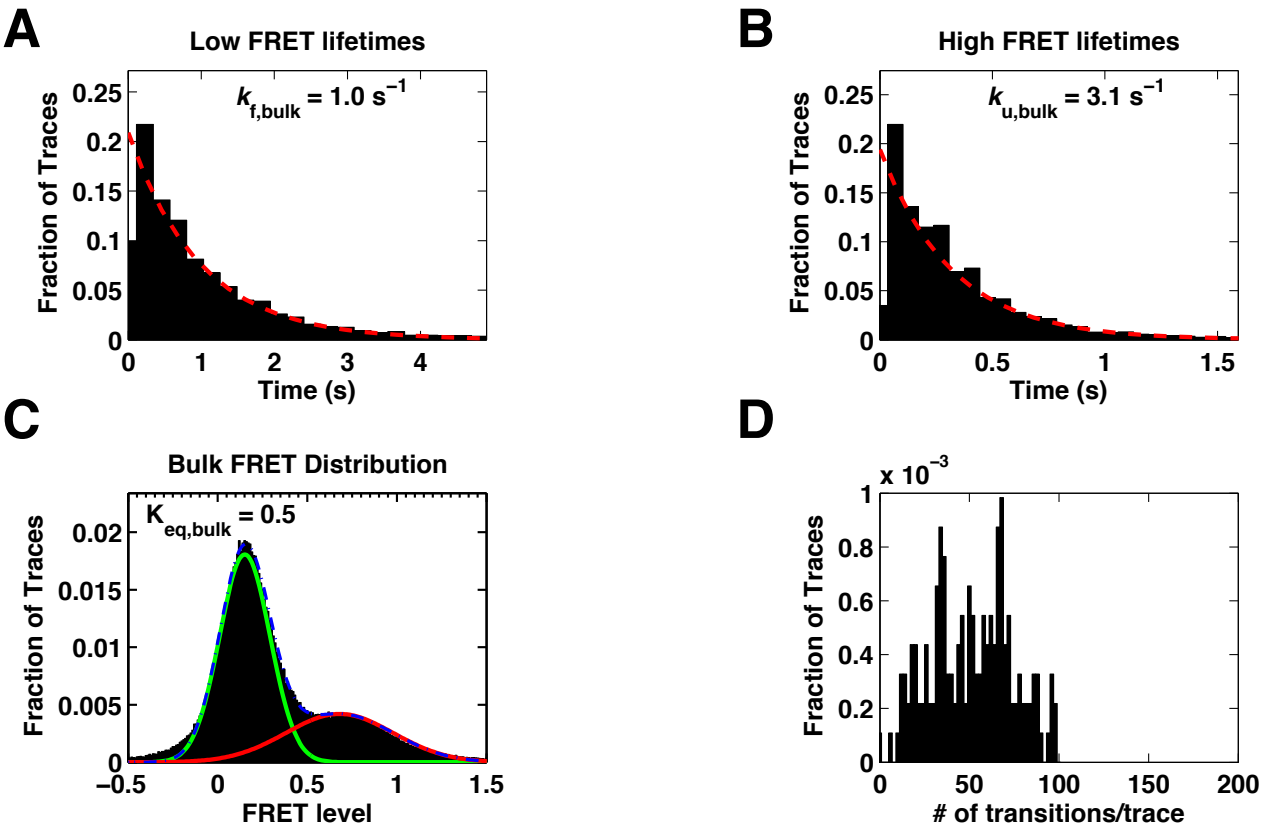
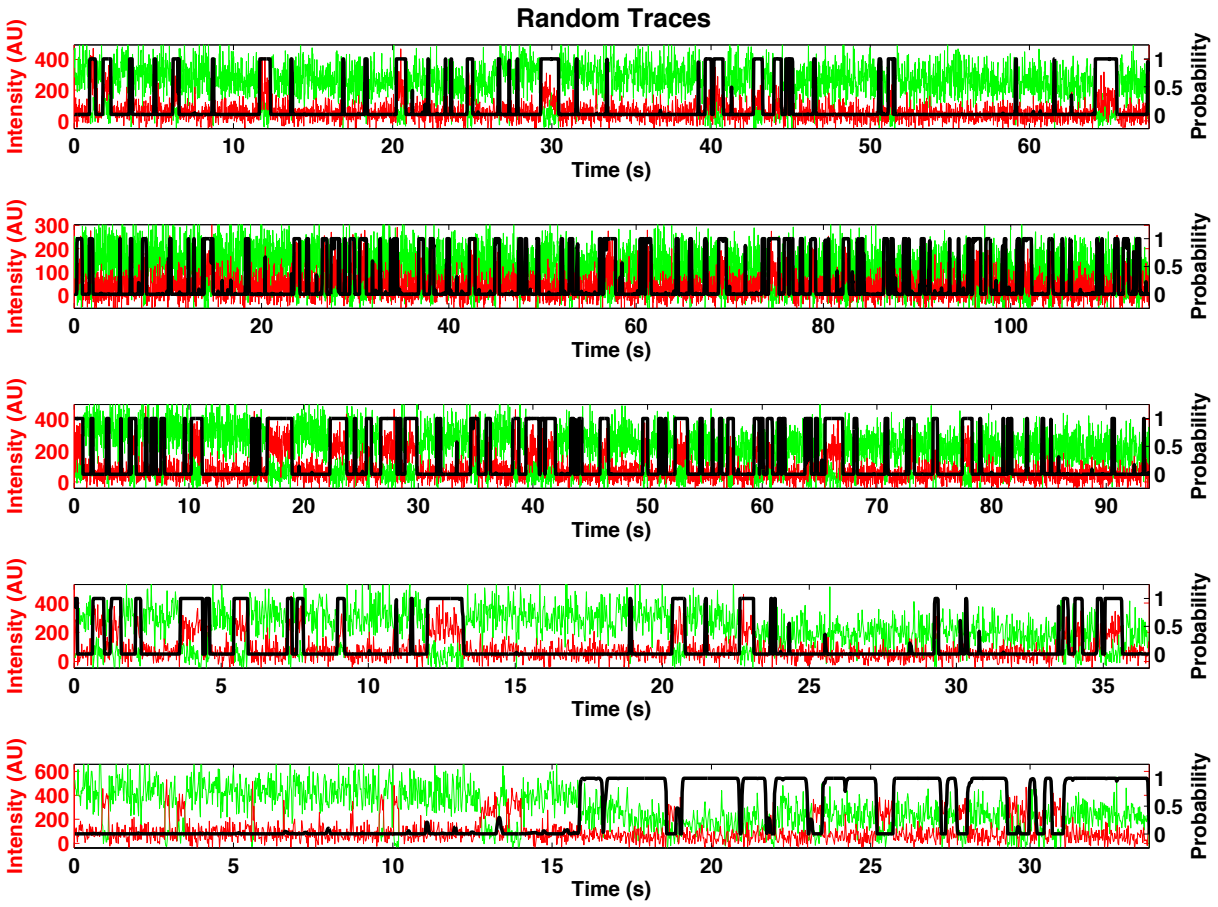


Figure S11-2. smFRET data assesment of aggregate data for WT. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.



FigureS11-3. Randomly selected FRET traces of WT. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S12-1. Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	0.0
$BaCl_2$ (mM)	5.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	94

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S12-2. Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	1.1	1.0 - 1.3	1.7
	$k_u(s^{-1})$	3.8	3.5 - 4.0	1.5
	K_{eq}	0.3	0.3 - 0.3	1.9
	SNR green	2.8	2.7 - 3.2	1.0
	SNR red	2.6	2.5 - 2.8	0.7
	ΔG(kcal/mol)	0.7	0.6 - 0.8	0.4
Fits from Cumulative Data[2]	Lifetime (s)	31.6	26.1 - 39.1	31.6
	$k_{f,\ bulk}(s^{-1})$	1.3	1.3 - 1.2	1.2
	$k_{u,\ bulk}(s^{-1})$	3.9	4.0 - 3.7	3.7
	$K_{eq,bulk}$	0.4	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.6	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

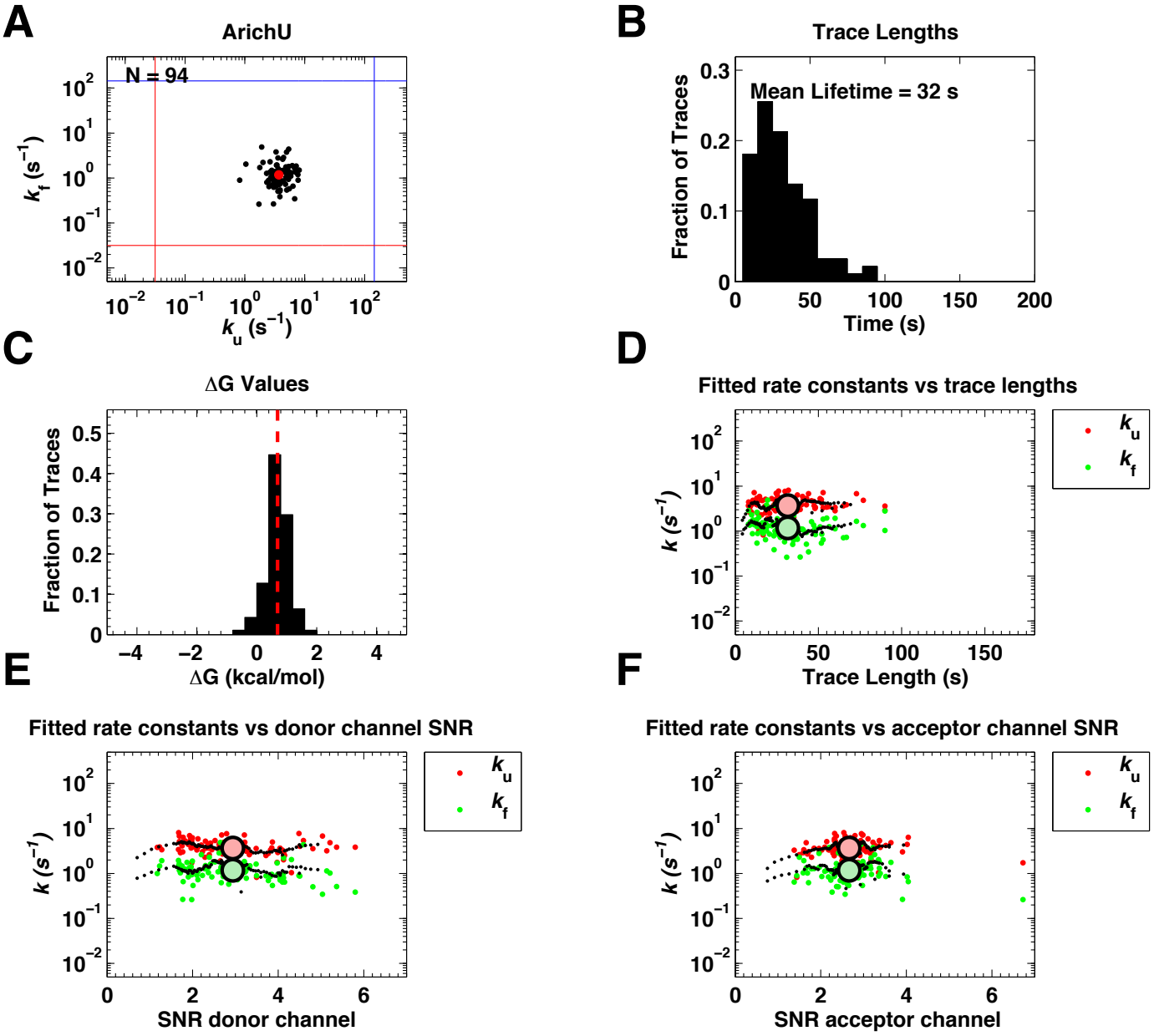


Figure S12-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

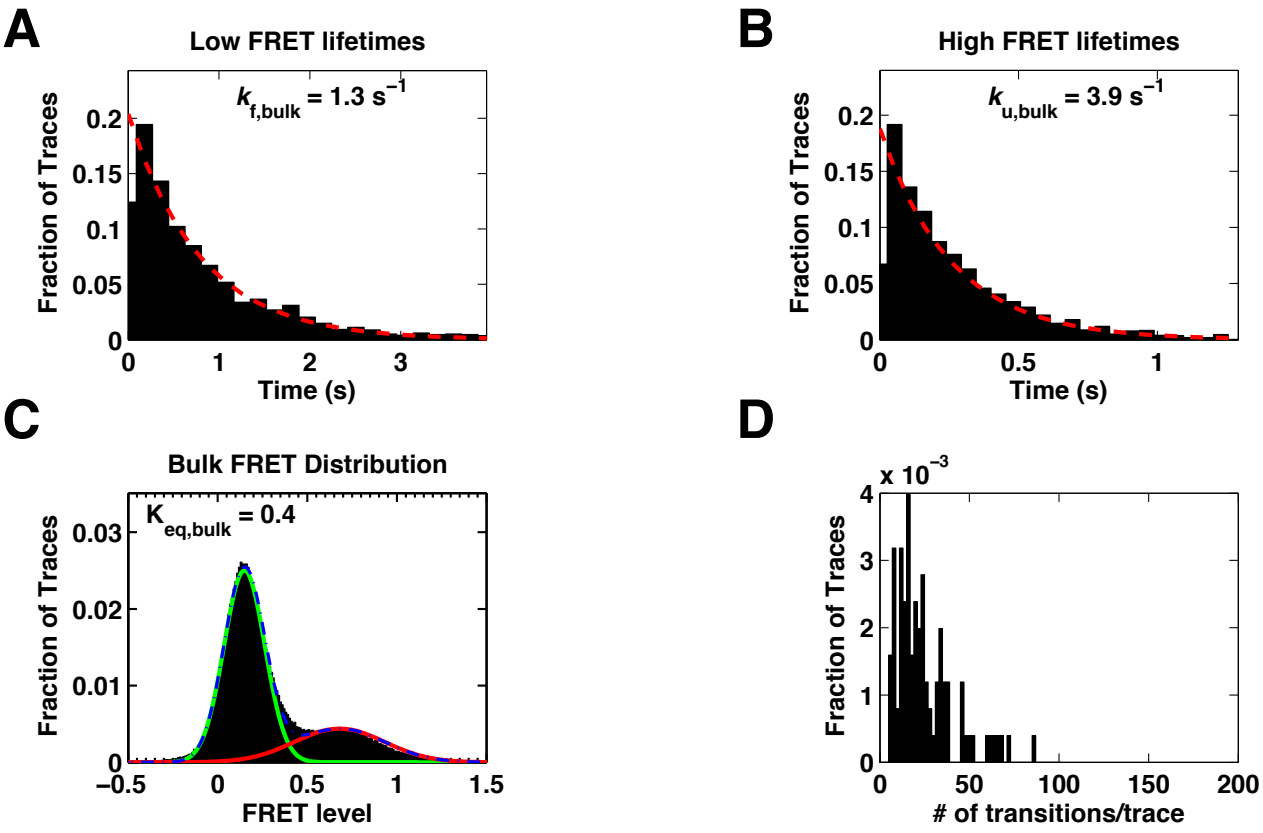


Figure S12-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

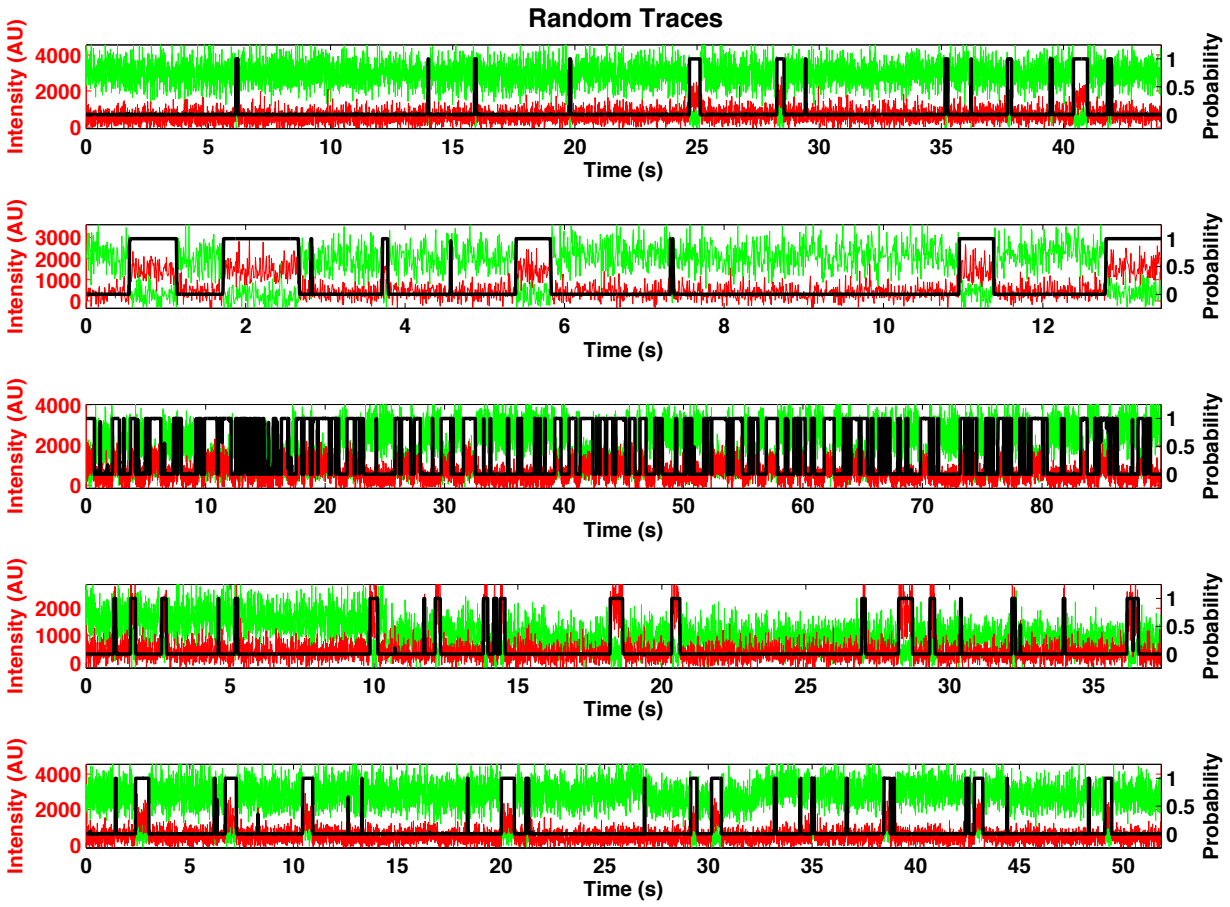


Figure S12-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S13-1. Variant and Conditions

Variant:	A186U
$MgCl_2$ (mM)	0.0
$BaCl_2$ (mM)	5.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	131

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S13-2. Folding parameters of smFRET the variant A186U inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.9	0.8 - 0.9	1.6
	$k_u(s^{-1})$	4.3	4.0 - 4.5	1.4
	K_{eq}	0.2	0.2 - 0.2	1.8
	SNR green	3.7	3.5 - 3.8	1.0
	SNR red	3.0	2.9 - 3.1	0.8
	ΔG(kcal/mol)	0.9	0.9 - 1.0	0.3
Fits from Cumulative Data[2]	Lifetime (s)	46.0	39.1 - 55.1	46.0
	$k_{f,\ bulk}(s^{-1})$	1.0	1.0 - 0.9	0.9
	$k_{u,\ bulk}(s^{-1})$	4.3	4.5 - 4.2	4.1
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

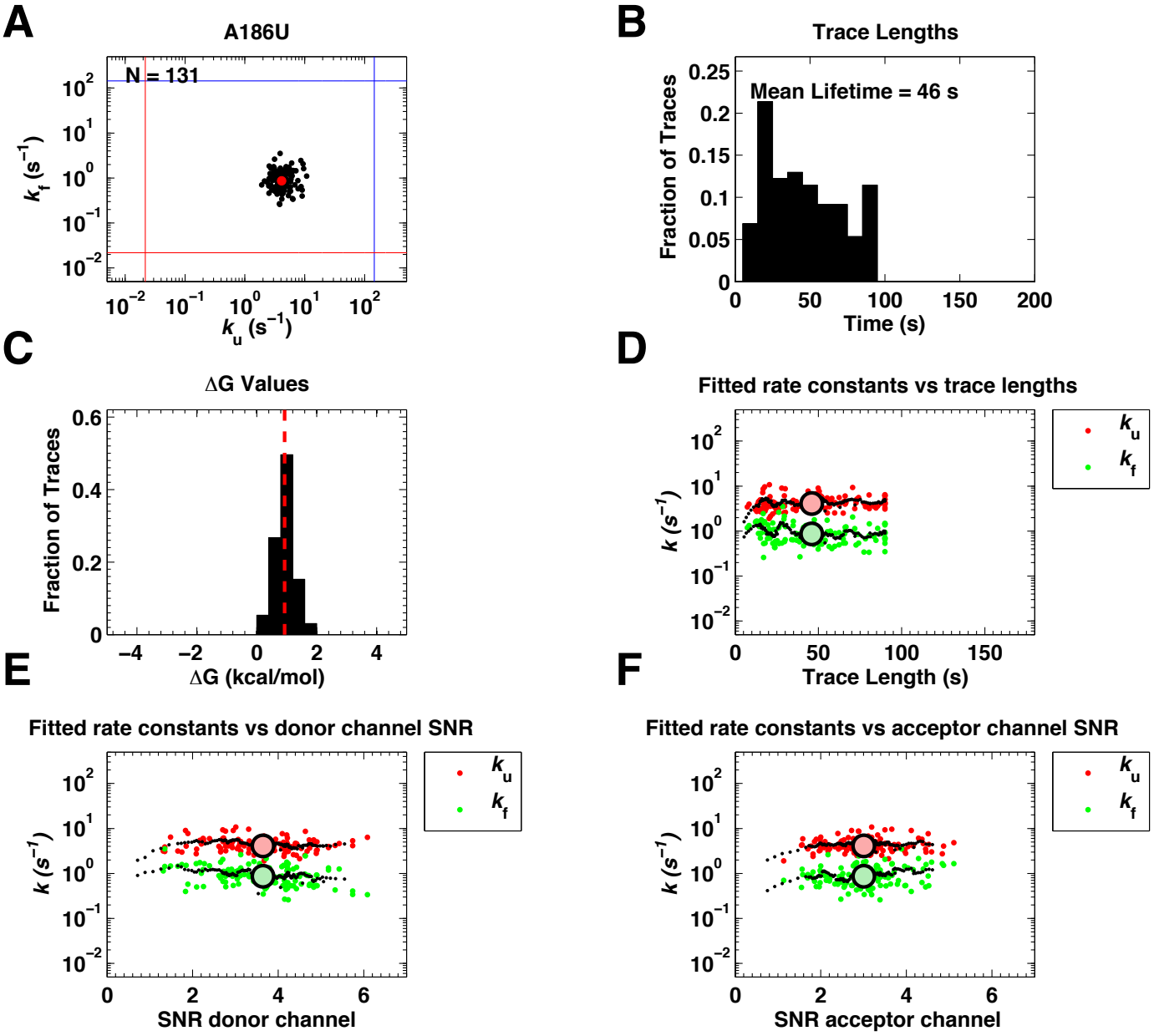


Figure S13-1. smFRET data assessment for A186U. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

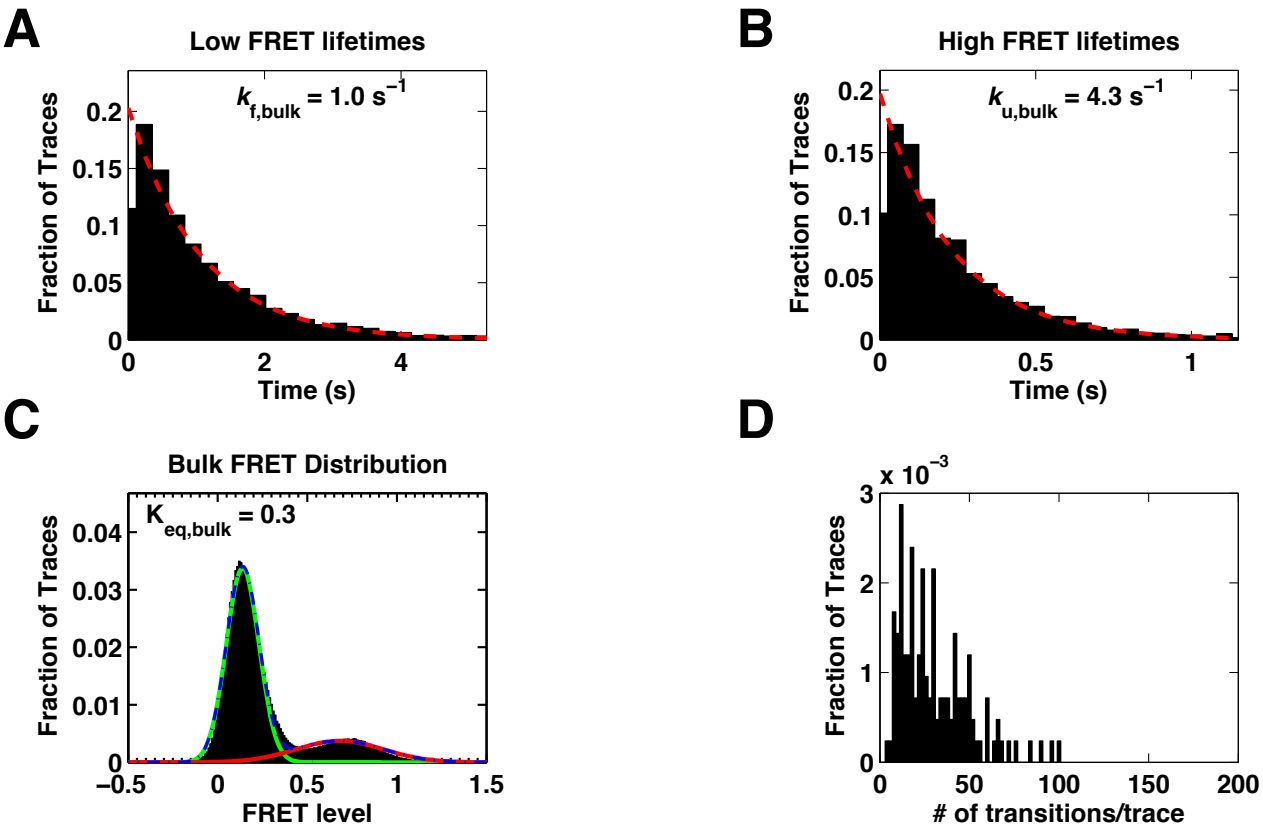


Figure S13-2. smFRET data assesment of aggregate data for A186U. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

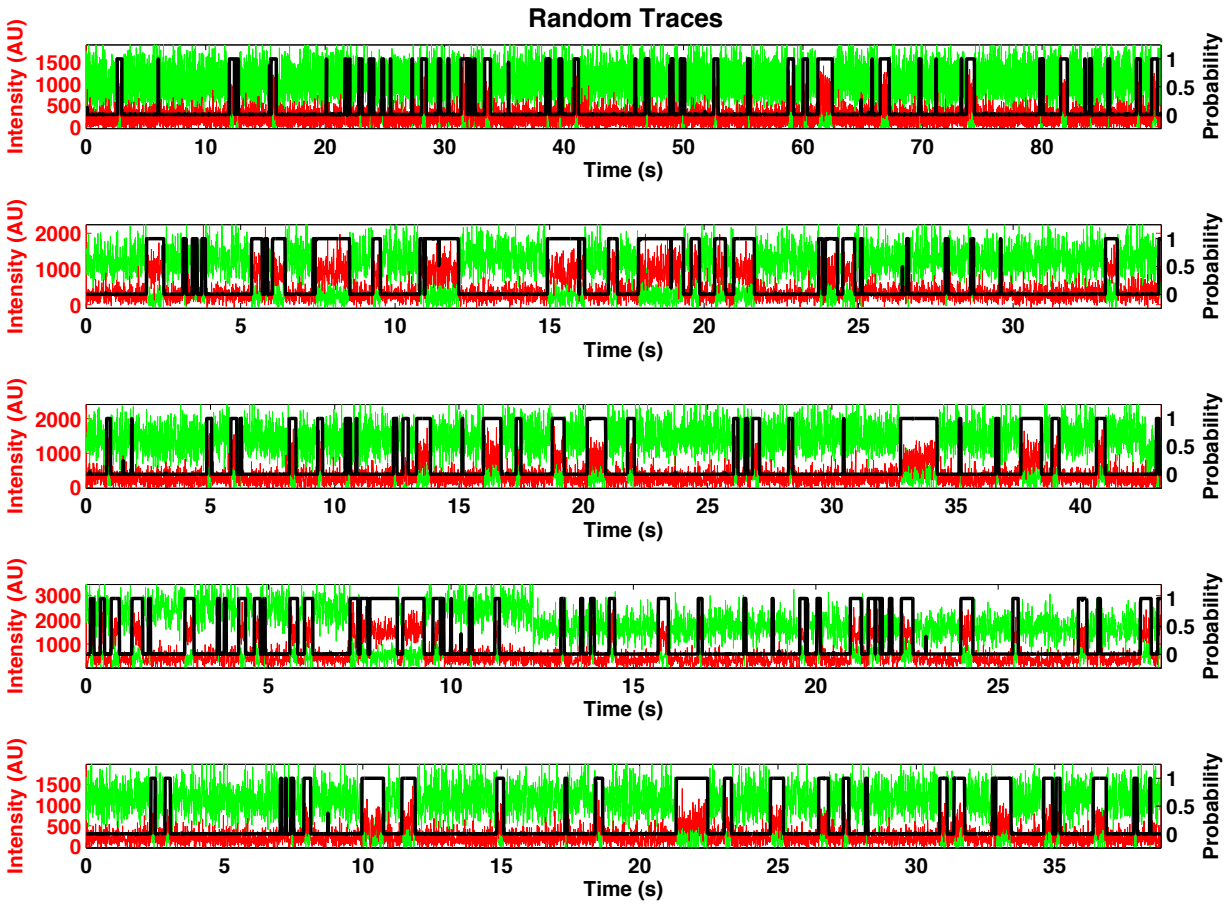


Figure S13-3. Randomly selected FRET traces of A186U. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S14-1.Variant and Conditions

Variant:	Alt+2
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	92
SNR Threshold[2]	0.50
Number of Traces	129

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S14-2.Folding parameters of smFRET the variant Alt+2 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	1.0	1.0 - 1.1	1.5
	$k_u(s^{-1})$	3.7	3.5 - 3.9	1.4
	K_{eq}	0.3	0.2 - 0.3	1.8
	SNR green	2.6	2.6 - 2.7	0.5
	SNR red	1.5	1.5 - 1.6	0.3
	ΔG(kcal/mol)	0.8	0.7 - 0.8	0.4
Fits from Cumulative Data[2]	Lifetime (s)	58.8	49.9 - 70.5	58.8
	$k_{f,\ bulk}(s^{-1})$	1.0	1.1 - 1.0	1.0
	$k_{u,\ bulk}(s^{-1})$	3.6	3.7 - 3.5	3.4
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

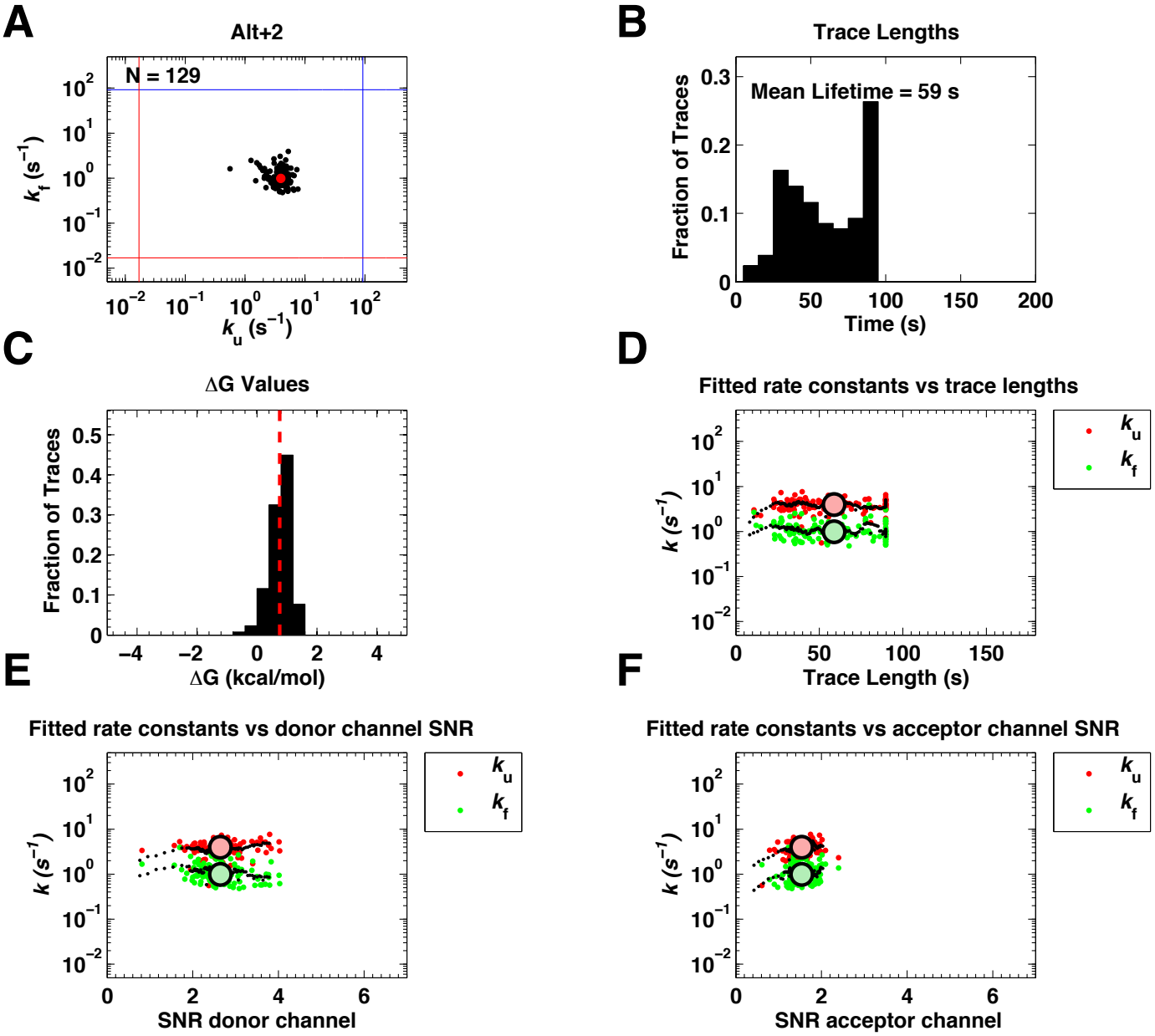


Figure S14-1. smFRET data assessment for Alt+2. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

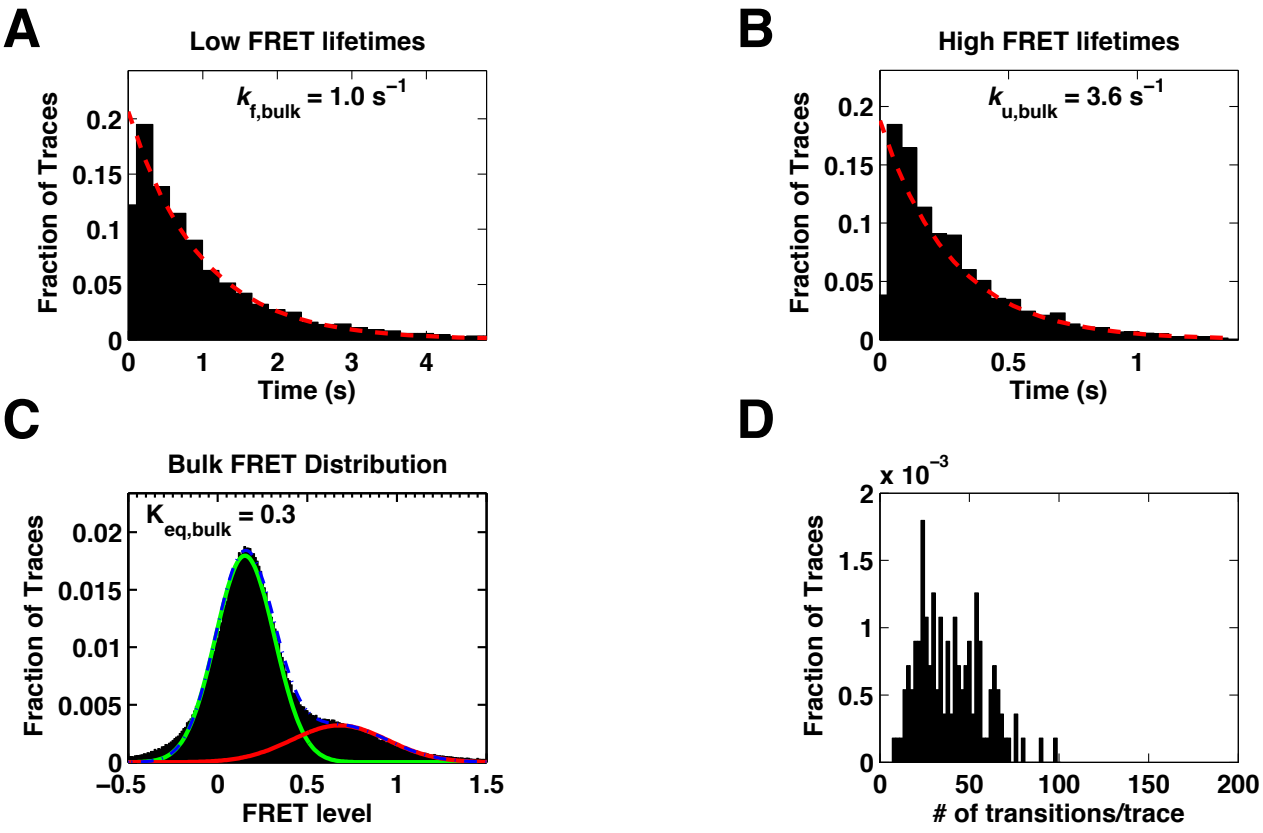


Figure S14-2. smFRET data assesment of aggregate data for Alt+2. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

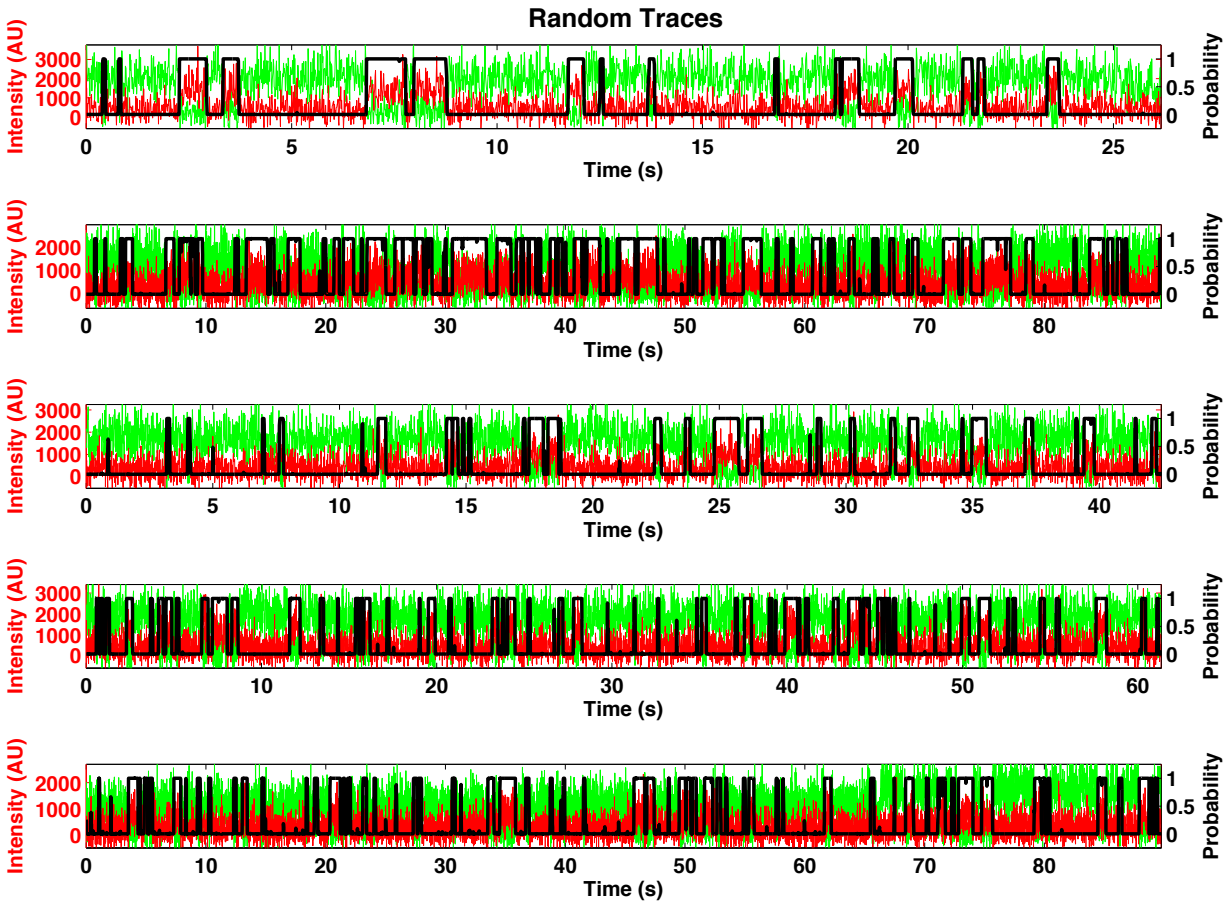


Figure S14-3. Randomly selected FRET traces of Alt+2. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S15-1. Variant and Conditions

Variant:	G212U
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	154
SNR Threshold[2]	0.75
Number of Traces	113

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S15-2. Folding parameters of smFRET the variant G212U inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	1.2	1.1 - 1.3	1.6
	$k_u(s^{-1})$	11.0	10.4 - 11.6	1.4
	K_{eq}	0.1	0.1 - 0.1	1.7
	SNR green	2.8	2.6 - 2.9	0.8
	SNR red	2.3	2.2 - 2.4	0.4
	ΔG(kcal/mol)	1.3	1.2 - 1.3	0.3
Fits from Cumulative Data[2]	Lifetime (s)	36.9	30.9 - 44.7	36.9
	$k_{f,\ bulk}(s^{-1})$	1.2	1.3 - 1.2	1.2
	$k_{u,\ bulk}(s^{-1})$	10.4	10.7 - 10.1	9.8
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	1.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

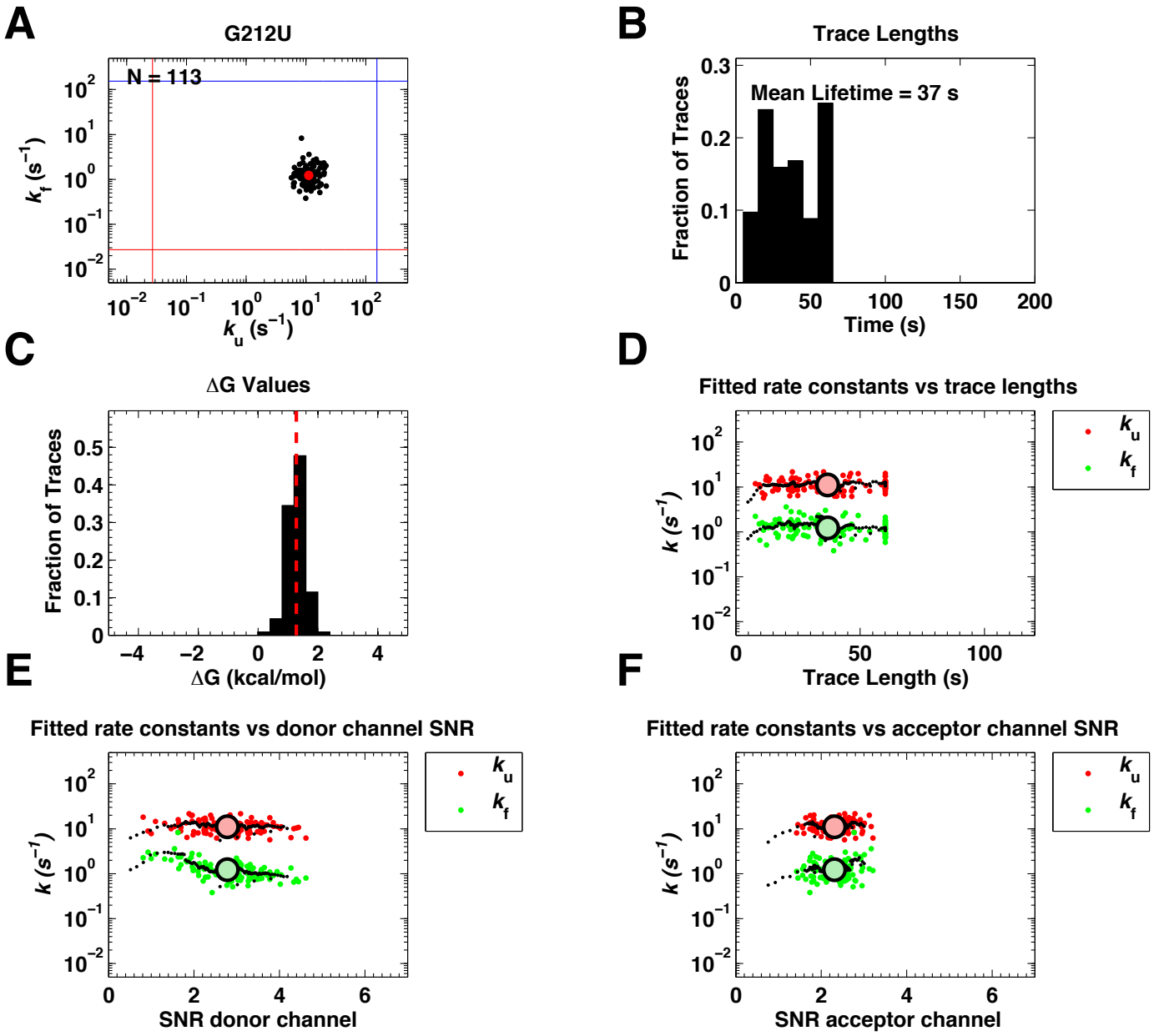


Figure S15-1. smFRET data assessment for G212U. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

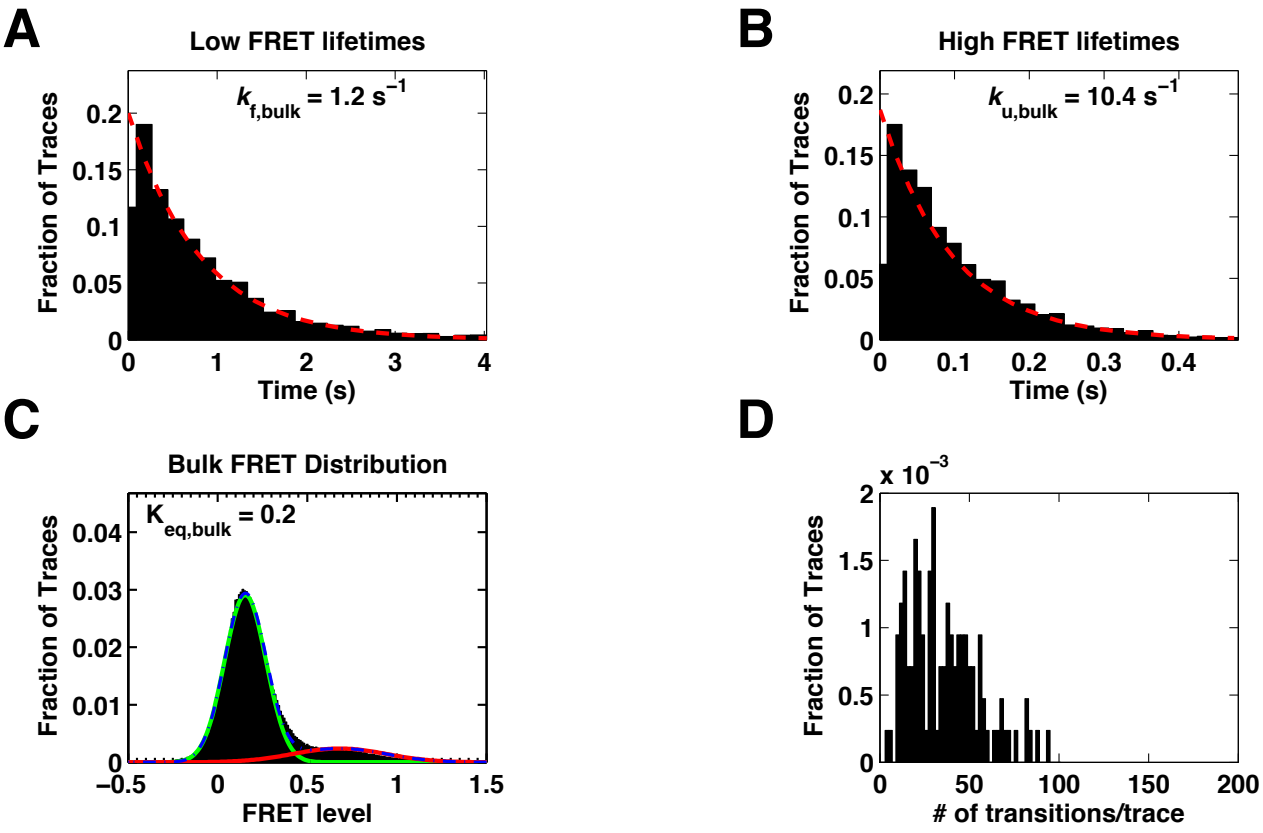


Figure S15-2. smFRET data assesment of aggregate data for G212U. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

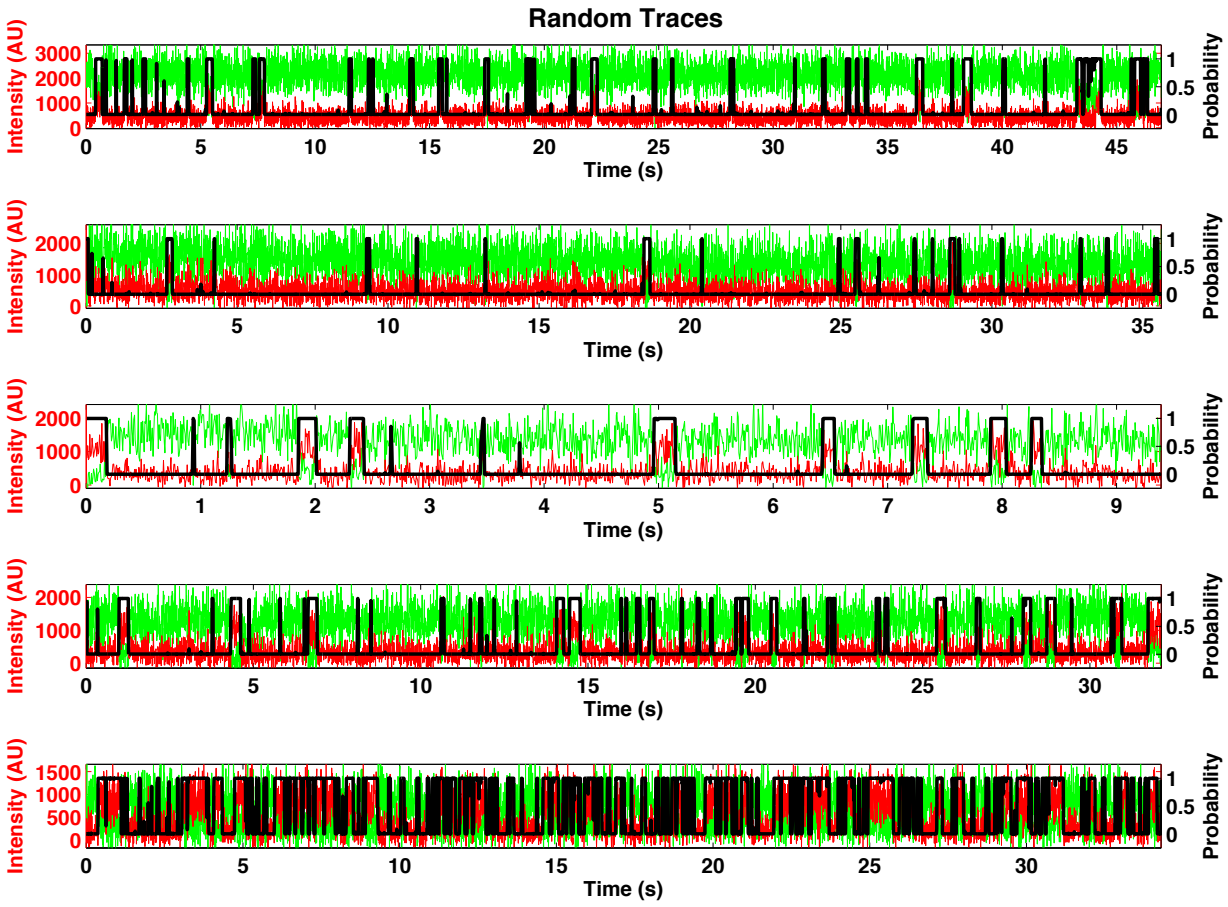


Figure S15-3. Randomly selected FRET traces of G212U. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S16-1.Variant and Conditions

Variant:	G212C
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	48
SNR Threshold[2]	0.75
Number of Traces	410

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S16-2. Folding parameters of smFRET the variant G212C inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.9	0.9 - 1.1	2.9
	$k_u(s^{-1})$	2.5	2.3 - 2.8	2.7
	K_{eq}	0.4	0.3 - 0.4	5.6
	SNR green	3.2	3.1 - 3.3	1.0
	SNR red	2.9	2.8 - 3.0	0.7
	ΔG(kcal/mol)	0.2	0.5 - 0.7	1.0
Fits from Cumulative Data[2]	Lifetime (s)	80.7	73.4 - 89.1	80.7
	$k_{f,\ bulk}(s^{-1})$	1.4	1.5 - 1.4	1.0
	$k_{u,\ bulk}(s^{-1})$	2.1	2.1 - 2.0	1.7
	$K_{eq,bulk}$	0.6	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.3	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

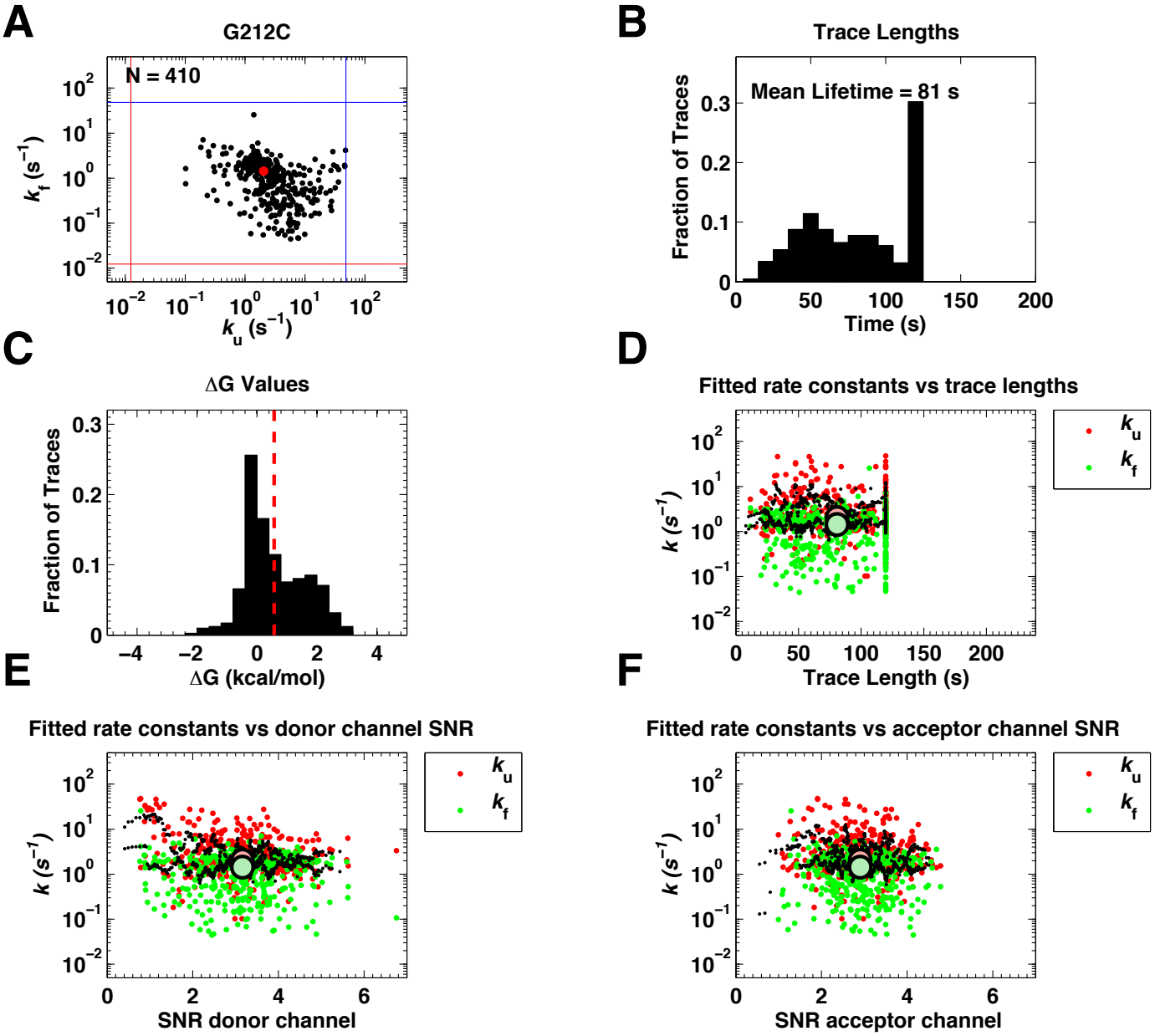


Figure S16-1. smFRET data assessment for G212C. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

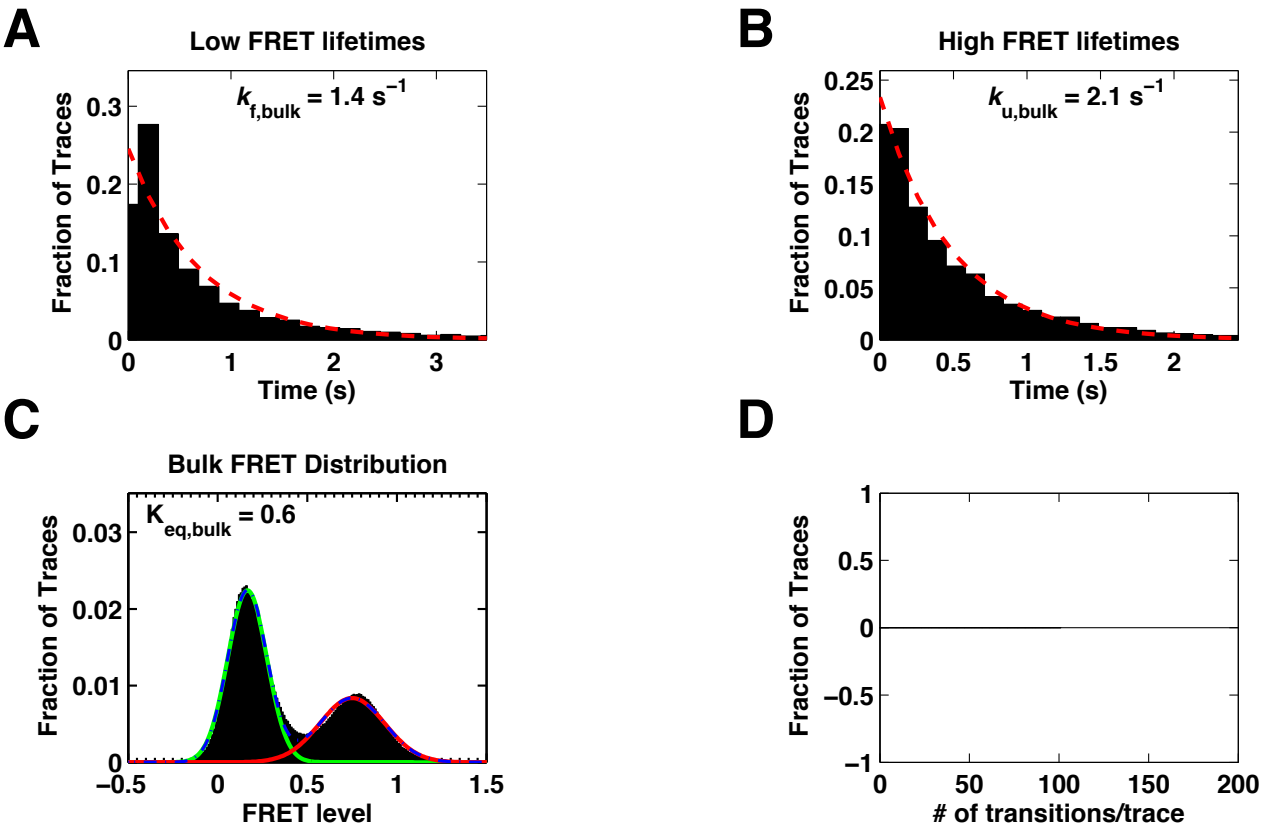


Figure S16-2. smFRET data assesment of aggregate data for G212C. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

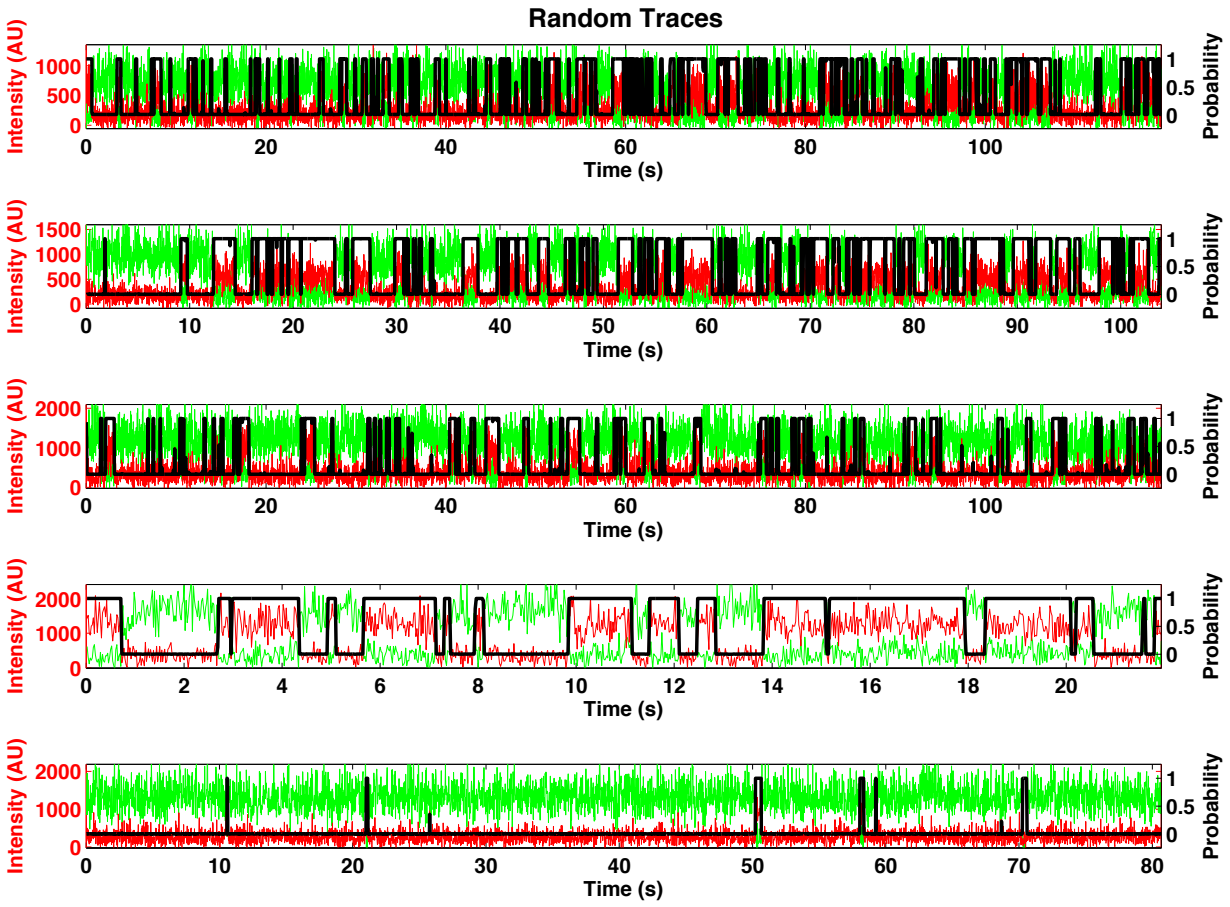


Figure S16-3. Randomly selected FRET traces of G212C. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S17-1.Variant and Conditions

Variant:	C109U
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	48
SNR Threshold[2]	0.75
Number of Traces	550

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S17-2. Folding parameters of smFRET the variant C109U inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	3.2	3.0 - 3.5	2.6
	$k_u(s^{-1})$	0.4	0.4 - 0.5	3.5
	K_{eq}	7.2	6.2 - 8.4	6.3
	SNR green	3.2	3.1 - 3.3	0.8
	SNR red	2.5	2.5 - 2.6	0.7
	ΔG(kcal/mol)	-1.5	-1.2 - -1.1	1.1
Fits from Cumulative Data[2]	Lifetime (s)	72.5	66.8 - 79.0	72.5
	$k_{f,\ bulk}(s^{-1})$	2.4	2.5 - 2.4	1.8
	$k_{u,\ bulk}(s^{-1})$	0.6	0.6 - 0.6	0.5
	$K_{eq,bulk}$	3.6	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

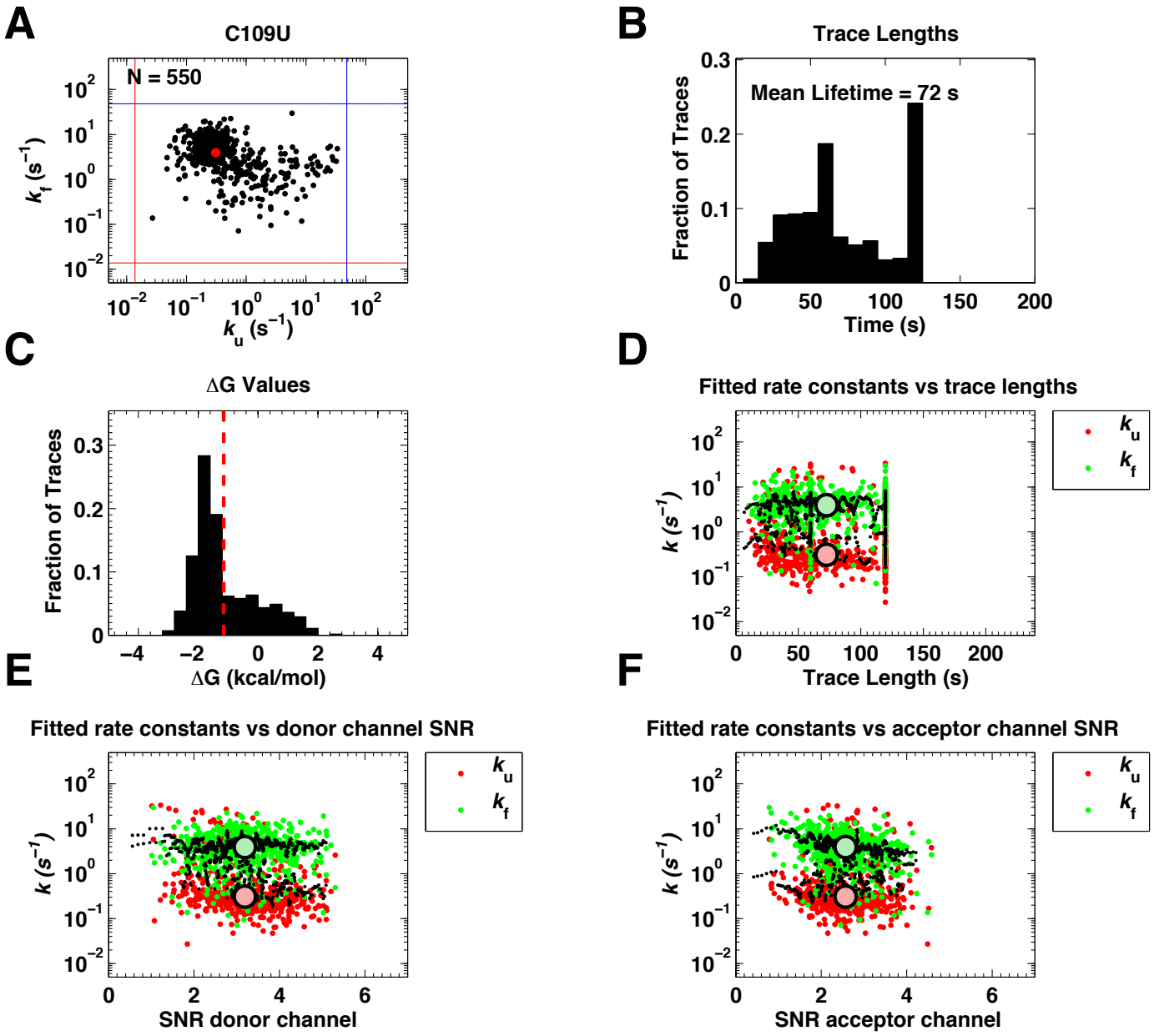


Figure S17-1. smFRET data assessment for C109U. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

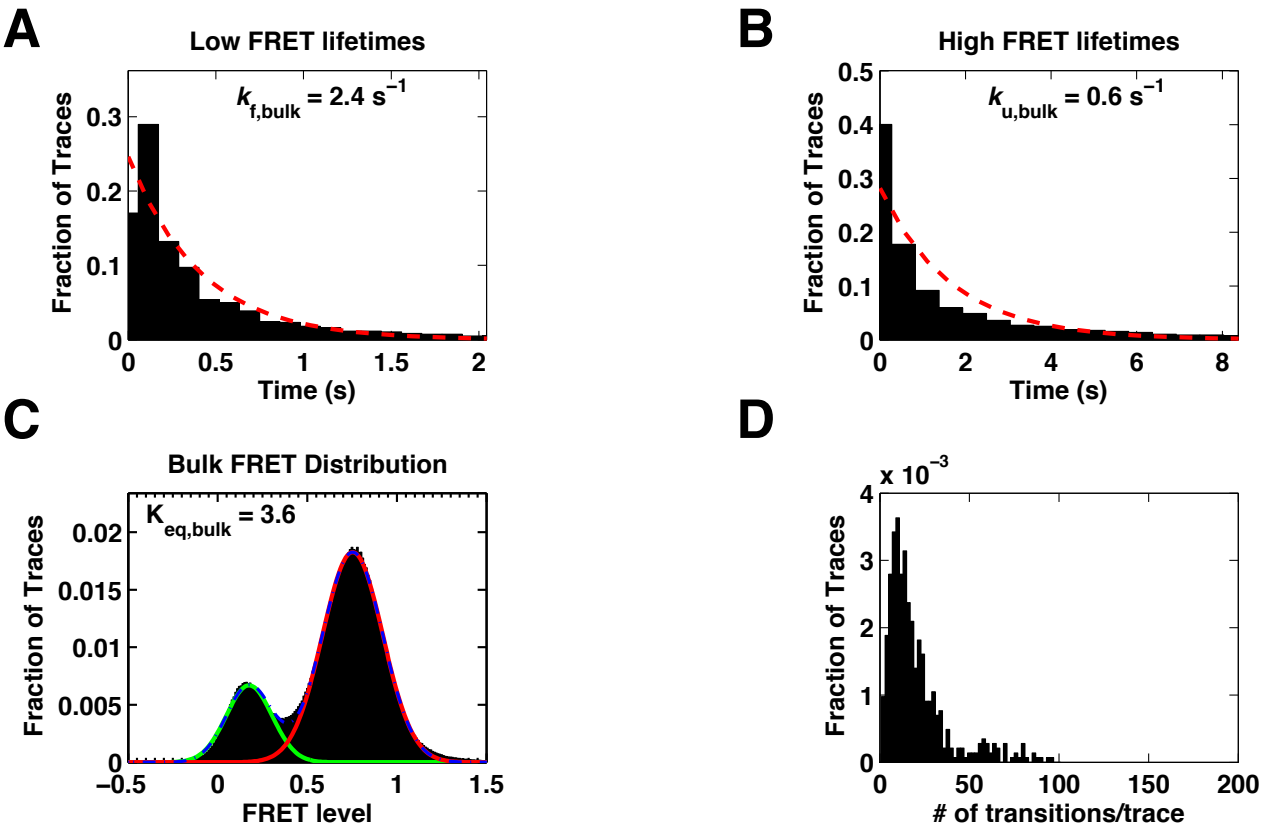


Figure S17-2. smFRET data assesment of aggregate data for C109U. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

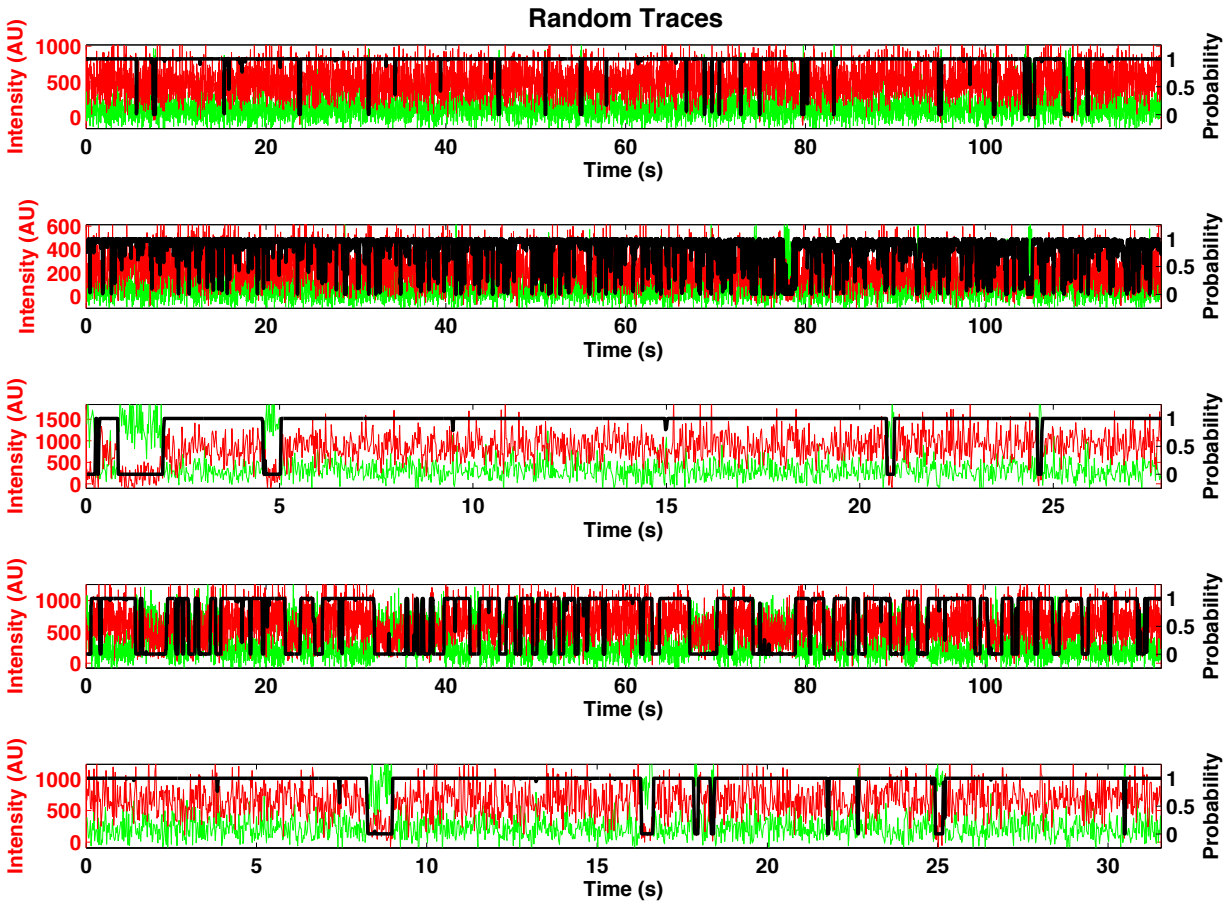


Figure S17-3. Randomly selected FRET traces of C109U. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S18-1. Variant and Conditions

Variant:	TL-AllU
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	124

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S18-2. Folding parameters of smFRET the variant TL-AllU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	5.8	4.6 - 6.8	3.0
	$k_u(s^{-1})$	45.5	37.9 - 52.7	2.6
	K_{eq}	0.1	0.1 - 0.1	2.2
	SNR green	0.7	0.7 - 0.8	0.2
	SNR red	1.4	1.3 - 1.5	0.5
	ΔG(kcal/mol)	1.2	1.1 - 1.3	0.5
Fits from Cumulative Data[2]	Lifetime (s)	6.7	5.7 - 8.1	6.7
	$k_{f,\ bulk}(s^{-1})$	5.8	6.0 - 5.6	4.6
	$k_{u,\ bulk}(s^{-1})$	56.5	58.5 - 54.5	38.4
	$K_{eq,bulk}$	0.9	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

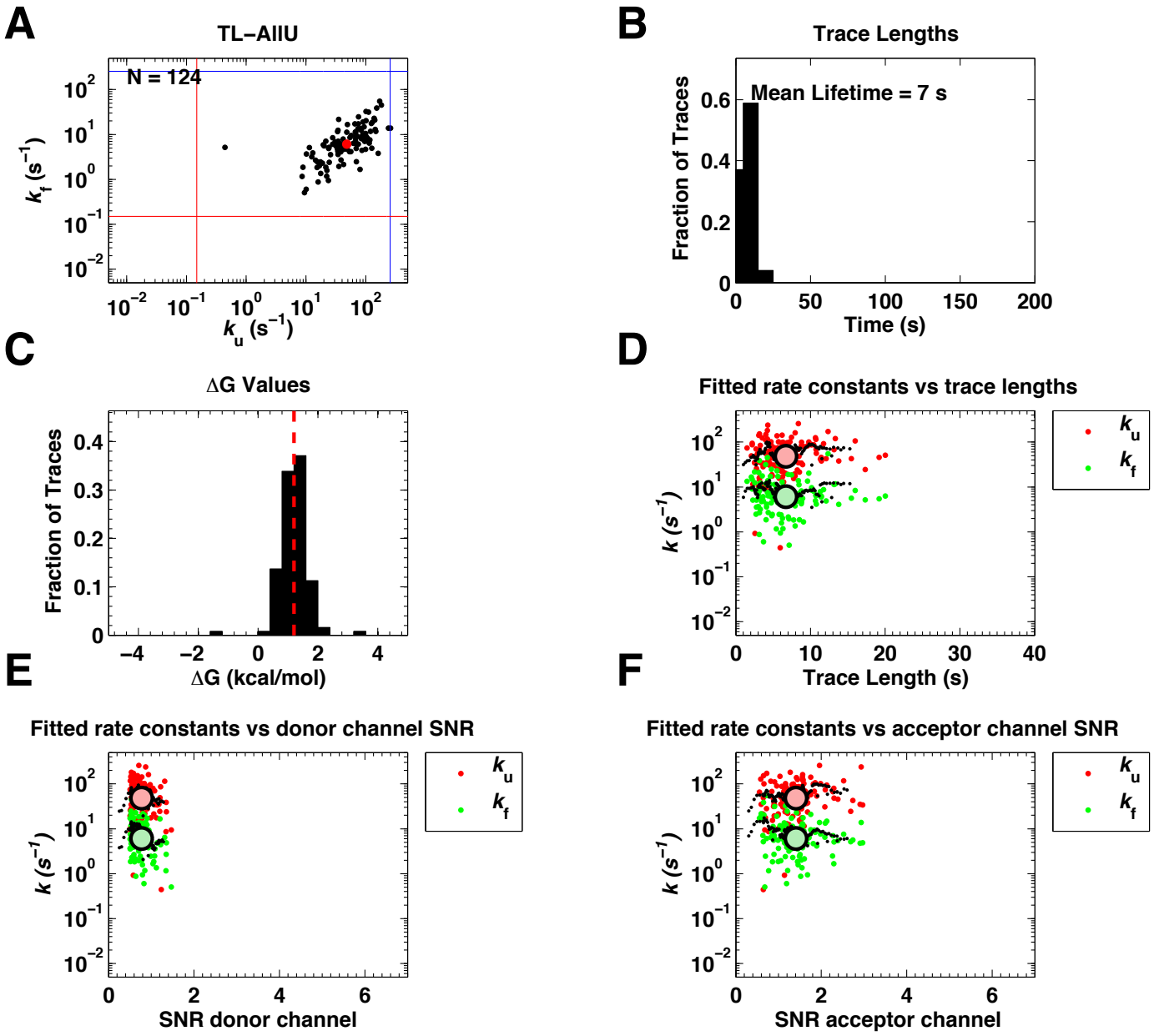


Figure S18-1. smFRET data assessment for TL-AllU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

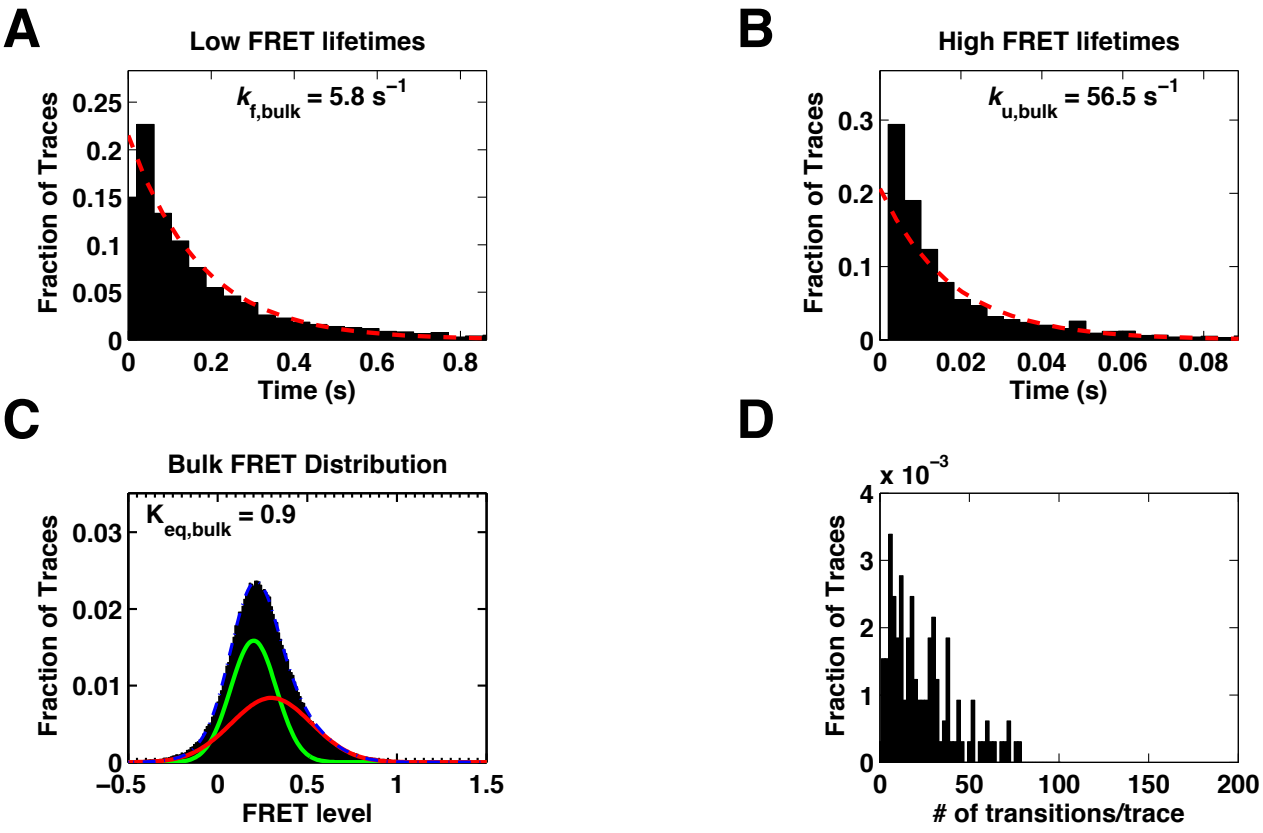


Figure S18-2. smFRET data assesment of aggregate data for TL-AllU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

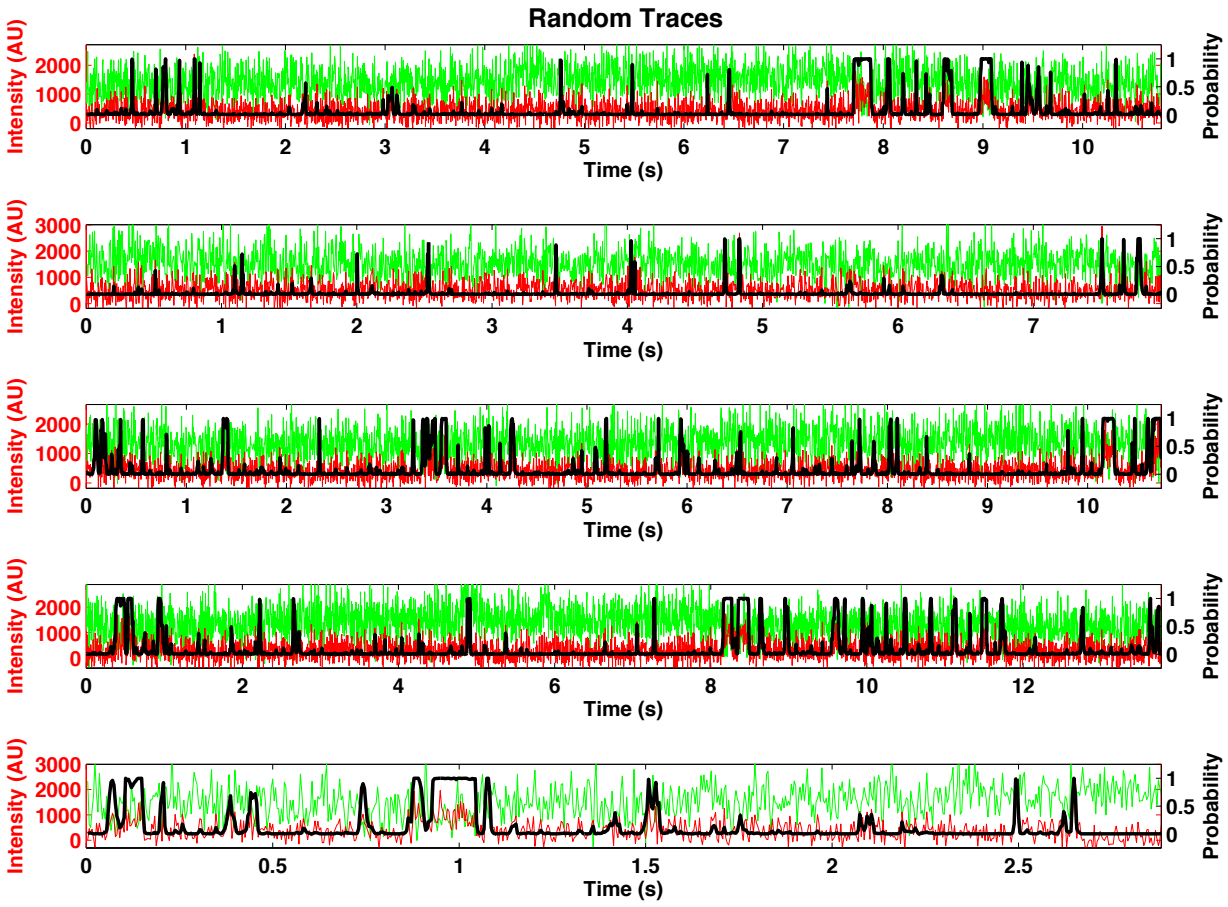


Figure S18-3. Randomly selected FRET traces of TL-AllU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table 1: Variant and Conditions

Variant:	L5B
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	83

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table 2: Folding parameters of smFRET the variant L5B inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	6.9	5.0 - 9.6	4.6
	$k_u(s^{-1})$	40.8	32.4 - 50.7	2.9
	K_{eq}	0.2	0.1 - 0.2	5.4
	SNR green	0.7	0.7 - 0.8	0.3
	SNR red	1.6	1.6 - 2.0	0.9
	ΔG(kcal/mol)	1.3	0.8 - 1.2	1.0
Fits from Cumulative Data[2]	Lifetime (s)	8.4	6.8 - 10.5	8.4
	$k_{f,\ bulk}(s^{-1})$	11.7	12.1 - 11.3	6.3
	$k_{u,\ bulk}(s^{-1})$	38.9	40.3 - 37.6	21.9
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

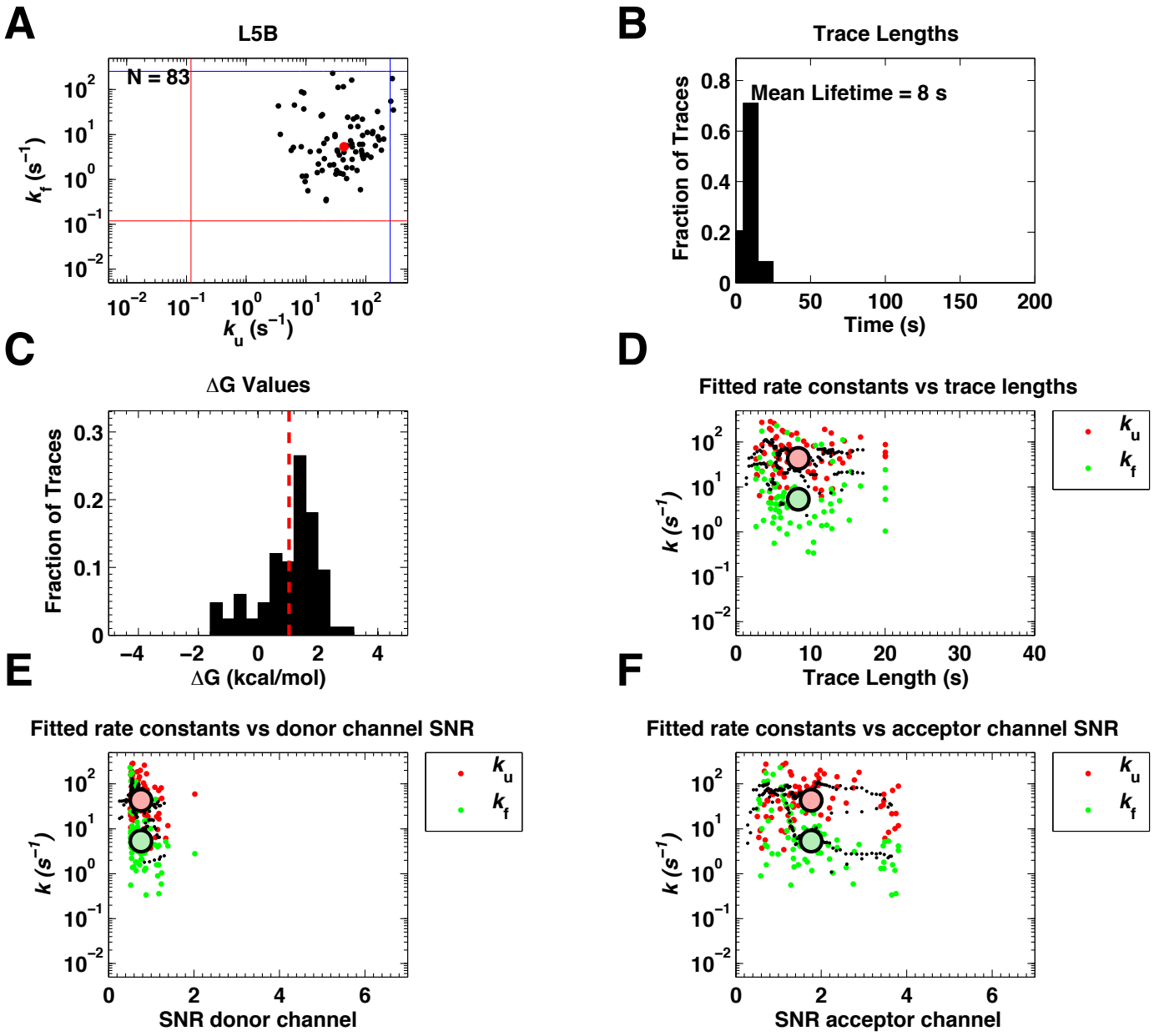


Figure 1. smFRET data assessment for L5B. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

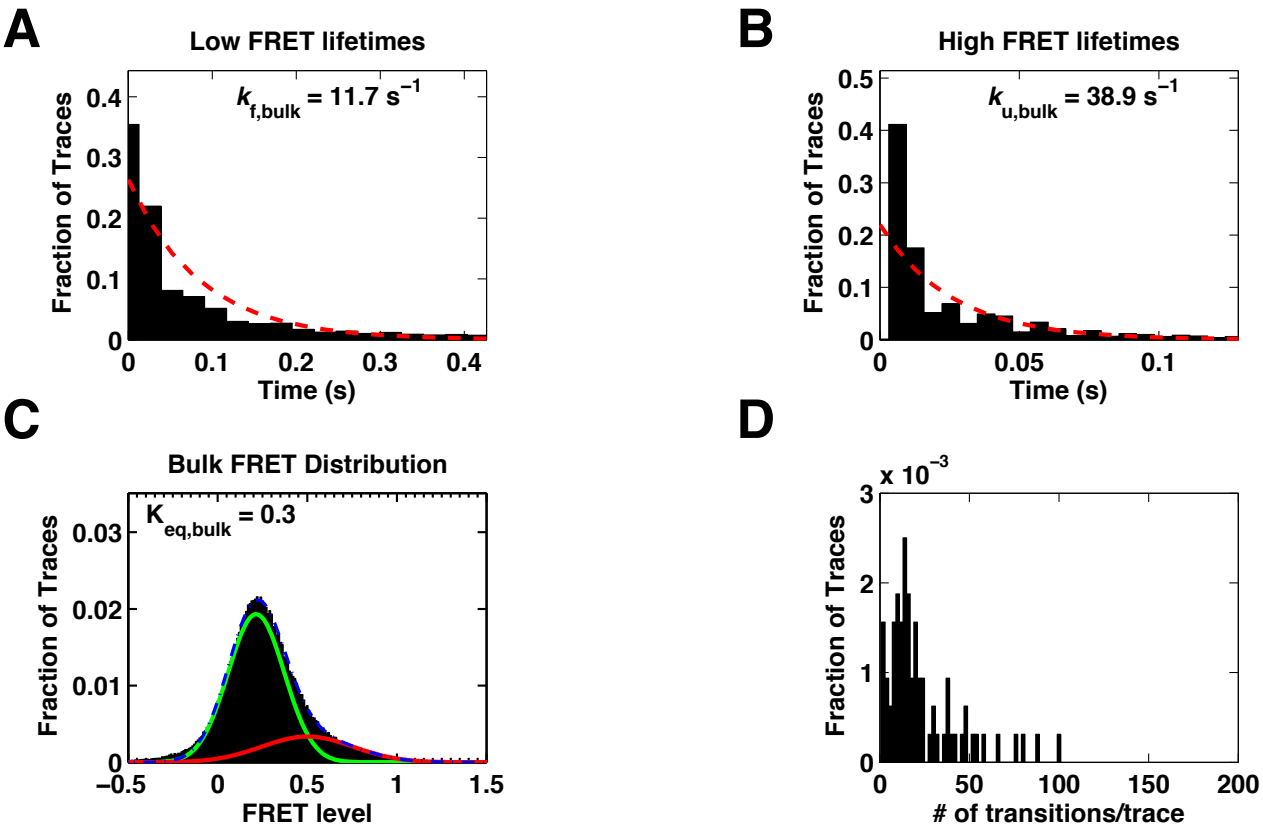


Figure 2. smFRET data assesment of aggregate data for L5B. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

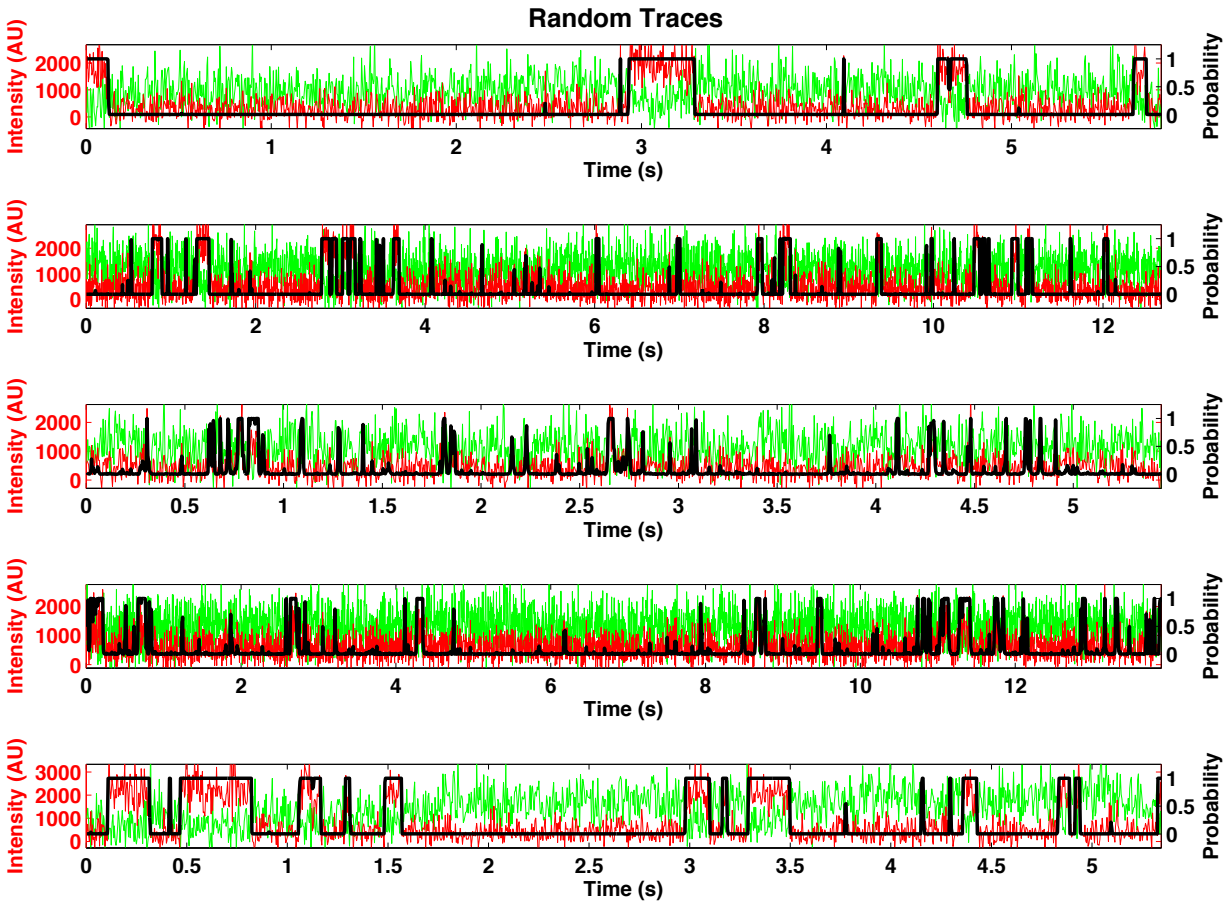


Figure 3. Randomly selected FRET traces of L5B. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S20-1.Variant and Conditions

Variant:	A225U/A226U/C223U
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	90

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S20-2. Folding parameters of smFRET the variant A225U/A226U/C223U inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	9.7	7.7 - 12.2	3.1
	$k_u(s^{-1})$	61.9	46.9 - 75.9	3.1
	K_{eq}	0.2	0.1 - 0.2	3.6
	SNR green	1.1	1.0 - 1.2	0.5
	SNR red	1.5	1.4 - 1.6	0.6
	ΔG(kcal/mol)	1.2	0.9 - 1.2	0.7
Fits from Cumulative Data[2]	Lifetime (s)	8.0	6.5 - 9.9	8.0
	$k_{f,\ bulk}(s^{-1})$	9.2	9.5 - 9.0	7.1
	$k_{u,\ bulk}(s^{-1})$	68.3	70.5 - 66.2	37.1
	$K_{eq,bulk}$	0.5	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.4	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

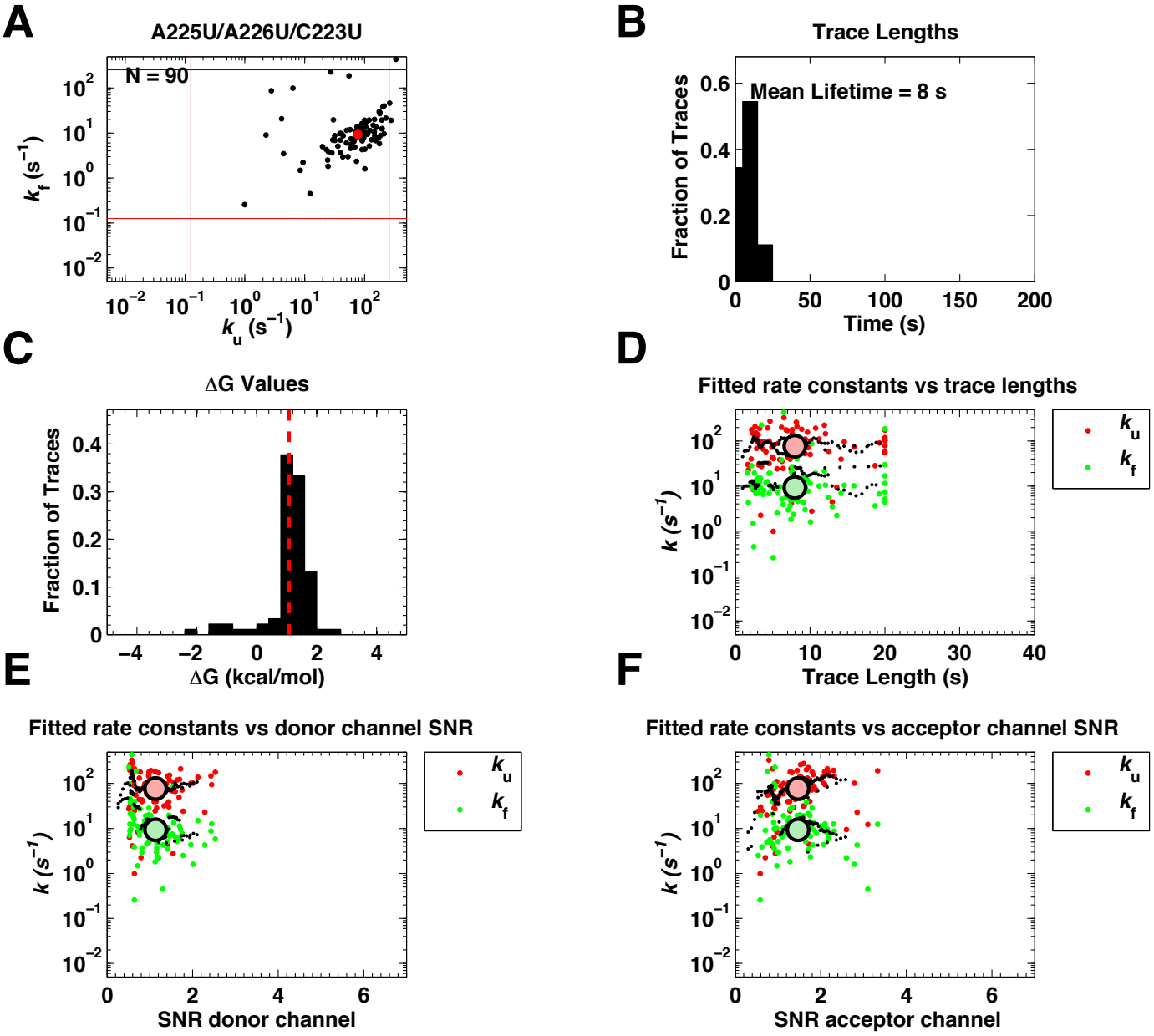


Figure S20-1. smFRET data assessment for A225U/A226U/C223U. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

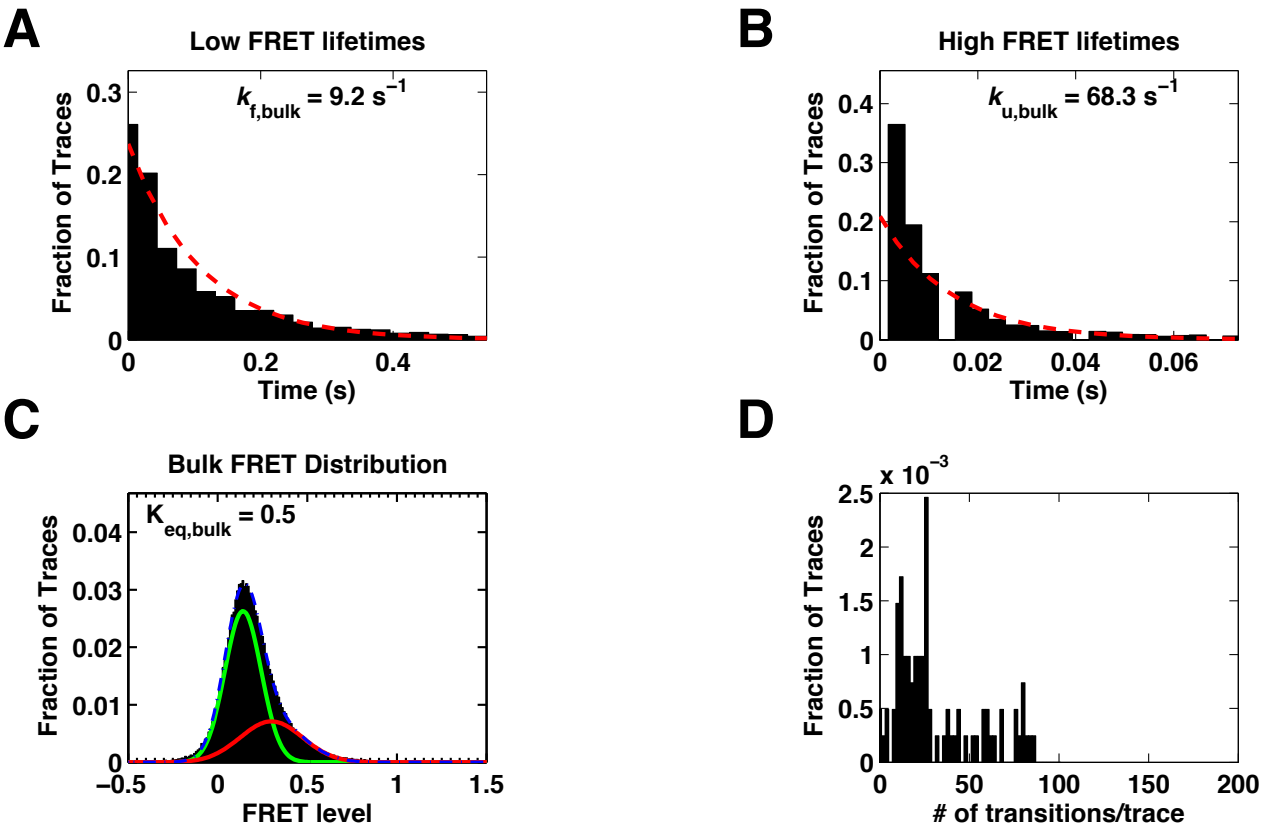


Figure S20-2. smFRET data assesment of aggregate data for A225U/A226U/C223U. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

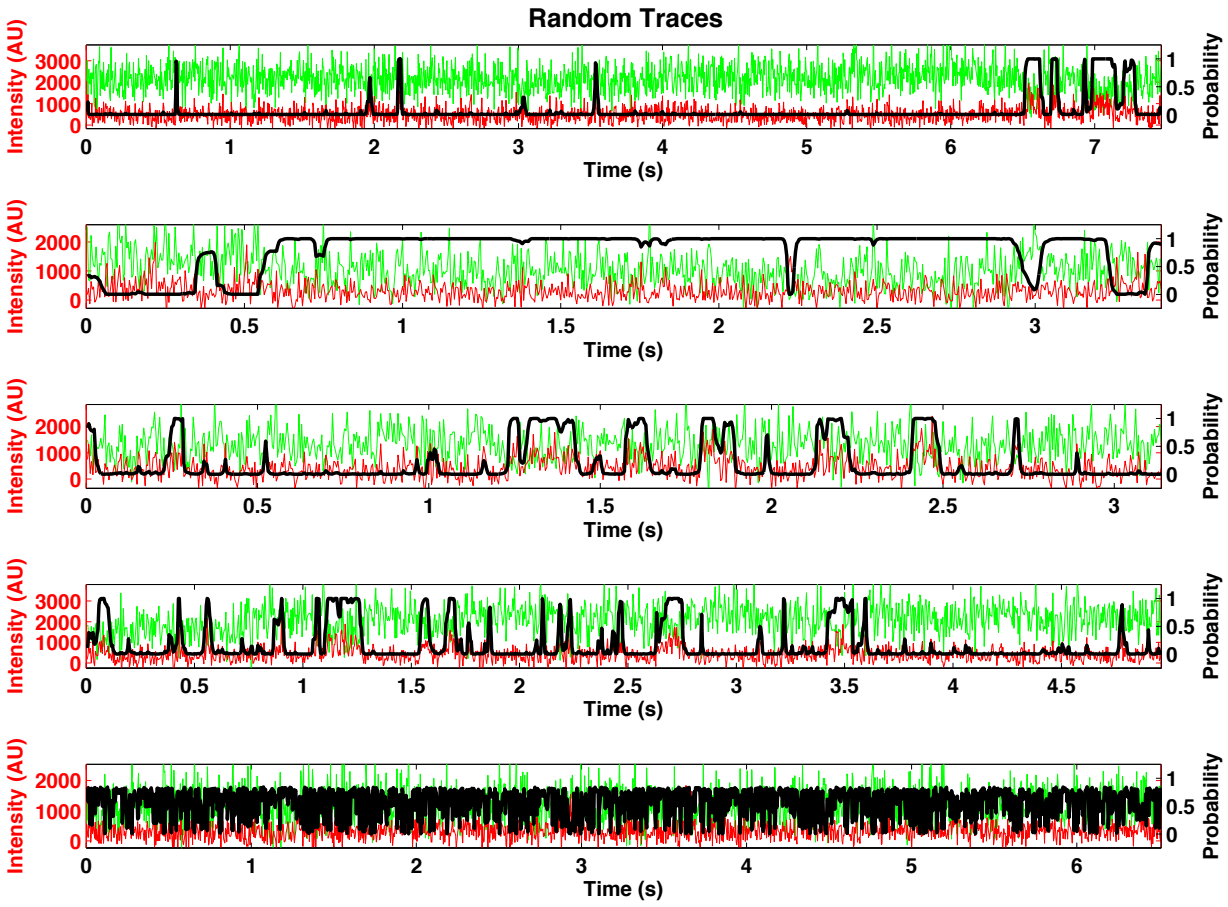


Figure S20-3. Randomly selected FRET traces of A225U/A226U/C223U. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S21-1. Variant and Conditions

Variant:	J6/6a BP
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	288
SNR Threshold[2]	0.50
Number of Traces	73

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S21-2. Folding parameters of smFRET the variant J6/6a BP inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	10.5	8.3 - 12.5	2.4
	$k_u(s^{-1})$	88.1	63.4 - 102.9	2.6
	K_{eq}	0.1	0.1 - 0.1	2.4
	SNR green	1.3	1.3 - 1.5	0.6
	SNR red	1.6	1.5 - 1.8	0.6
	ΔG(kcal/mol)	1.2	1.1 - 1.3	0.5
Fits from Cumulative Data[2]	Lifetime (s)	8.0	6.4 - 10.2	8.0
	$k_{f,\ bulk}(s^{-1})$	10.1	10.5 - 9.8	7.9
	$k_{u,\ bulk}(s^{-1})$	95.1	98.1 - 92.1	74.4
	$K_{eq,bulk}$	0.5	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.4	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

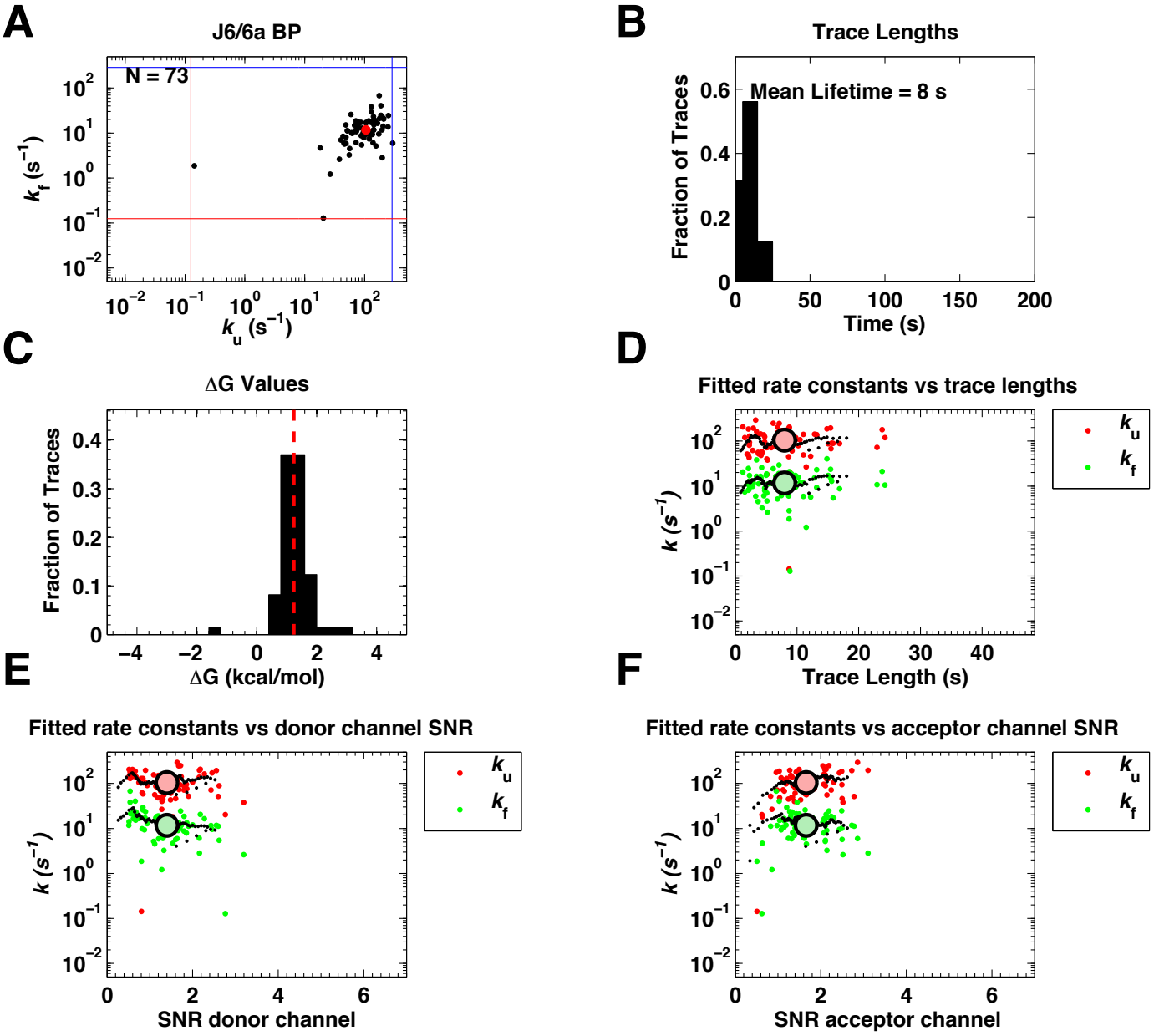


Figure S21-1. smFRET data assessment for J6/6a BP. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

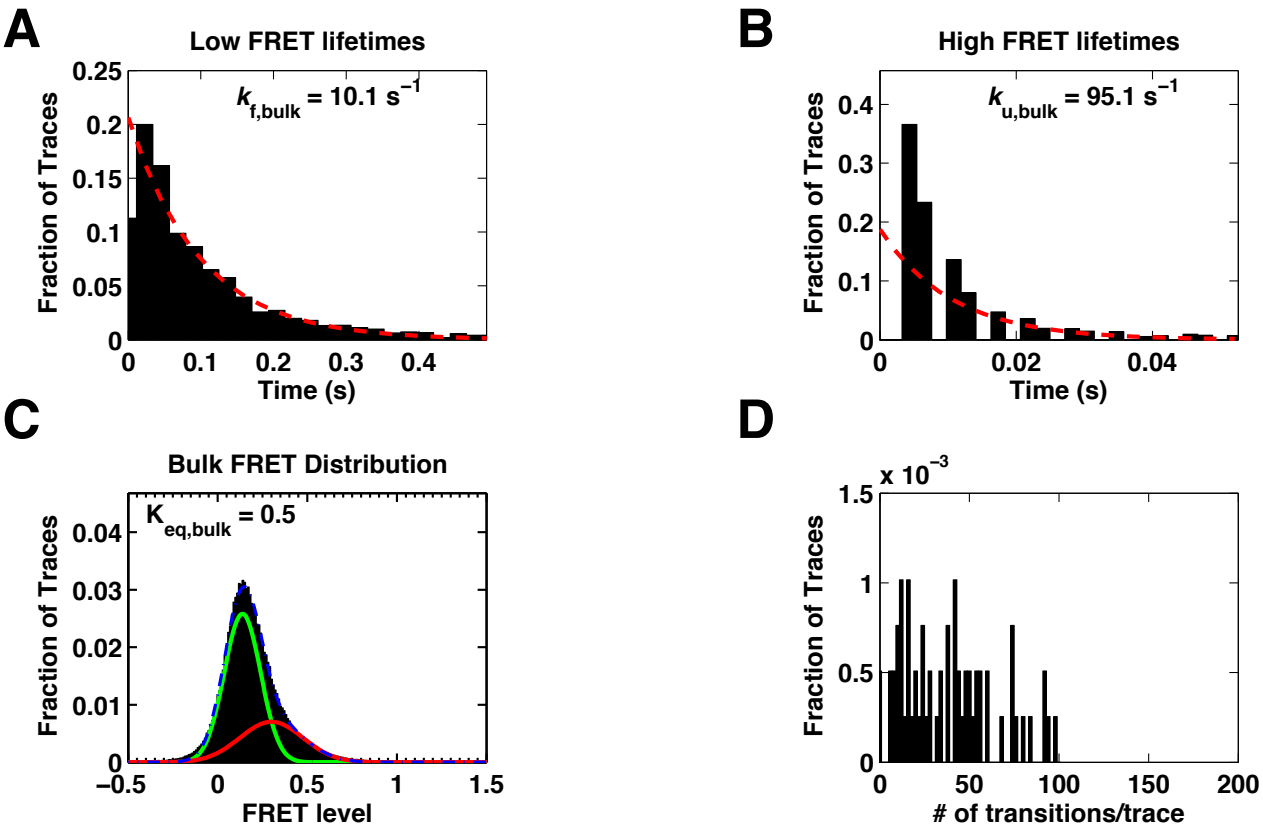


Figure S21-2. smFRET data assesment of aggregate data for J6/6a BP. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

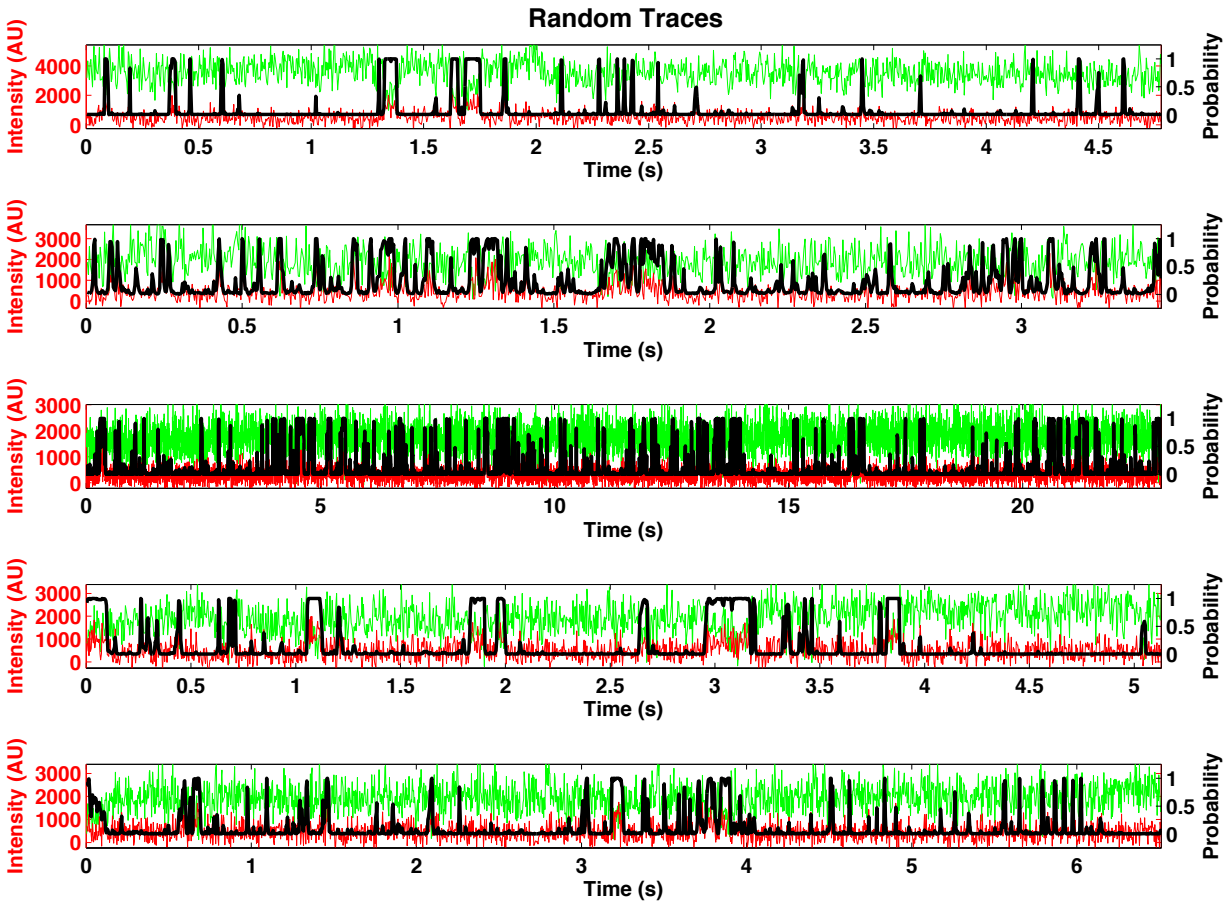


Figure S21-3. Randomly selected FRET traces of J6/6a BP. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S22-1. Variant and Conditions

Variant:	J6/6a BP Cy3b/Atto674N
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	265
SNR Threshold[2]	0.25
Number of Traces	93

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S22-2. Folding parameters of smFRET the variant J6/6a BP Cy3b/Atto674N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	14.0	11.5 - 16.0	2.2
	$k_u(s^{-1})$	104.0	59.6 - 122.7	3.9
	K_{eq}	0.1	0.1 - 0.2	2.5
	SNR green	1.9	1.7 - 2.0	0.6
	SNR red	1.9	1.8 - 2.0	0.4
	ΔG(kcal/mol)	1.2	1.0 - 1.2	0.5
Fits from Cumulative Data[2]	Lifetime (s)	13.8	11.4 - 17.1	13.8
	$k_{f,\ bulk}(s^{-1})$	12.9	13.2 - 12.7	10.3
	$k_{u,\ bulk}(s^{-1})$	103.2	105.2 - 101.3	84.4
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.9	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

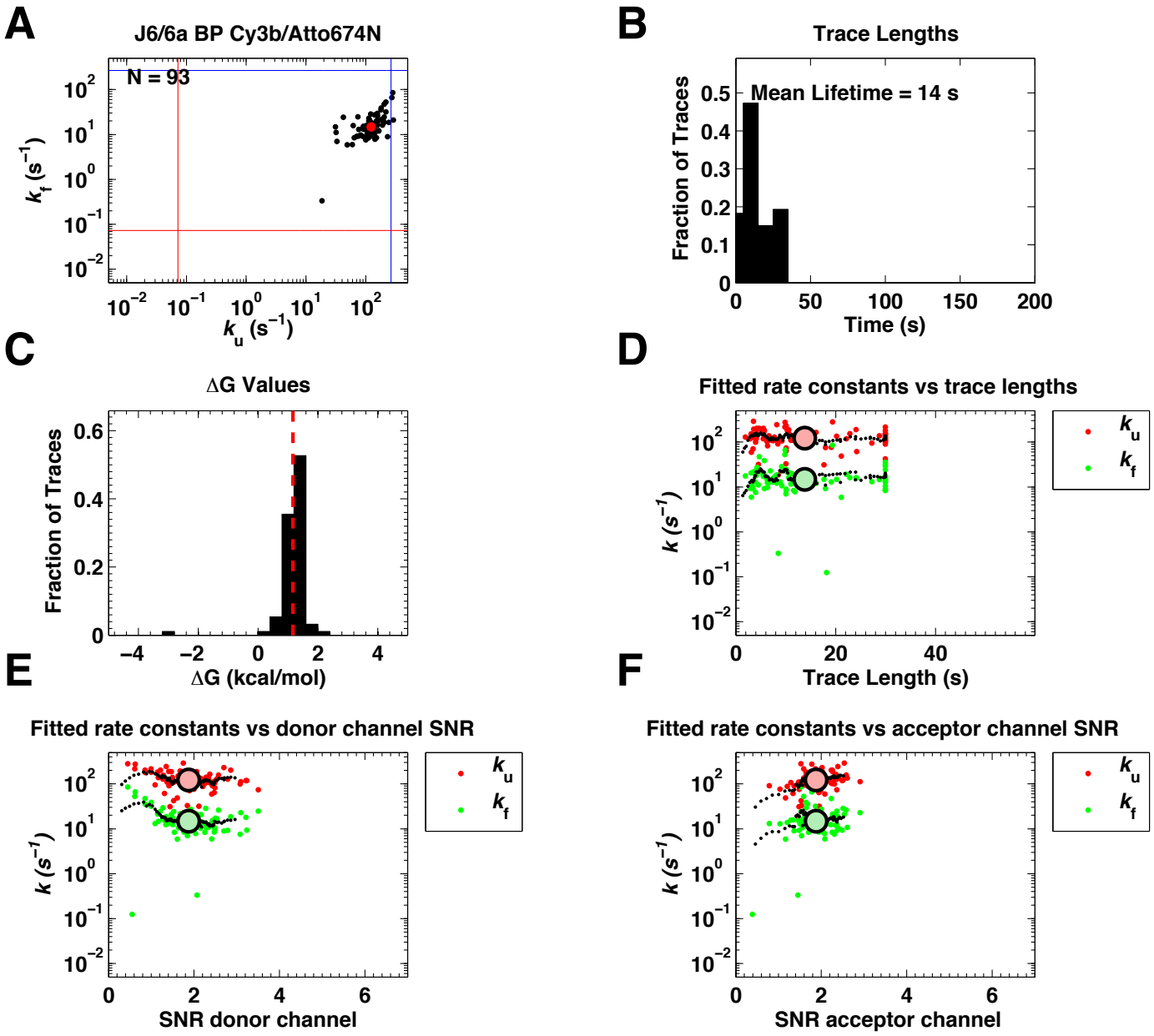


Figure S22-1. smFRET data assessment for J6/6a BP Cy3b/Atto674N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

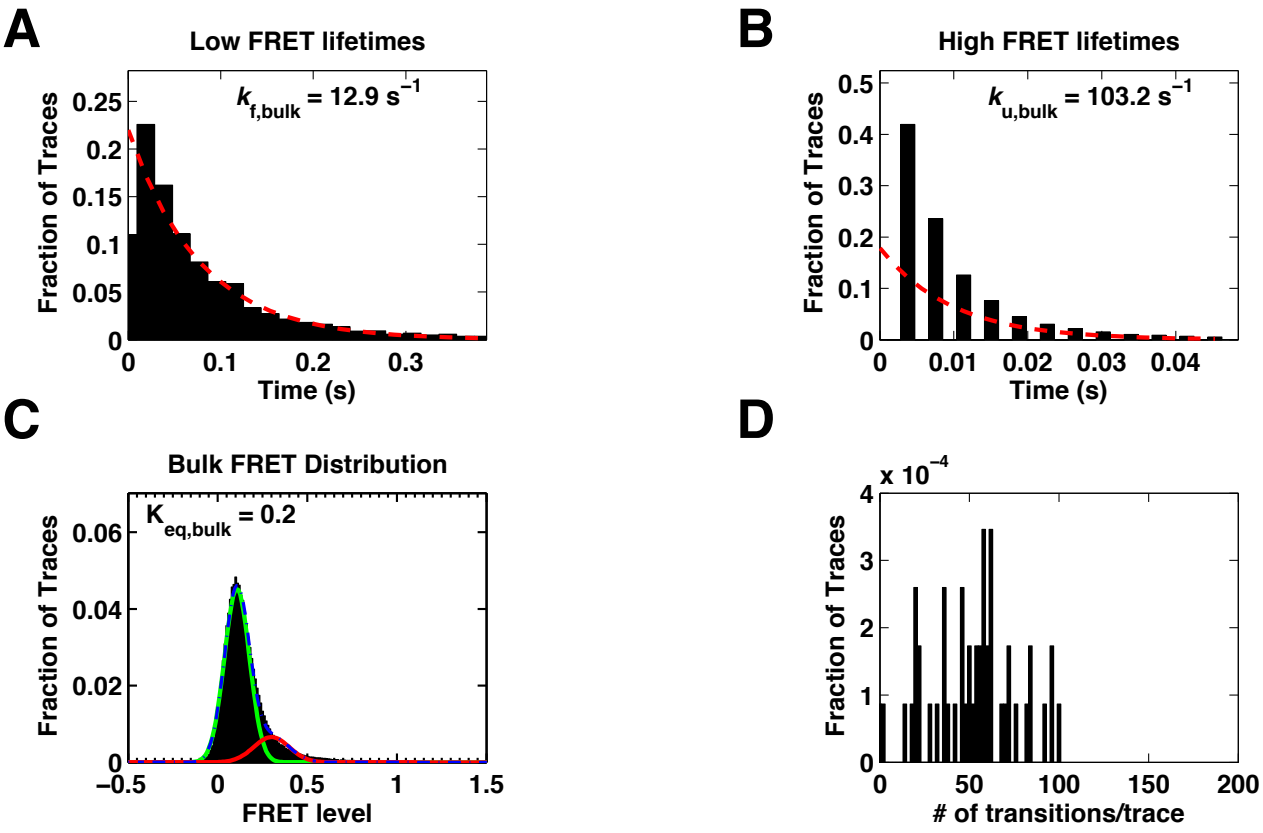


Figure S22-2. smFRET data assesment of aggregate data for J6/6a BP Cy3b/Atto674N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

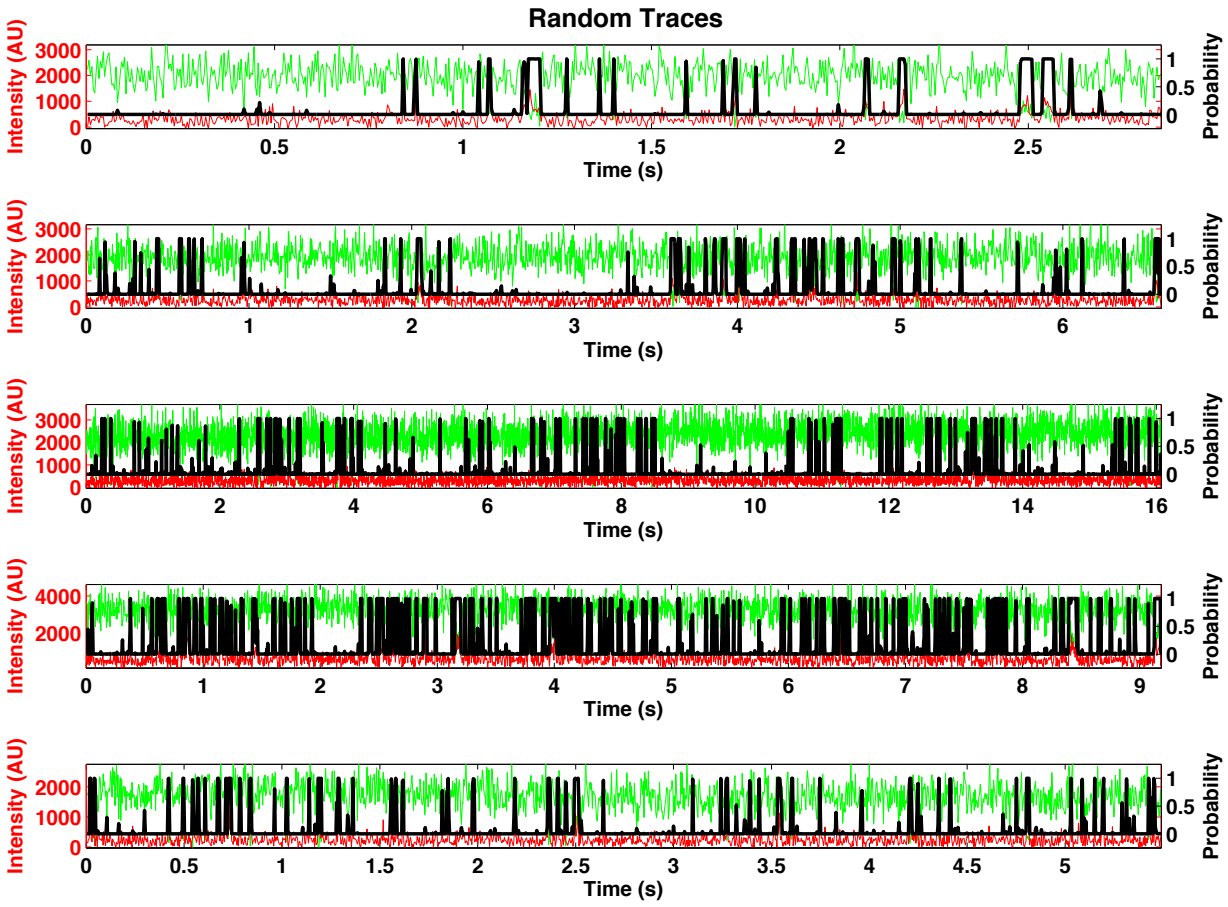


Figure S22-3. Randomly selected FRET traces of J6/6a BP Cy3b/Atto674N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S23-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	111

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S23-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	16.6	15.2 - 18.0	1.5
	$k_u(s^{-1})$	5.2	4.9 - 5.6	1.4
	K_{eq}	3.2	2.8 - 3.5	1.9
	SNR green	3.4	3.4 - 3.7	0.8
	SNR red	1.5	1.6 - 1.7	0.4
	ΔG(kcal/mol)	-0.7	-0.7 - -0.6	0.4
Fits from Cumulative Data[2]	Lifetime (s)	6.8	5.7 - 8.3	6.8
	$k_{f,\ bulk}(s^{-1})$	15.9	16.5 - 15.3	14.6
	$k_{u,\ bulk}(s^{-1})$	5.0	5.2 - 4.8	4.8
	$K_{eq,bulk}$	3.7	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.8	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

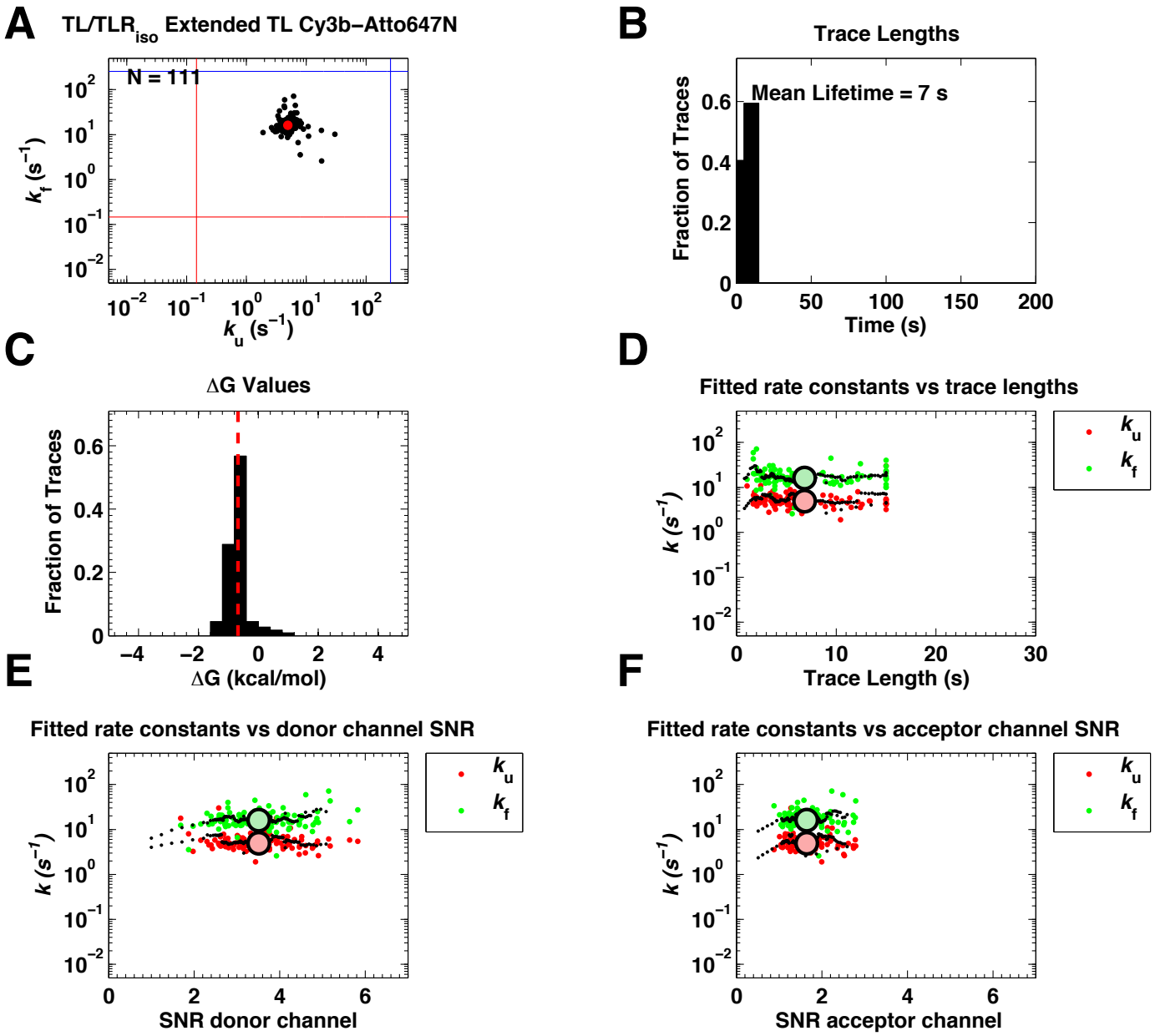


Figure S23-1. smFRET data assessment for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

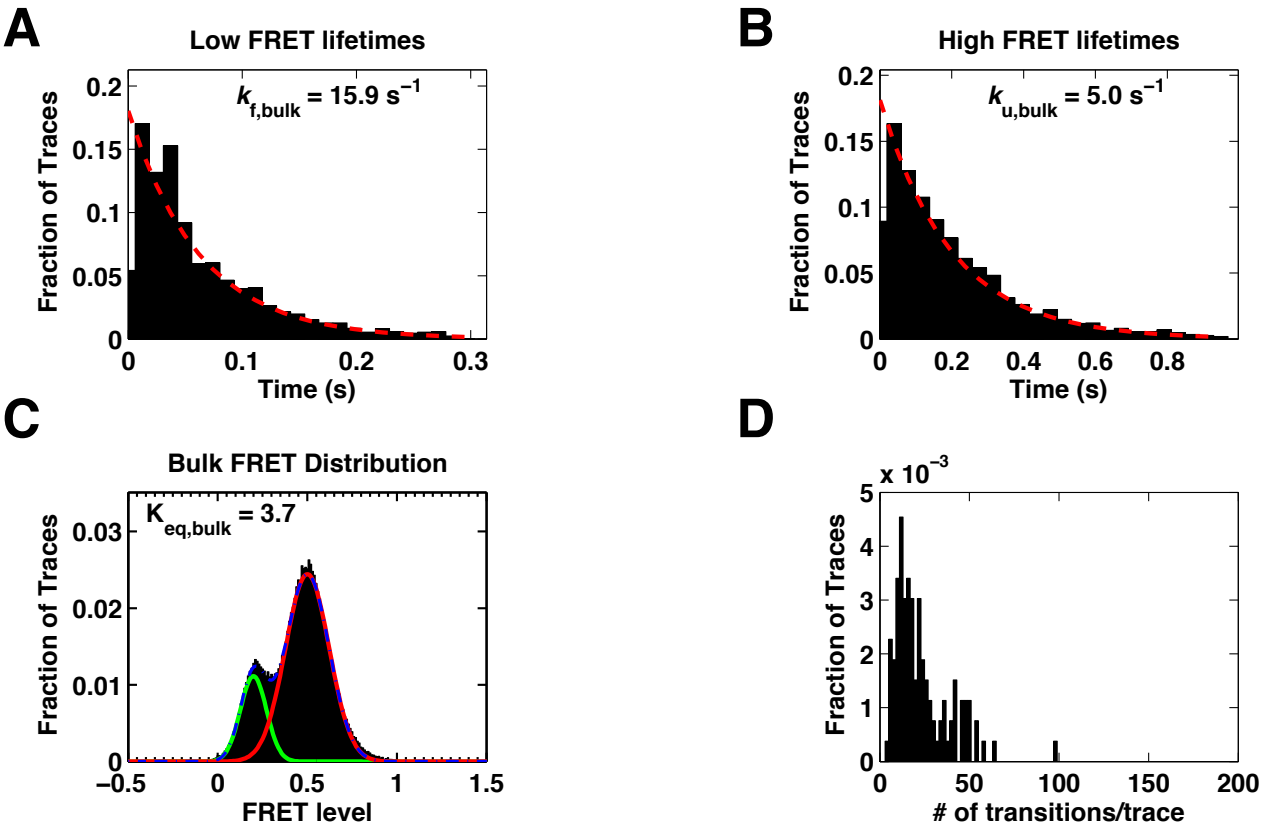


Figure S23-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

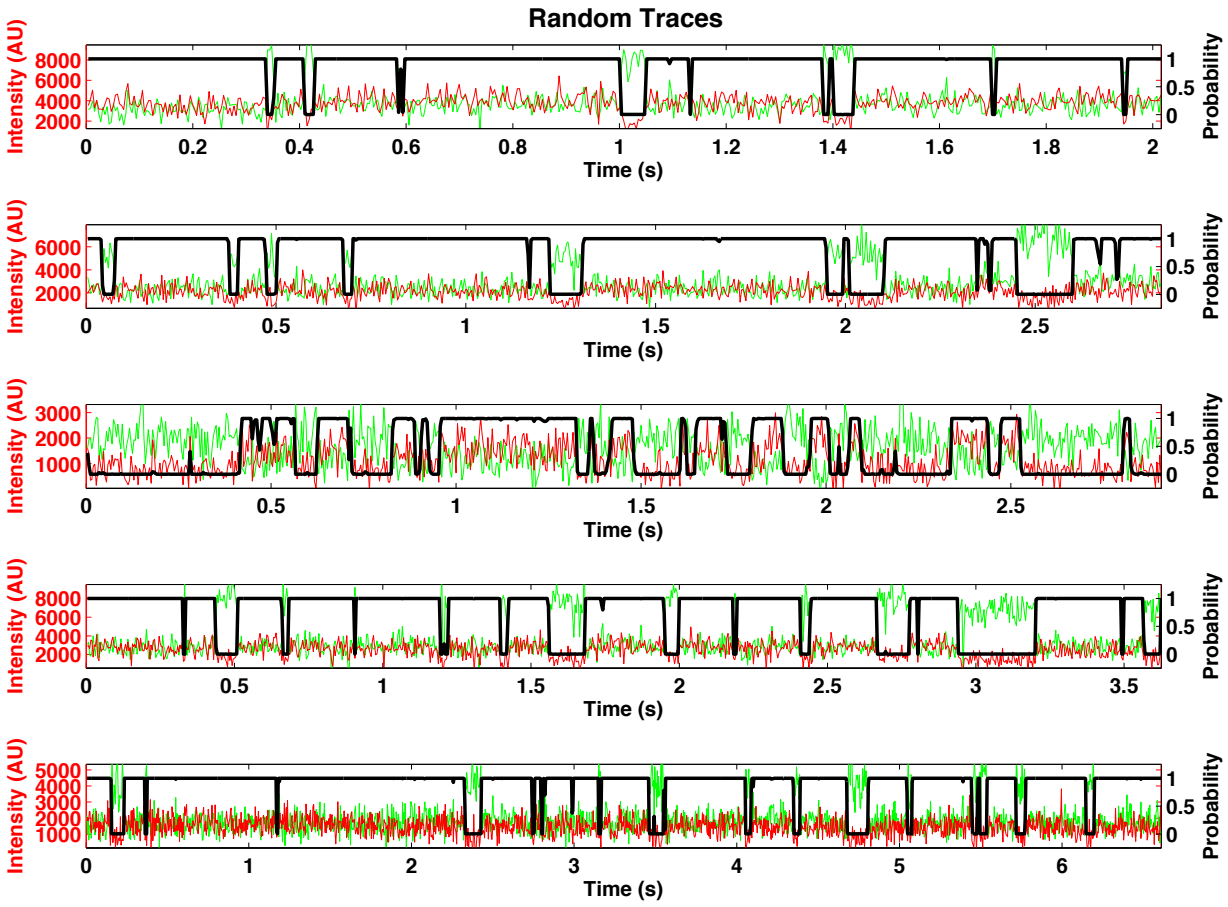


Figure S23-3. Randomly selected FRET traces of TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S24-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ T14
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	141

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S24-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ T14 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	54.7	48.6 - 59.6	1.8
	$k_u(s^{-1})$	8.9	8.3 - 9.6	1.5
	K_{eq}	6.1	5.3 - 6.8	2.1
	SNR green	2.4	2.2 - 2.4	0.5
	SNR red	2.4	2.3 - 2.5	0.6
	ΔG(kcal/mol)	-1.2	-1.1 - -1.0	0.4
Fits from Cumulative Data[2]	Lifetime (s)	5.2	4.5 - 6.2	5.2
	$k_{f,\ bulk}(s^{-1})$	46.2	47.7 - 44.7	34.1
	$k_{u,\ bulk}(s^{-1})$	6.8	7.0 - 6.6	6.5
	$K_{eq,bulk}$	5.5	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

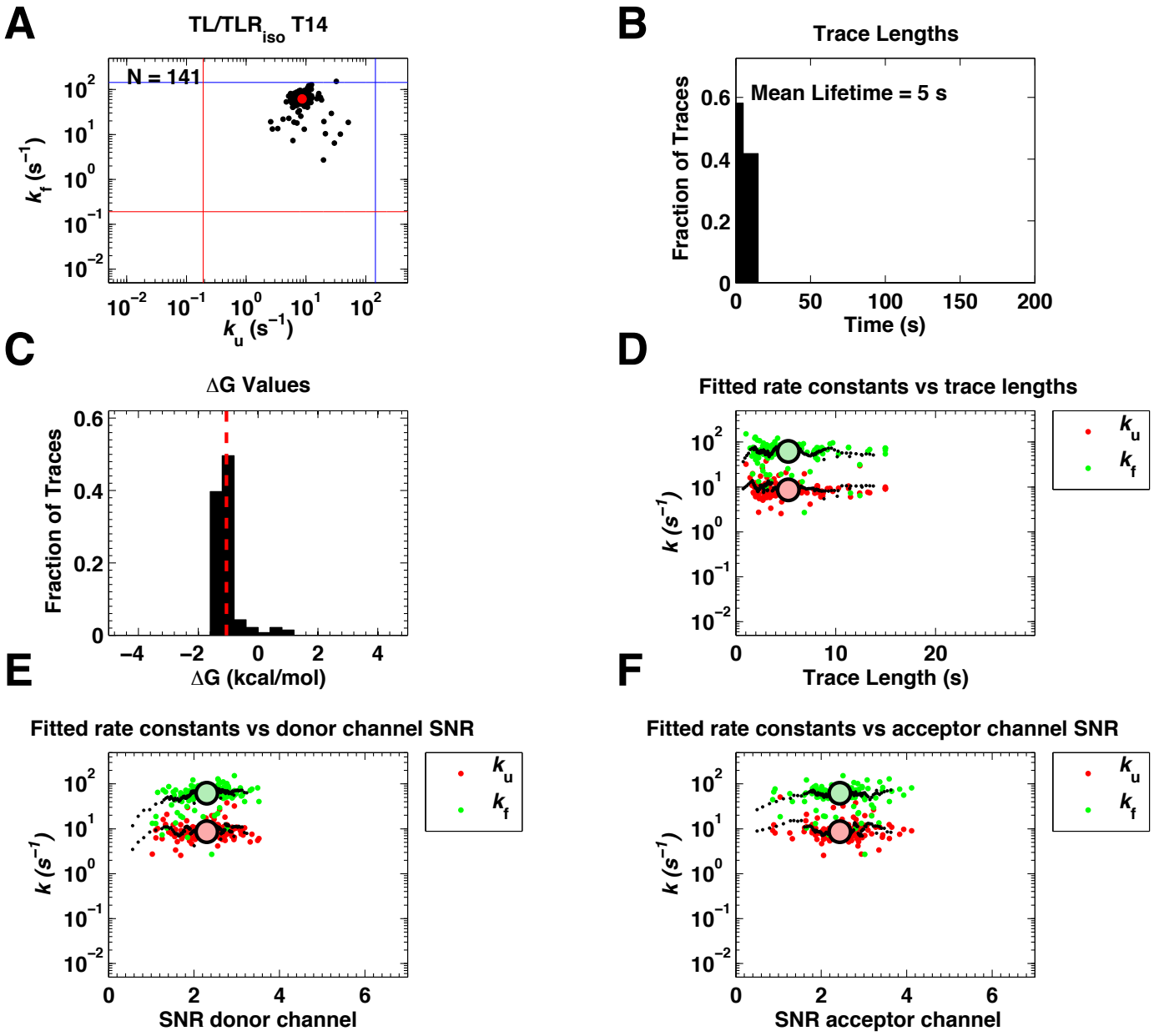


Figure S24-1. smFRET data assessment for TL/TLR$_{iso}$ T14. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

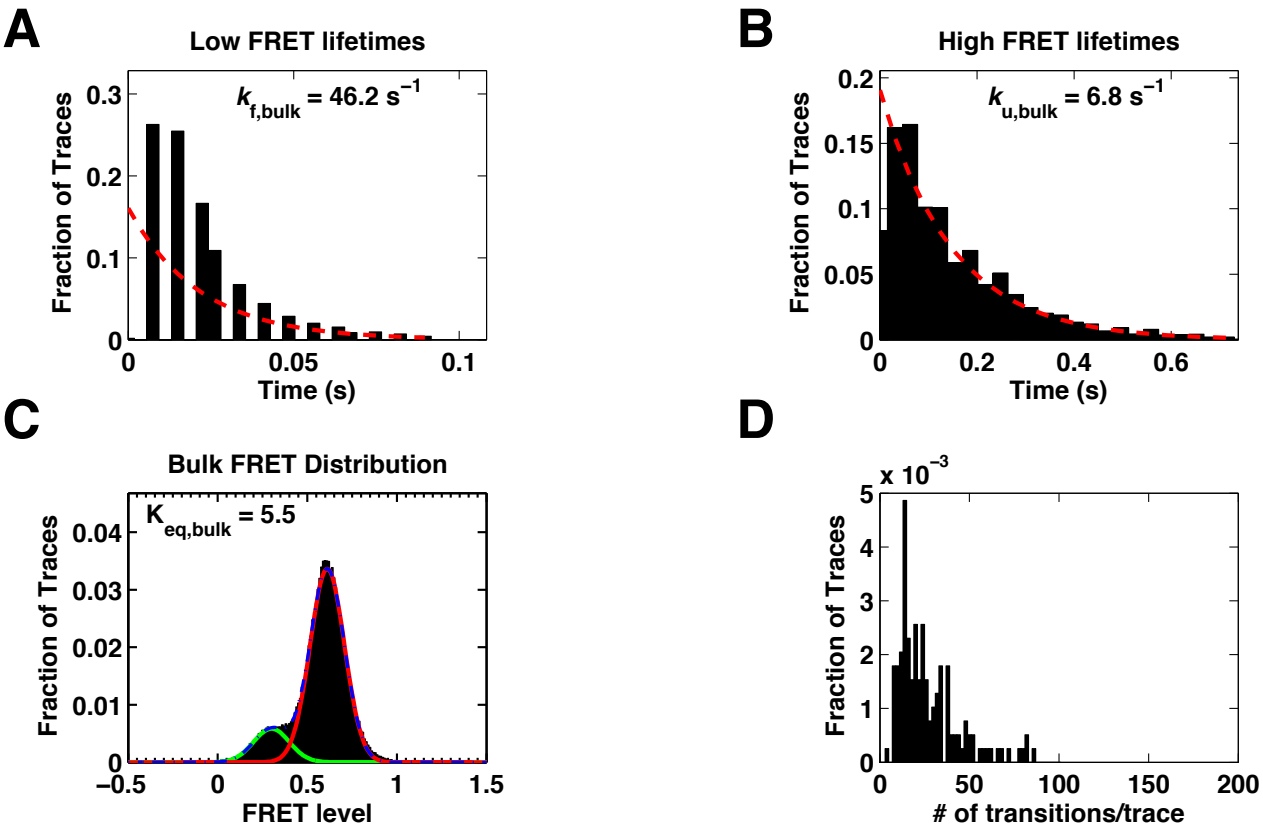


Figure S24-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ T14. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

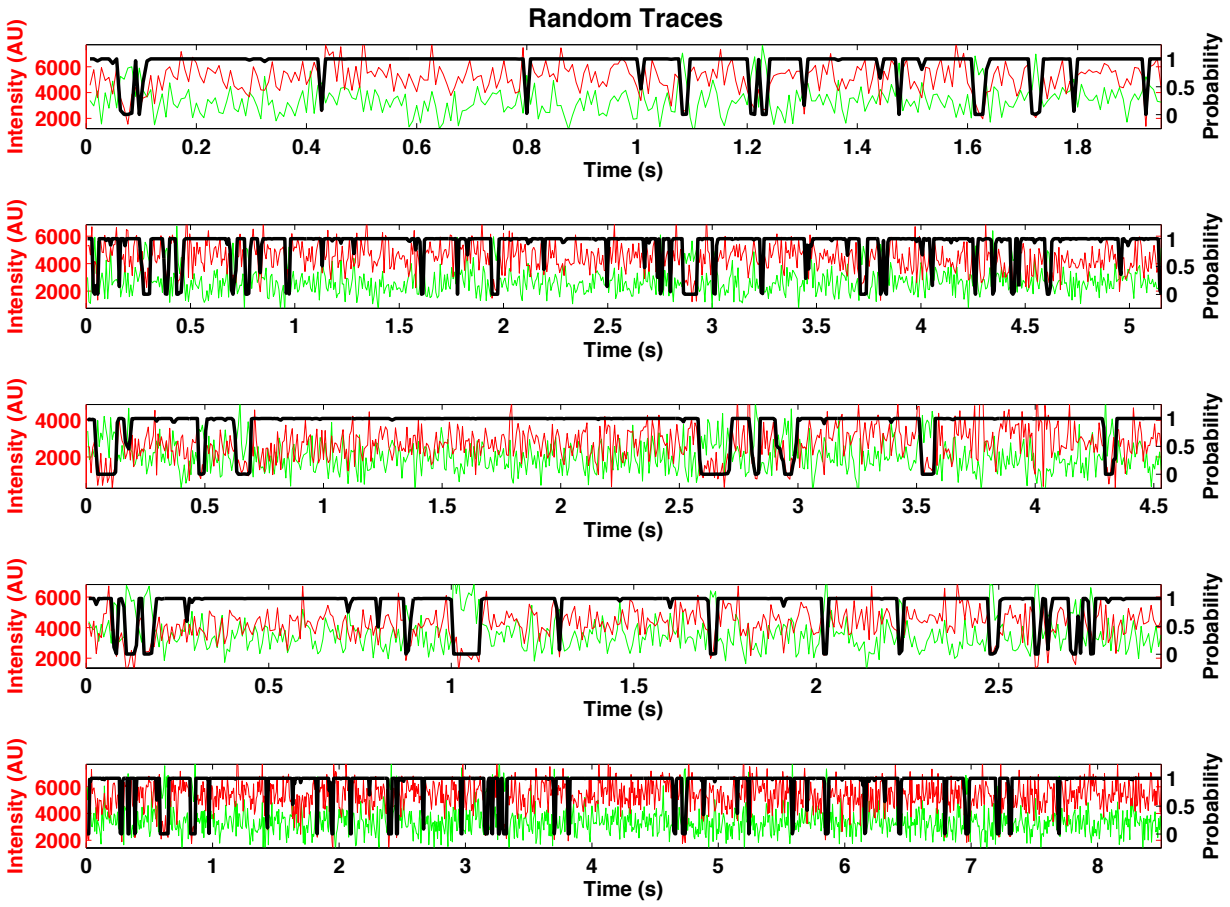


Figure S24-3. Randomly selected FRET traces of TL/TLR$_{iso}$ T14. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S25-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ A7 Cy3b/Atto647N
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	84

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S25-2.Folding parameters of smFRET the variant TL/TLR$_{iso}$ A7 Cy3b/Atto647N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	20.4	18.5 - 22.6	1.6
	$k_u(s^{-1})$	4.3	3.9 - 4.7	1.5
	K_{eq}	4.8	4.1 - 5.4	1.9
	SNR green	4.2	4.0 - 4.5	1.0
	SNR red	1.7	1.7 - 1.9	0.5
	ΔG(kcal/mol)	-0.9	-1.0 - -0.8	0.4
Fits from Cumulative Data[2]	Lifetime (s)	7.0	5.7 - 8.7	7.0
	$k_{f,\ bulk}(s^{-1})$	19.7	20.6 - 18.8	18.0
	$k_{u,\ bulk}(s^{-1})$	4.3	4.5 - 4.2	4.1
	$K_{eq,bulk}$	4.6	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.9	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

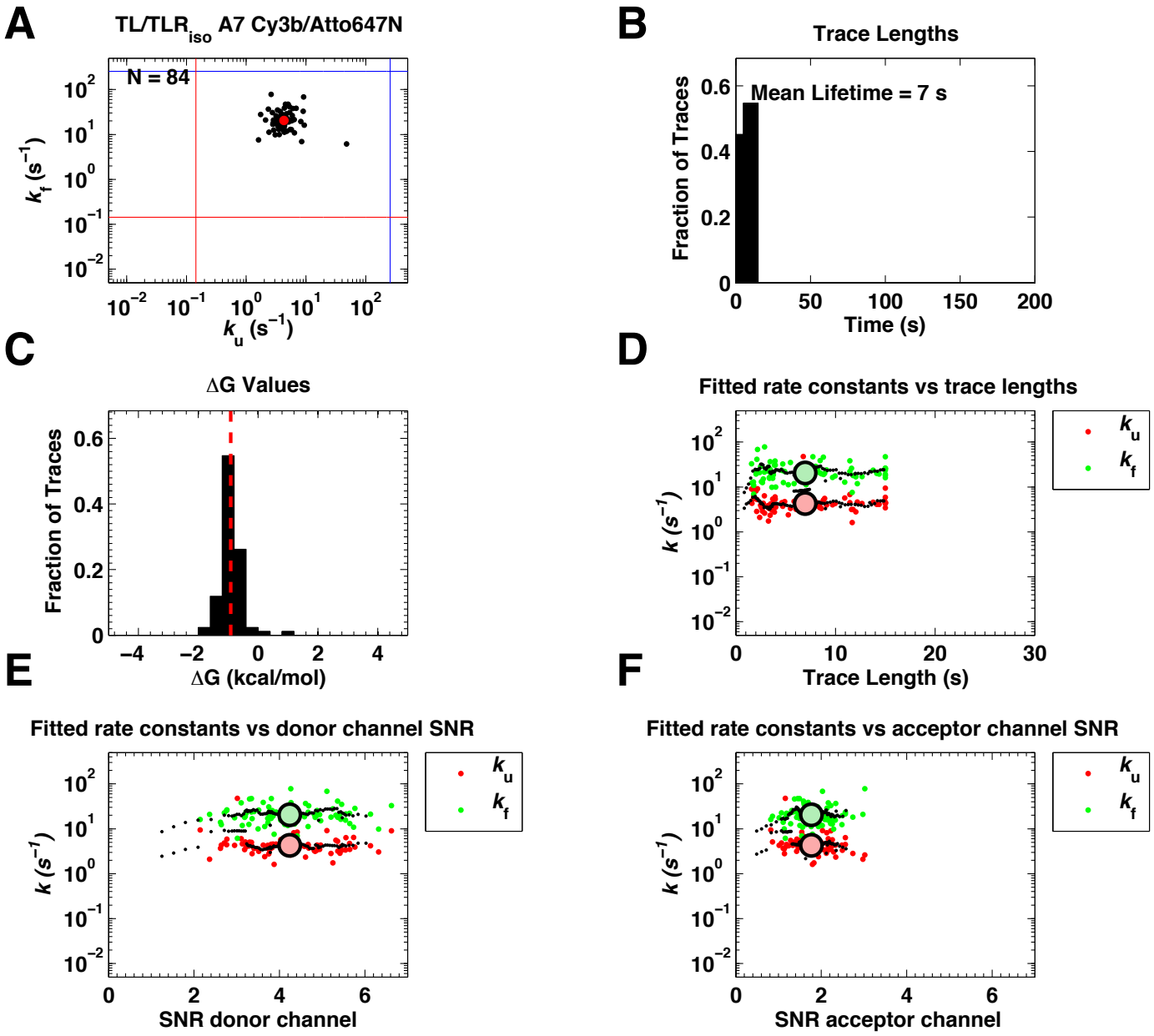


Figure S25-1. smFRET data assessment for TL/TLR$_{iso}$ A7 Cy3b/Atto647N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

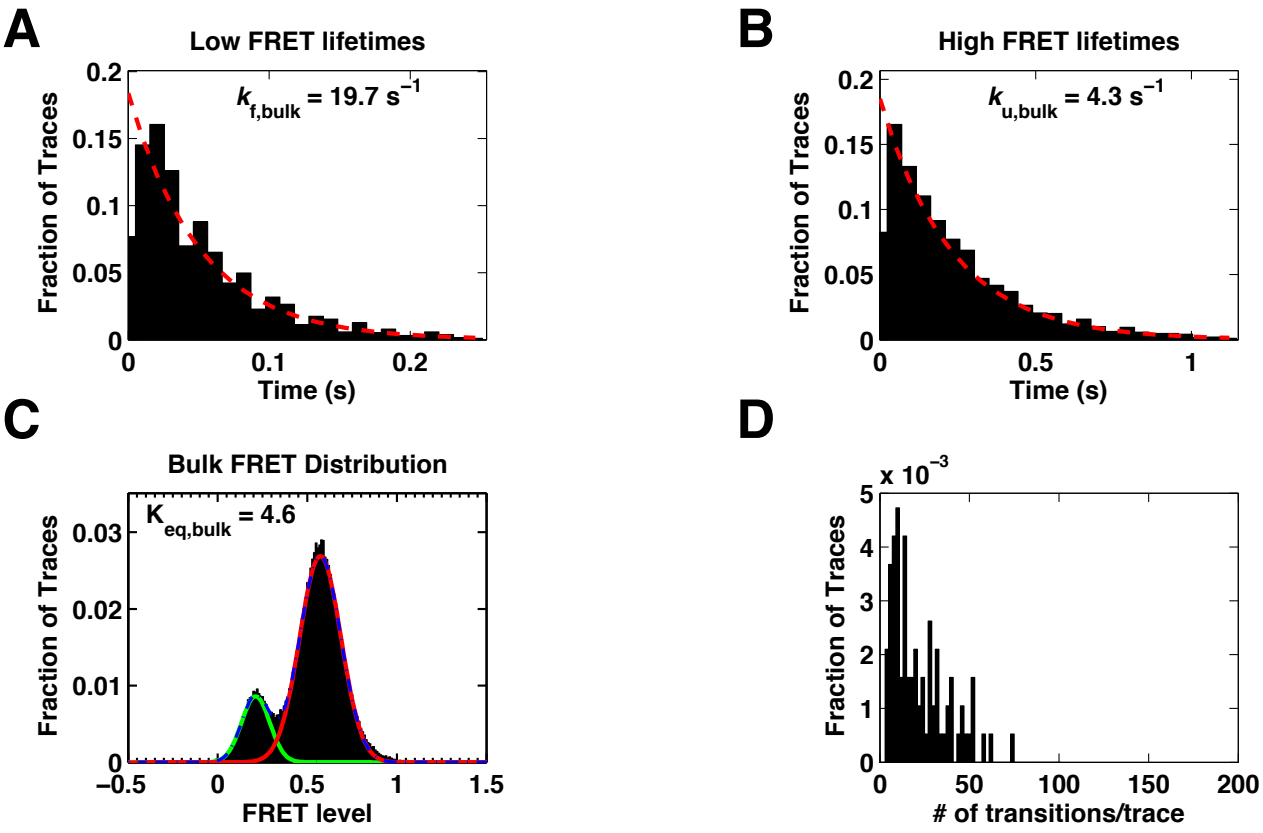


Figure S25-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ A7 Cy3b/Atto647N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

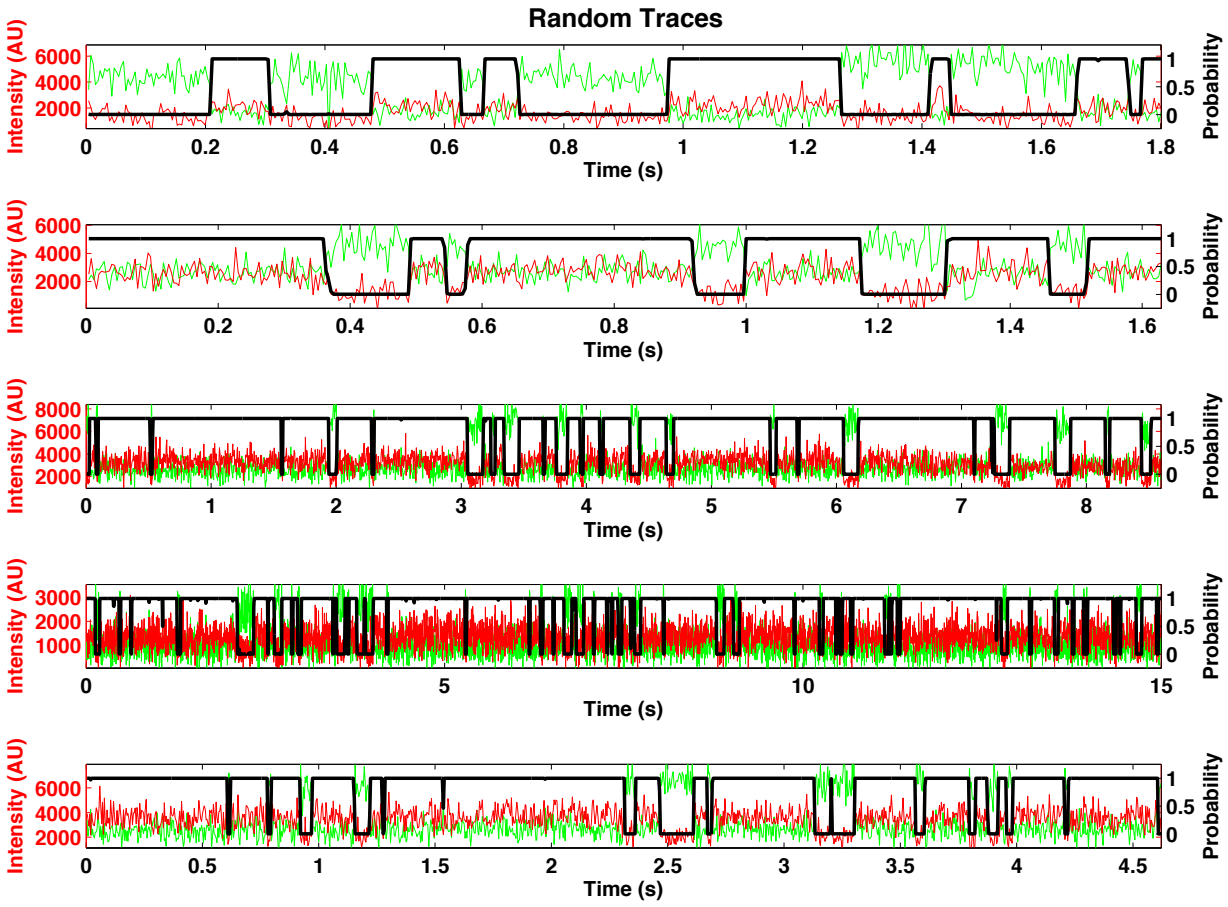


Figure S25-3. Randomly selected FRET traces of TL/TLR$_{iso}$ A7 Cy3b/Atto647N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S26-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ P4P6 Helical Context
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	99

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S26-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ P4P6 Helical Context inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	13.2	11.6 - 14.7	1.8
	$k_u(s^{-1})$	5.6	4.9 - 6.3	1.8
	K_{eq}	2.4	1.9 - 2.8	2.6
	SNR green	1.3	1.3 - 1.4	0.3
	SNR red	2.2	2.1 - 2.3	0.5
	ΔG(kcal/mol)	-0.6	-0.6 - -0.4	0.6
Fits from Cumulative Data[2]	Lifetime (s)	5.3	4.4 - 6.5	5.3
	$k_{f,\ bulk}(s^{-1})$	12.2	12.9 - 11.6	9.6
	$k_{u,\ bulk}(s^{-1})$	5.0	5.3 - 4.8	4.6
	$K_{eq,bulk}$	2.9	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.6	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

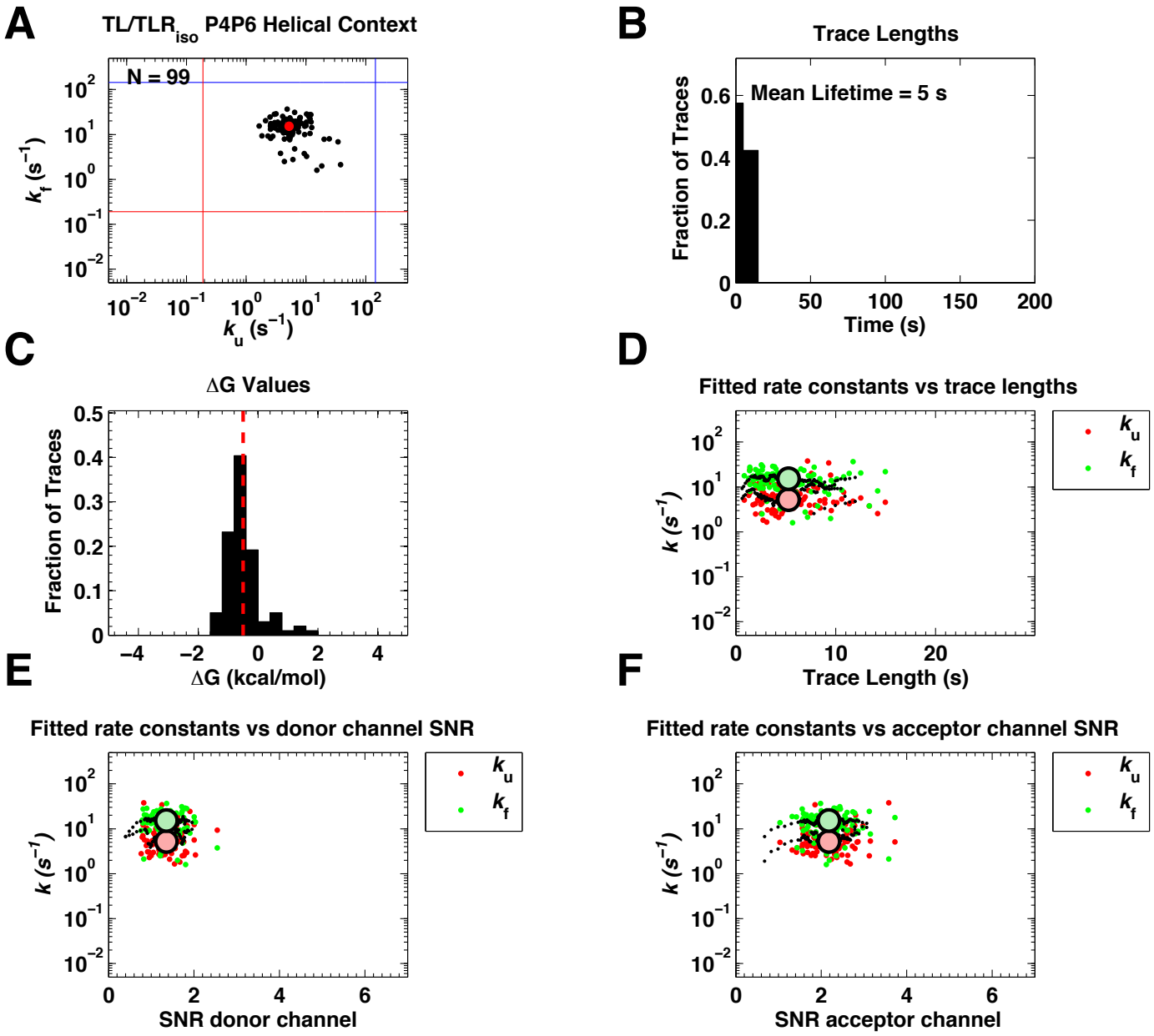


Figure S26-1. smFRET data assessment for TL/TLR$_{iso}$ P4P6 Helical Context. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

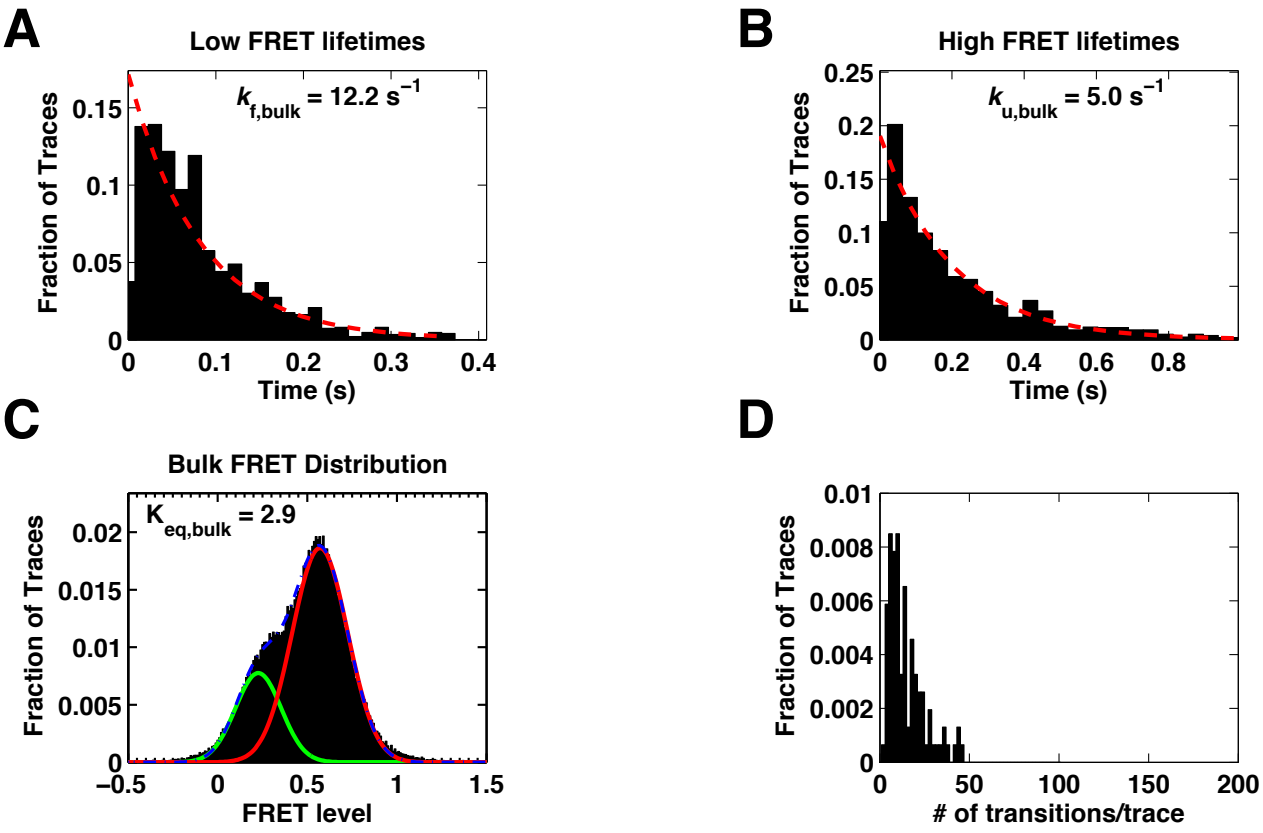


Figure S26-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ P4P6 Helical Context. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

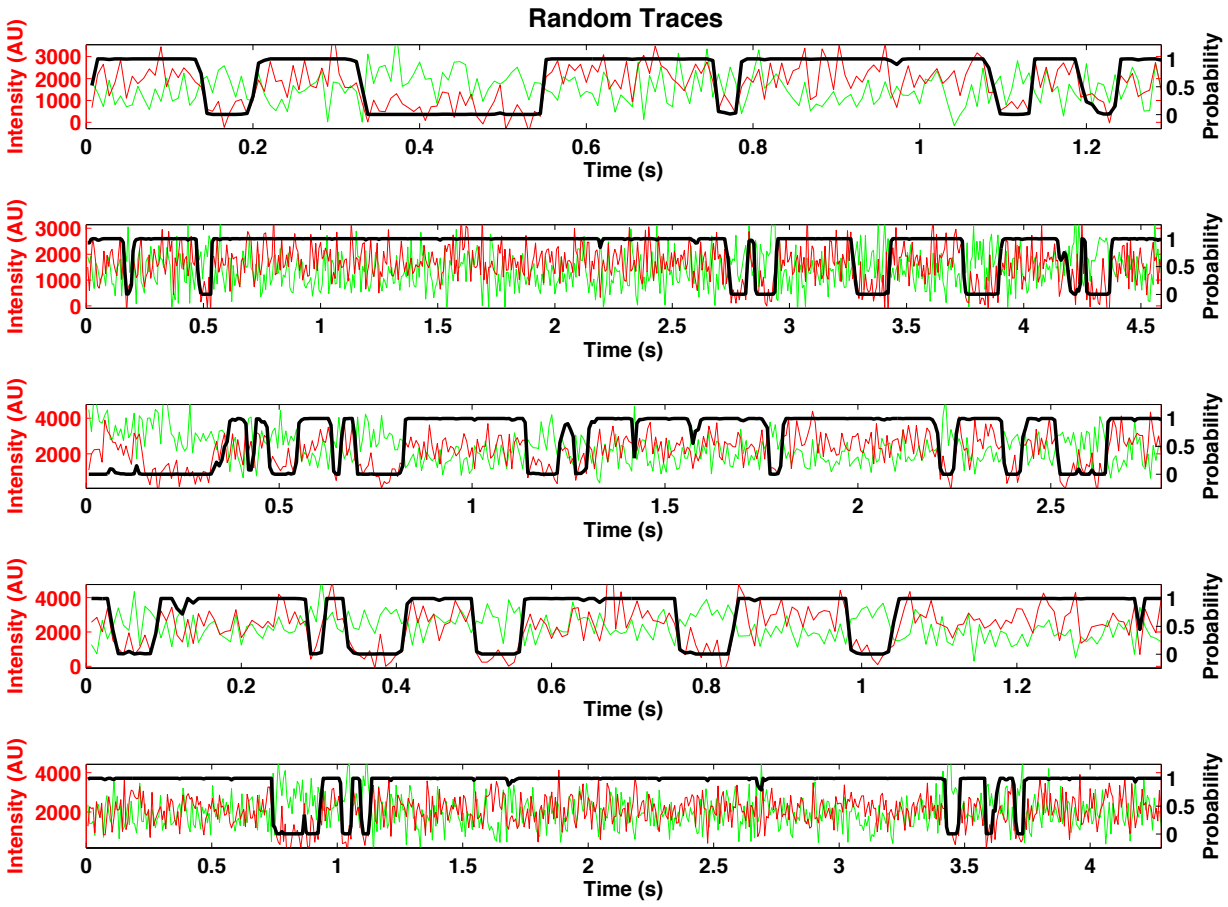


Figure S26-3. Randomly selected FRET traces of TL/TLR$_{iso}$ P4P6 Helical Context. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S27-1.Variant and Conditions

Variant:	TL/TLR$_{iso}$ A7
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	206

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S27-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ A7 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	26.5	25.4 - 27.4	1.3
	$k_u(s^{-1})$	3.6	3.5 - 3.8	1.4
	K_{eq}	7.3	6.9 - 7.7	1.5
	SNR green	1.0	1.1 - 1.2	0.3
	SNR red	3.7	3.7 - 3.9	0.8
	ΔG(kcal/mol)	-1.2	-1.2 - -1.1	0.2
Fits from Cumulative Data[2]	Lifetime (s)	9.8	8.5 - 11.2	9.8
	$k_{f,\ bulk}(s^{-1})$	22.7	23.3 - 22.1	21.9
	$k_{u,\ bulk}(s^{-1})$	3.3	3.4 - 3.2	3.2
	$K_{eq,bulk}$	8.0	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.2	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

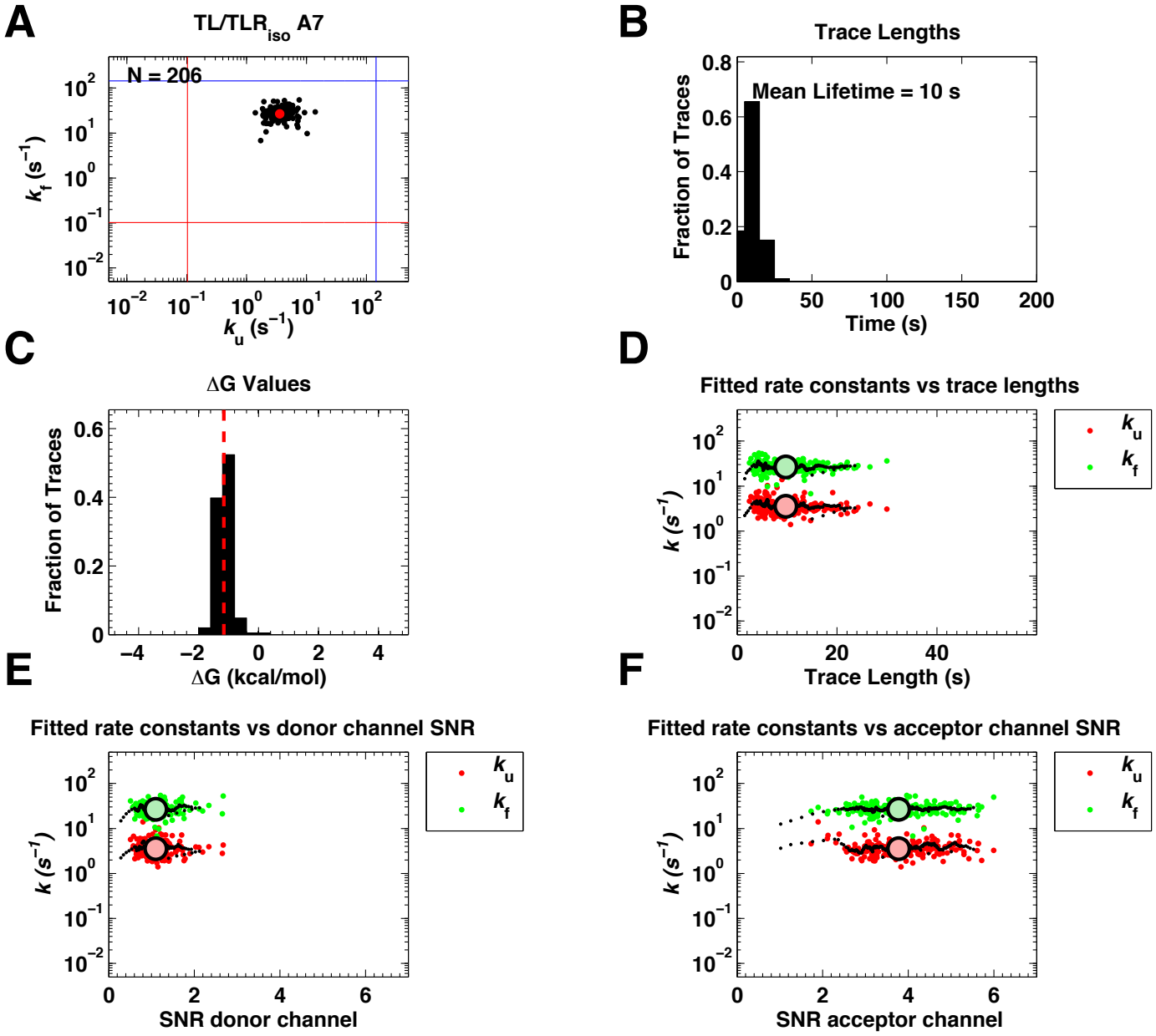


Figure S27-1. smFRET data assessment for TL/TLR$_{iso}$ A7. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

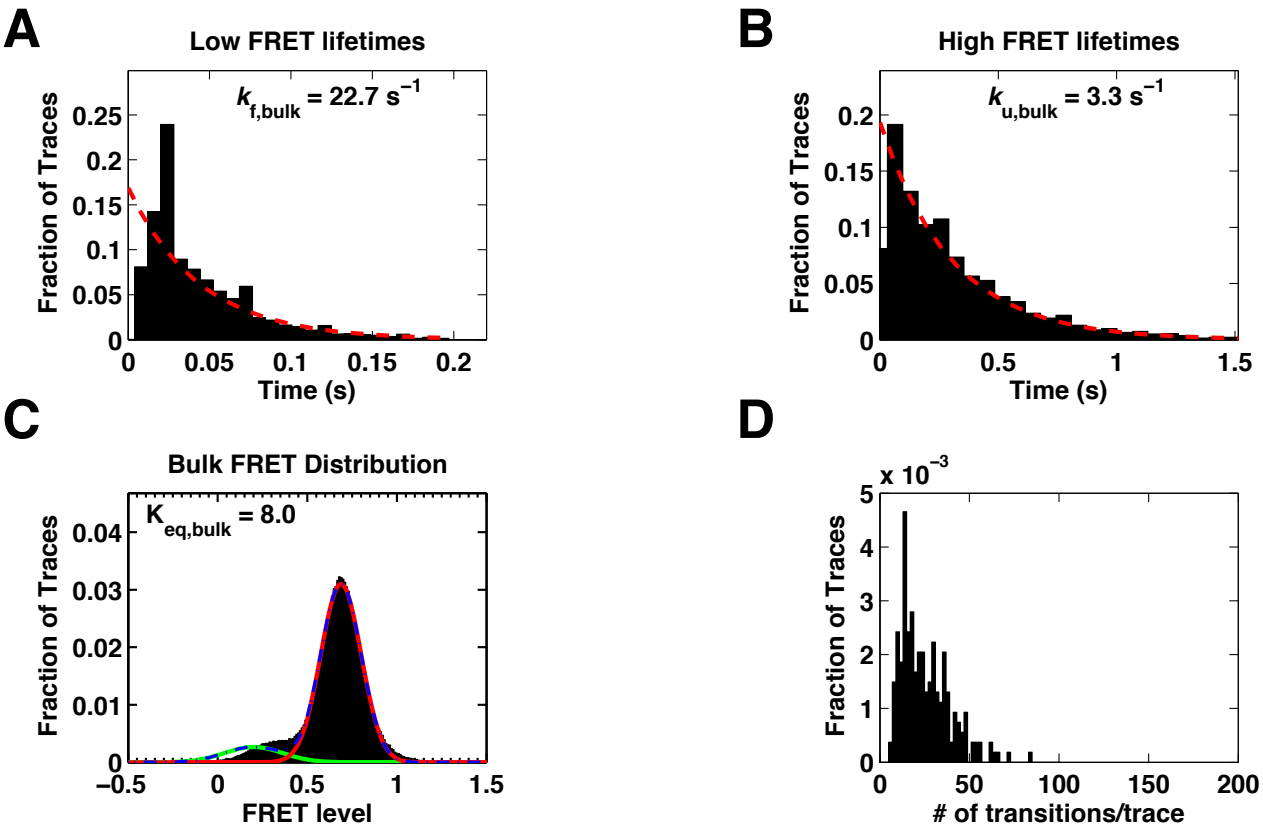


Figure S27-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ A7. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

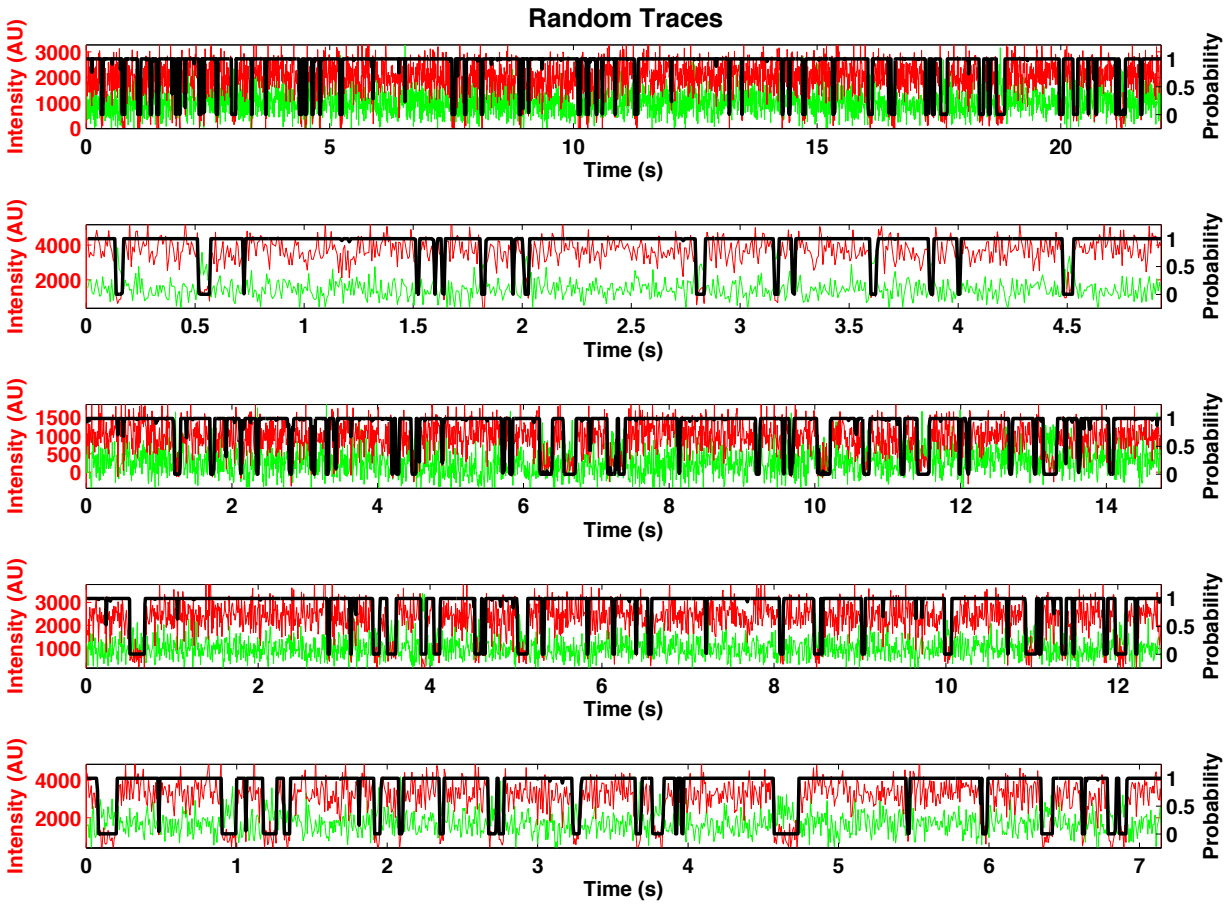


Figure S27-3. Randomly selected FRET traces of TL/TLR$_{iso}$ A7. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S28-1. Variant and Conditions

Variant:	J5/5a-AllU/ArichU
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	48
SNR Threshold[2]	0.50
Number of Traces	175

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S28-2. Folding parameters of smFRET the variant J5/5a-AllU/ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.2	0.2 - 0.3	7.9
	$k_u(s^{-1})$	4.7	4.1 - 5.2	2.3
	K_{eq}	0.1	0.0 - 0.1	9.7
	SNR green	2.7	2.7 - 3.1	1.3
	SNR red	2.7	2.7 - 3.0	1.1
	ΔG(kcal/mol)	2.2	1.5 - 1.9	1.3
Fits from Cumulative Data[2]	Lifetime (s)	47.9	41.5 - 55.8	47.9
	$k_{f,\ bulk}(s^{-1})$	2.9	3.0 - 2.8	0.6
	$k_{u,\ bulk}(s^{-1})$	3.7	3.8 - 3.6	3.0
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

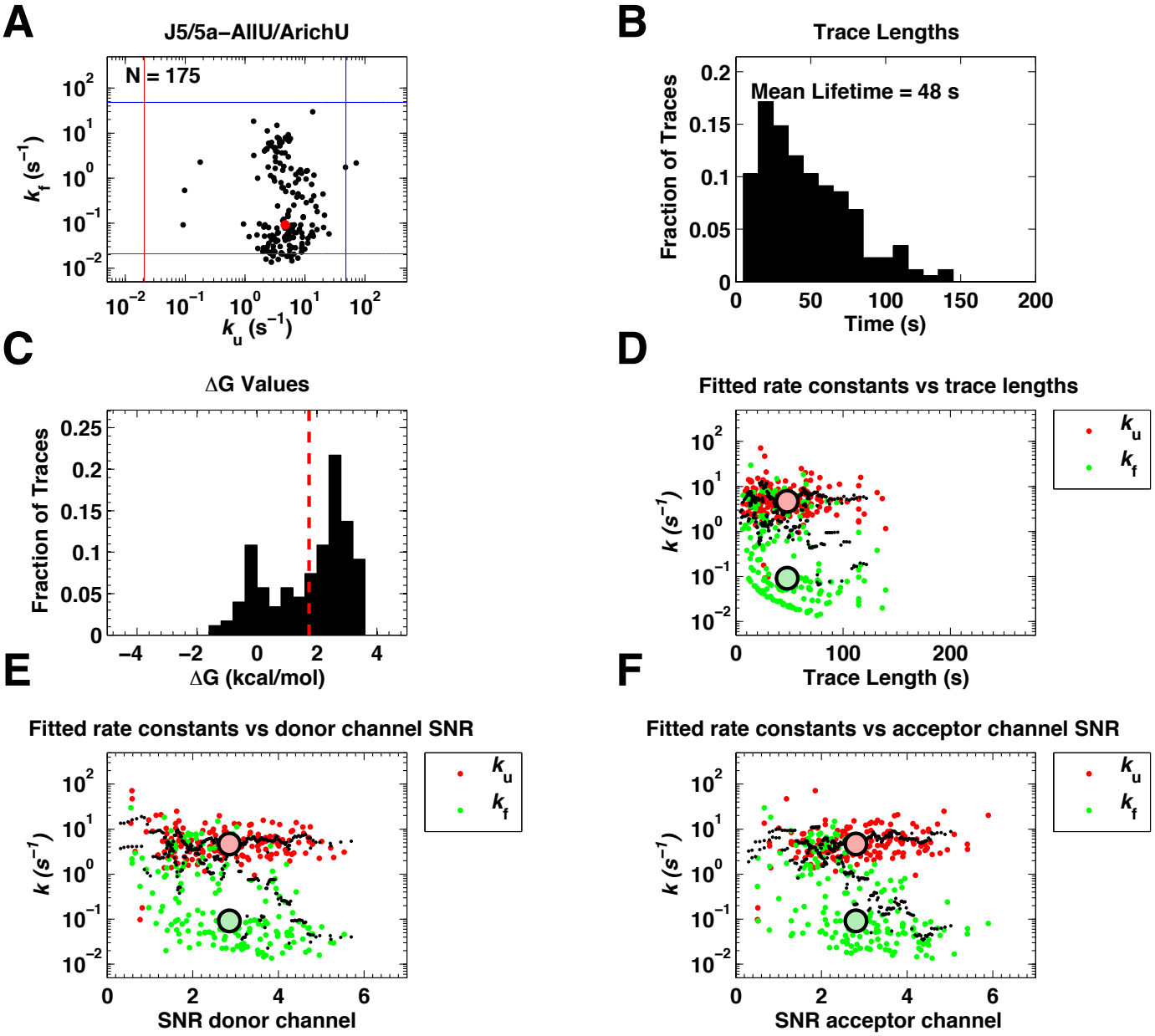


Figure S28-1. smFRET data assessment for J5/5a-AllU/ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

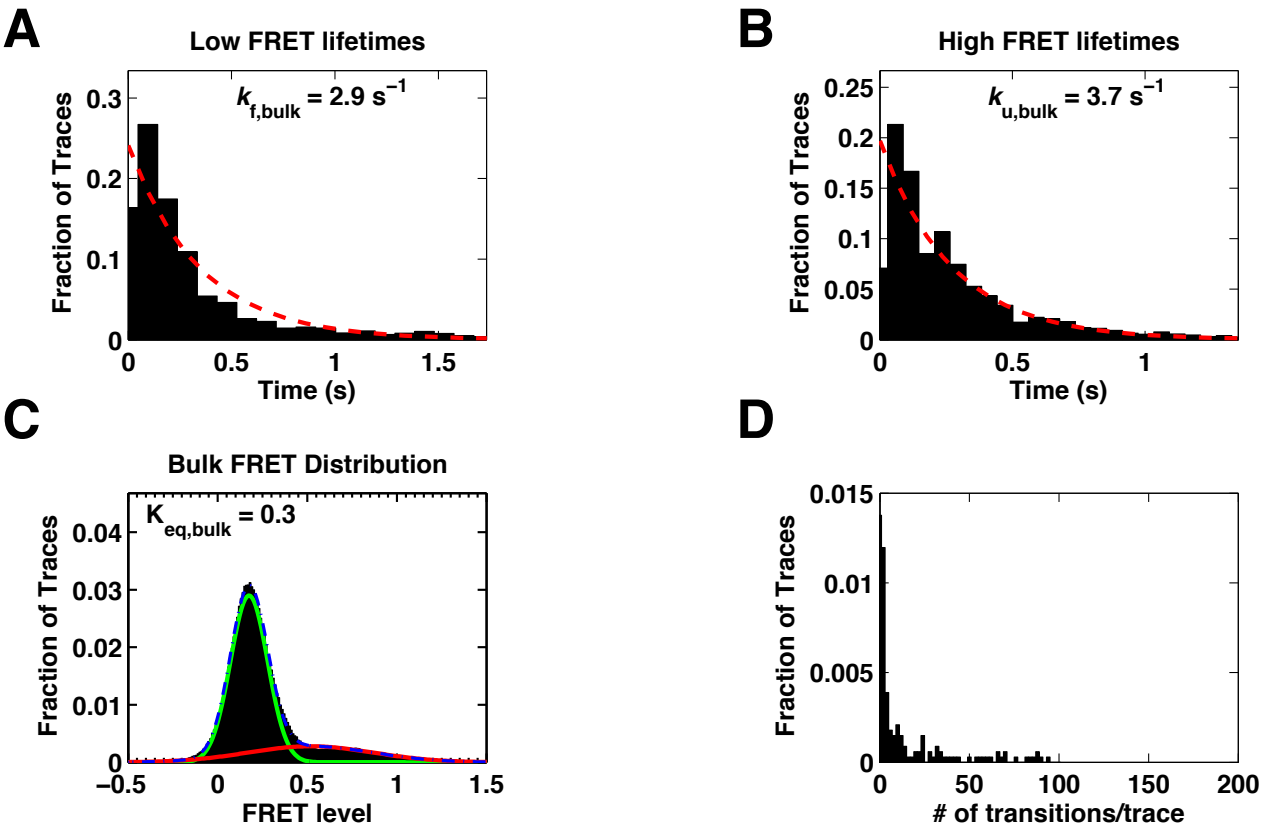


Figure S28-2. smFRET data assesment of aggregate data for J5/5a-AllU/ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

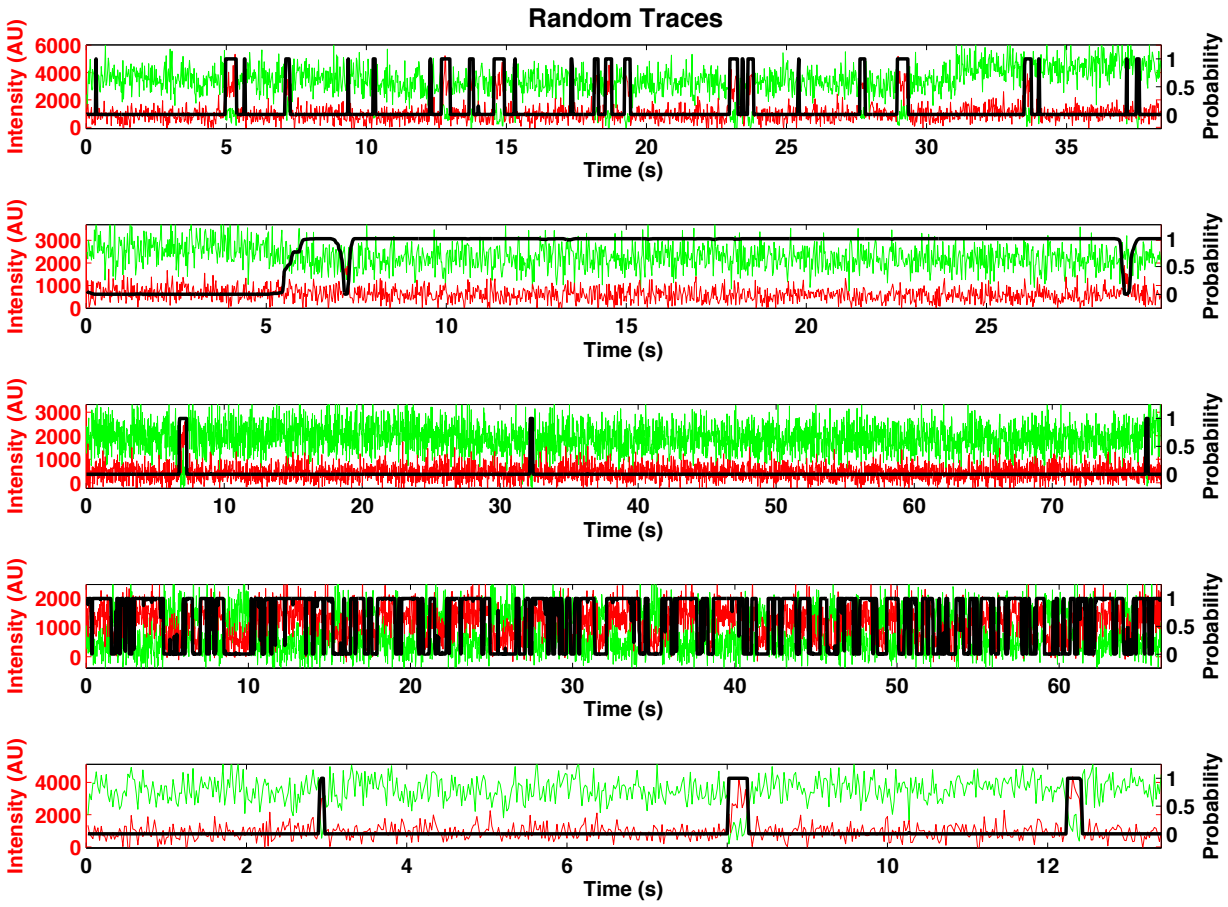


Figure S28-3. Randomly selected FRET traces of J5/5a-AllU/ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S29-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ A7 Cy3b
$MgCl_2$ (mM)	0.0
$BaCl_2$ (mM)	5.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	156

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S29-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ A7 Cy3b inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	20.1	18.5 - 21.4	1.6
	$k_u(s^{-1})$	2.4	2.2 - 2.6	1.6
	K_{eq}	8.5	7.4 - 9.3	2.0
	SNR green	3.6	3.5 - 3.7	0.7
	SNR red	1.4	1.4 - 1.4	0.3
	ΔG(kcal/mol)	-1.3	-1.3 - -1.2	0.4
Fits from Cumulative Data[2]	Lifetime (s)	9.3	8.0 - 10.9	9.3
	$k_{f,\ bulk}(s^{-1})$	19.4	20.1 - 18.7	16.2
	$k_{u,\ bulk}(s^{-1})$	2.4	2.4 - 2.3	2.3
	$K_{eq,bulk}$	7.7	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.2	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

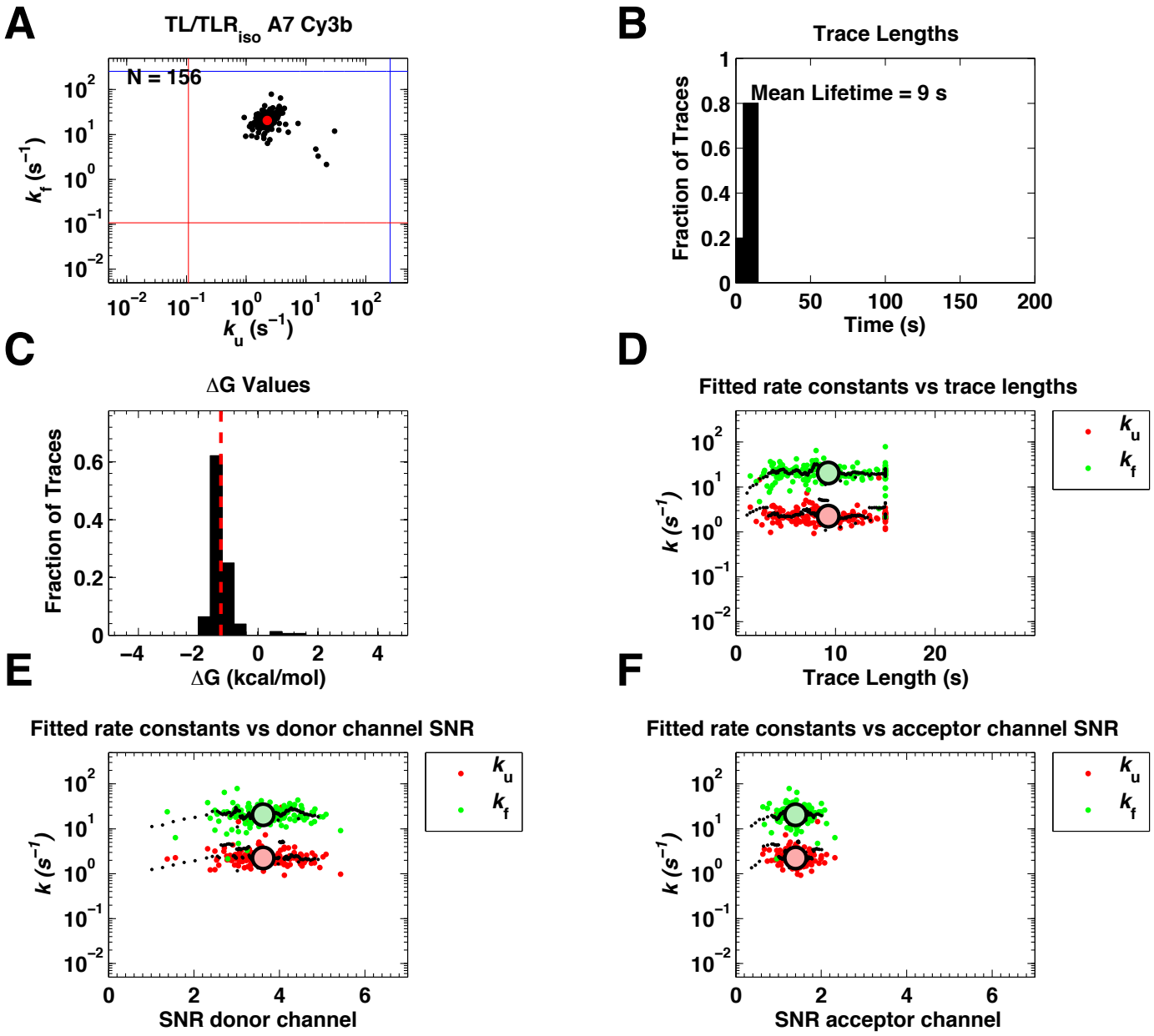


Figure S29-1. smFRET data assessment for TL/TLR$_{iso}$ A7 Cy3b. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

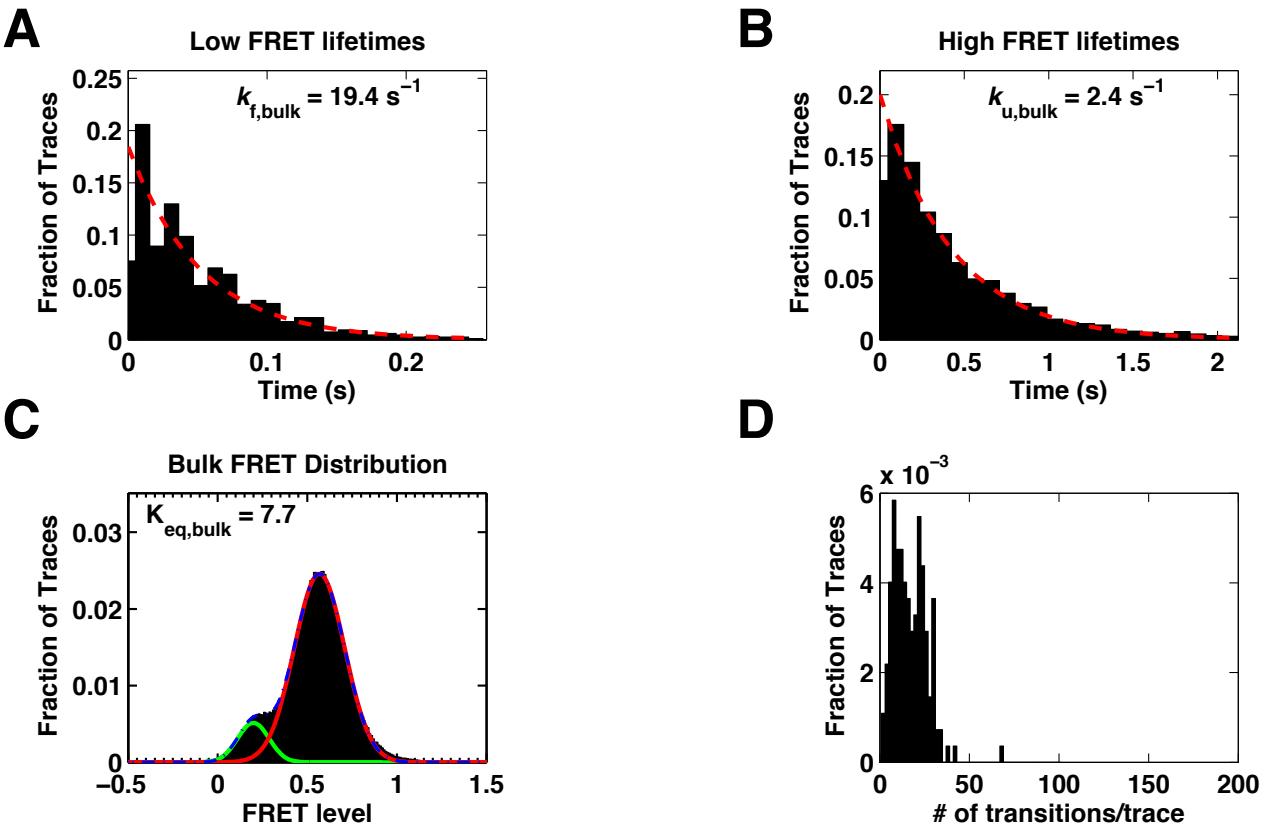


Figure S29-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ A7 Cy3b. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

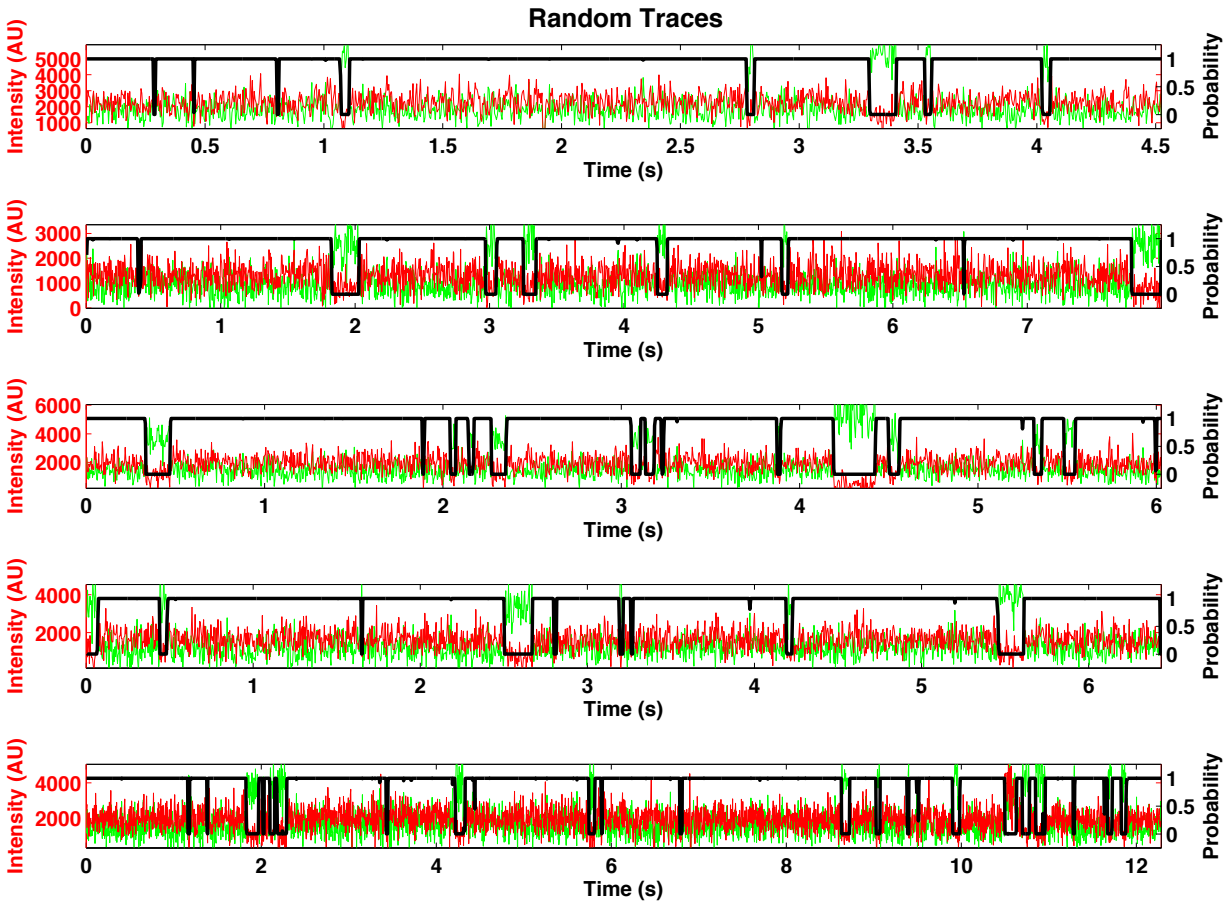


Figure S9-3. Randomly selected FRET traces of TL/TLR$_{iso}$ A7 Cy3b. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S30-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ Extended TL Cy3b/Atto647N
$MgCl_2$ (mM)	0.0
$BaCl_2$ (mM)	5.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	104

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S30-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ Extended TL Cy3b/Atto647N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	13.9	13.0 - 15.0	1.5
	$k_u(s^{-1})$	1.5	1.4 - 1.8	2.0
	K_{eq}	9.2	7.7 - 10.6	2.3
	SNR green	3.2	3.2 - 3.4	0.6
	SNR red	1.3	1.3 - 1.4	0.3
	ΔG(kcal/mol)	-1.4	-1.4 - -1.2	0.5
Fits from Cumulative Data[2]	Lifetime (s)	10.7	8.9 - 13.1	10.7
	$k_{f,\ bulk}(s^{-1})$	12.3	12.9 - 11.7	11.6
	$k_{u,\ bulk}(s^{-1})$	1.7	1.7 - 1.6	1.5
	$K_{eq,bulk}$	5.5	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

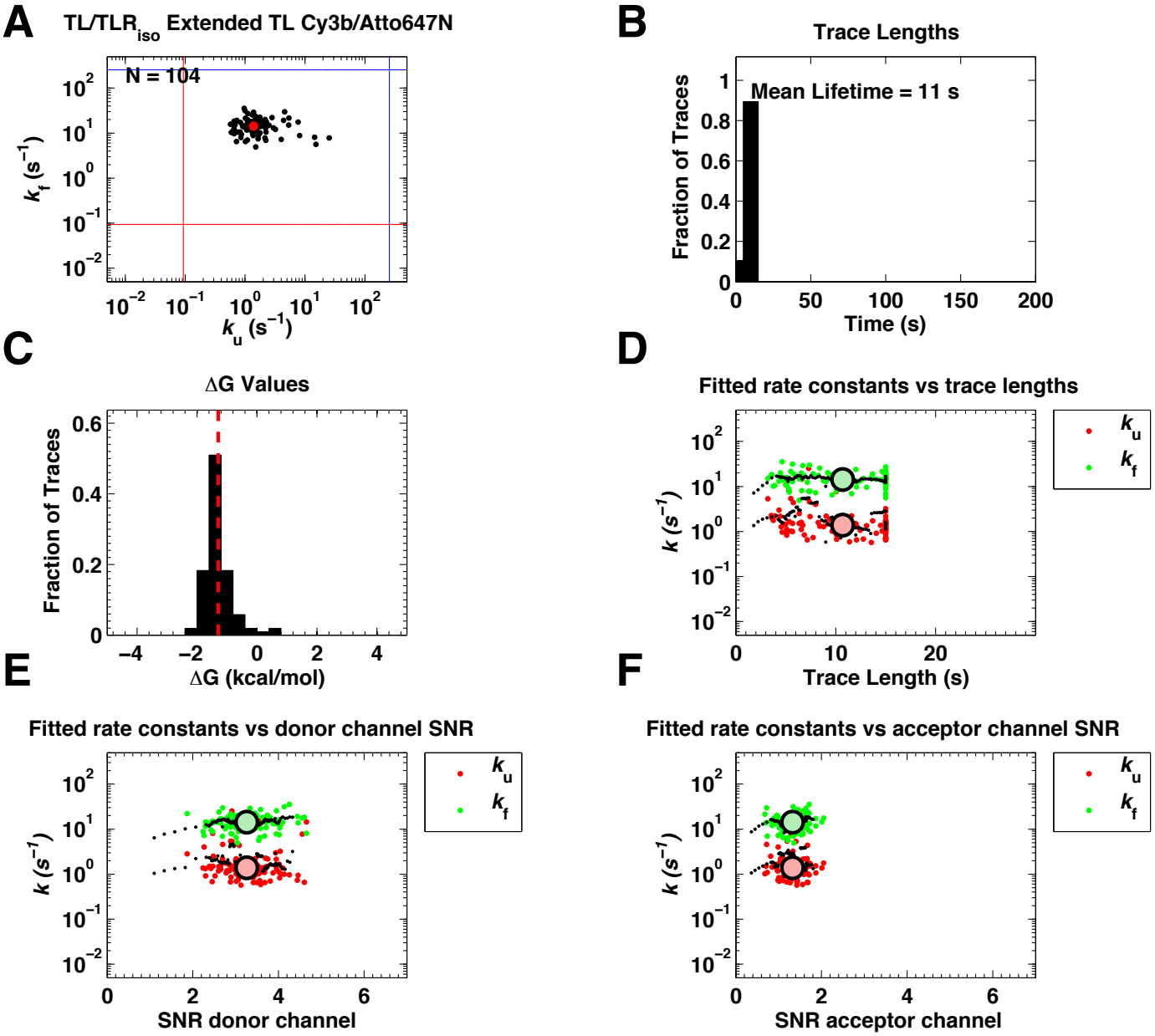


Figure S30-1. smFRET data assessment for TL/TLR$_{iso}$ Extended TL Cy3b/Atto647N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

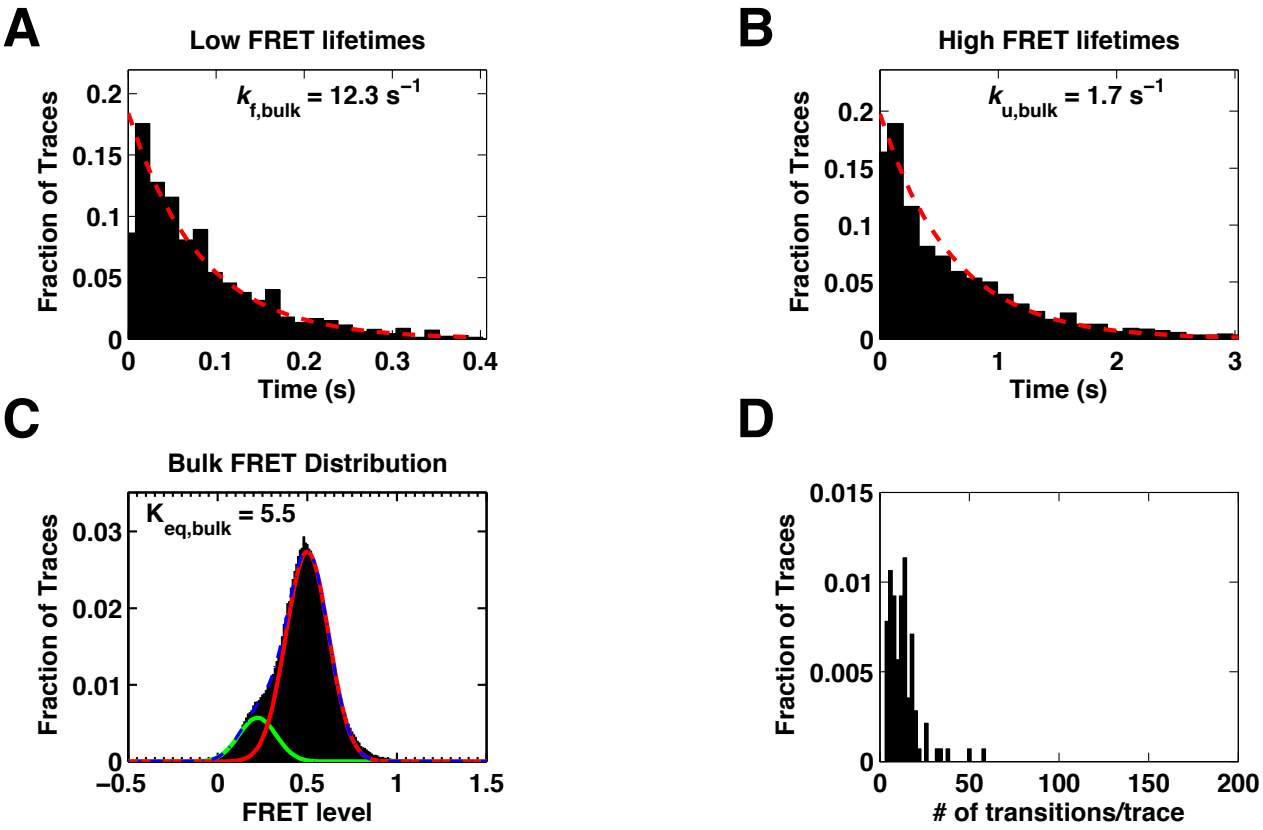


Figure S30-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ Extended TL Cy3b/Atto647N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

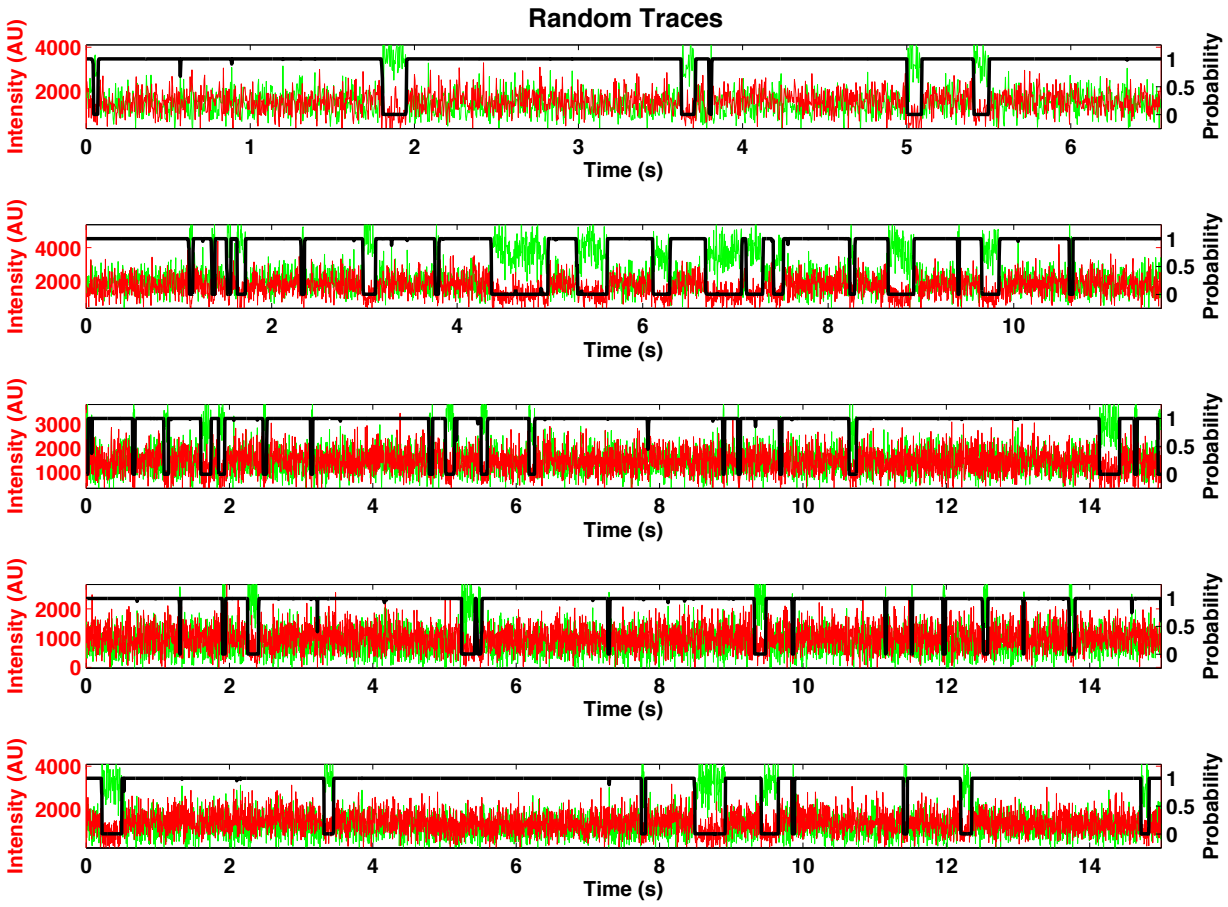


Figure S30-3. Randomly selected FRET traces of TL/TLR$_{iso}$ Extended TL Cy3b/Atto647N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S31-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ A7
$MgCl_2$ (mM)	0.0
$BaCl_2$ (mM)	5.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	59

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S31-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ A7 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	32.4	25.8 - 38.4	2.1
	$k_u(s^{-1})$	2.6	2.1 - 3.5	2.6
	K_{eq}	12.3	8.1 - 15.9	3.7
	SNR green	1.1	1.1 - 1.2	0.4
	SNR red	2.7	2.5 - 2.9	0.7
	ΔG(kcal/mol)	-1.7	-1.6 - -1.2	0.8
Fits from Cumulative Data[2]	Lifetime (s)	11.5	9.0 - 15.1	11.5
	$k_{f,\ bulk}(s^{-1})$	31.6	33.2 - 30.1	19.5
	$k_{u,\ bulk}(s^{-1})$	4.0	4.2 - 3.8	3.0
	$K_{eq,bulk}$	9.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.3	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

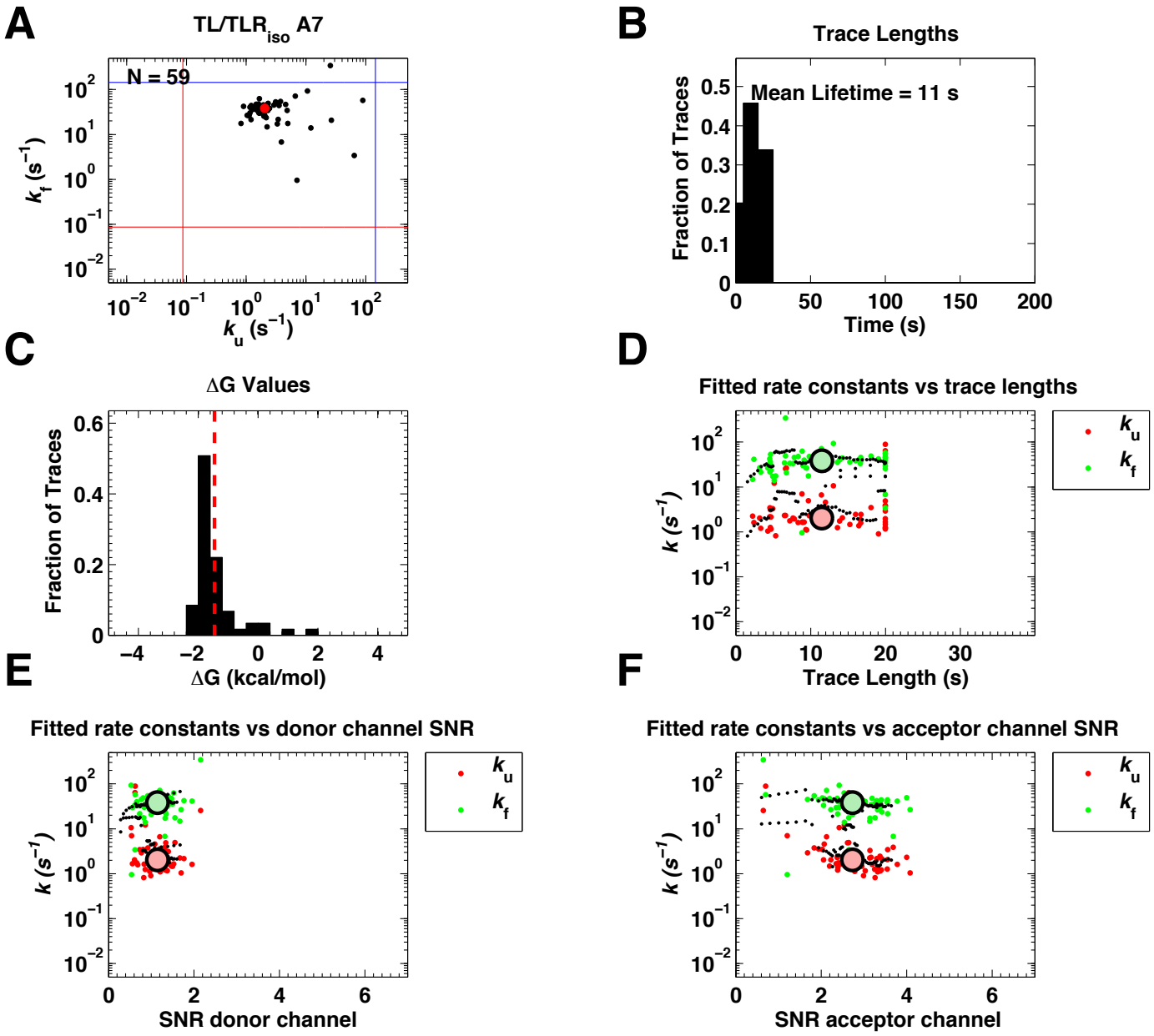


Figure S31-1. smFRET data assessment for TL/TLR$_{iso}$ A7. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

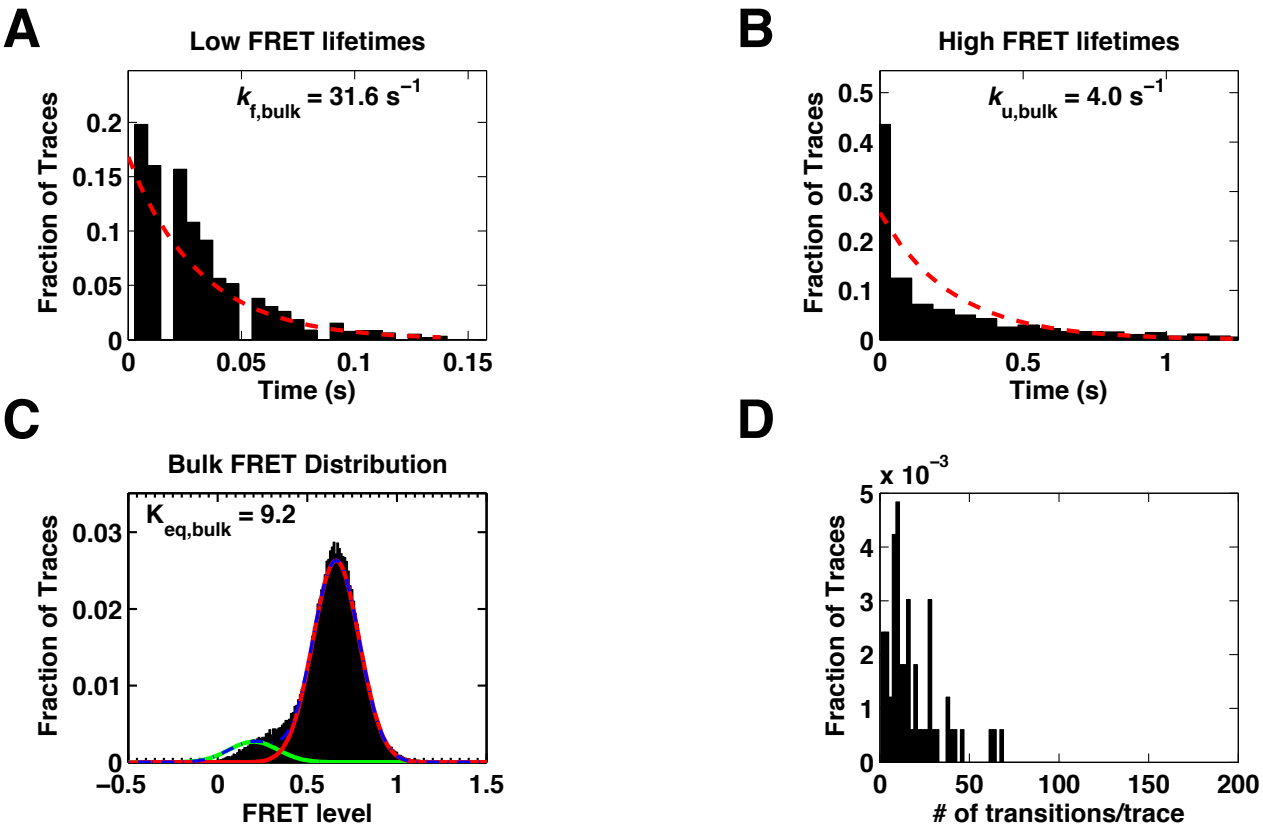


Figure S31-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ A7. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

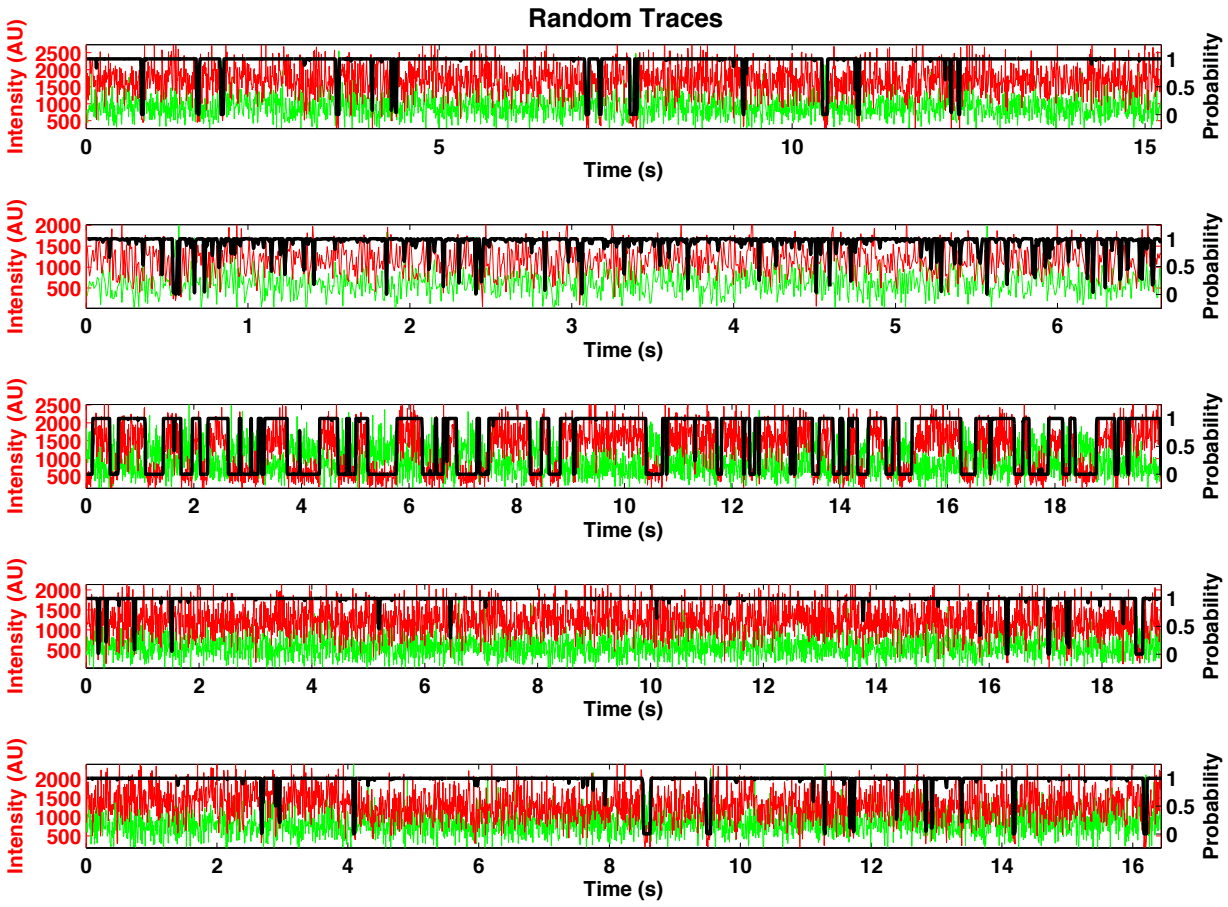


Figure S31- 3. Randomly selected FRET traces of TL/TLR$_{iso}$ A7. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S32-1. Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	2.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	12

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S32-2.Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.3	0.2 - 0.5	2.7
	$k_u(s^{-1})$	8.1	5.3 - 11.9	2.1
	K_{eq}	0.0	0.0 - 0.1	3.4
	SNR green	2.1	1.7 - 2.4	0.6
	SNR red	3.6	2.8 - 3.9	1.0
	ΔG(kcal/mol)	2.1	1.6 - 2.4	0.7
Fits from Cumulative Data[2]	Lifetime (s)	78.6	47.9 - 152.1	78.6
	$k_{f,\ bulk}(s^{-1})$	0.4	0.5 - 0.4	0.3
	$k_{u,\ bulk}(s^{-1})$	6.5	7.4 - 5.7	5.8
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	1.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

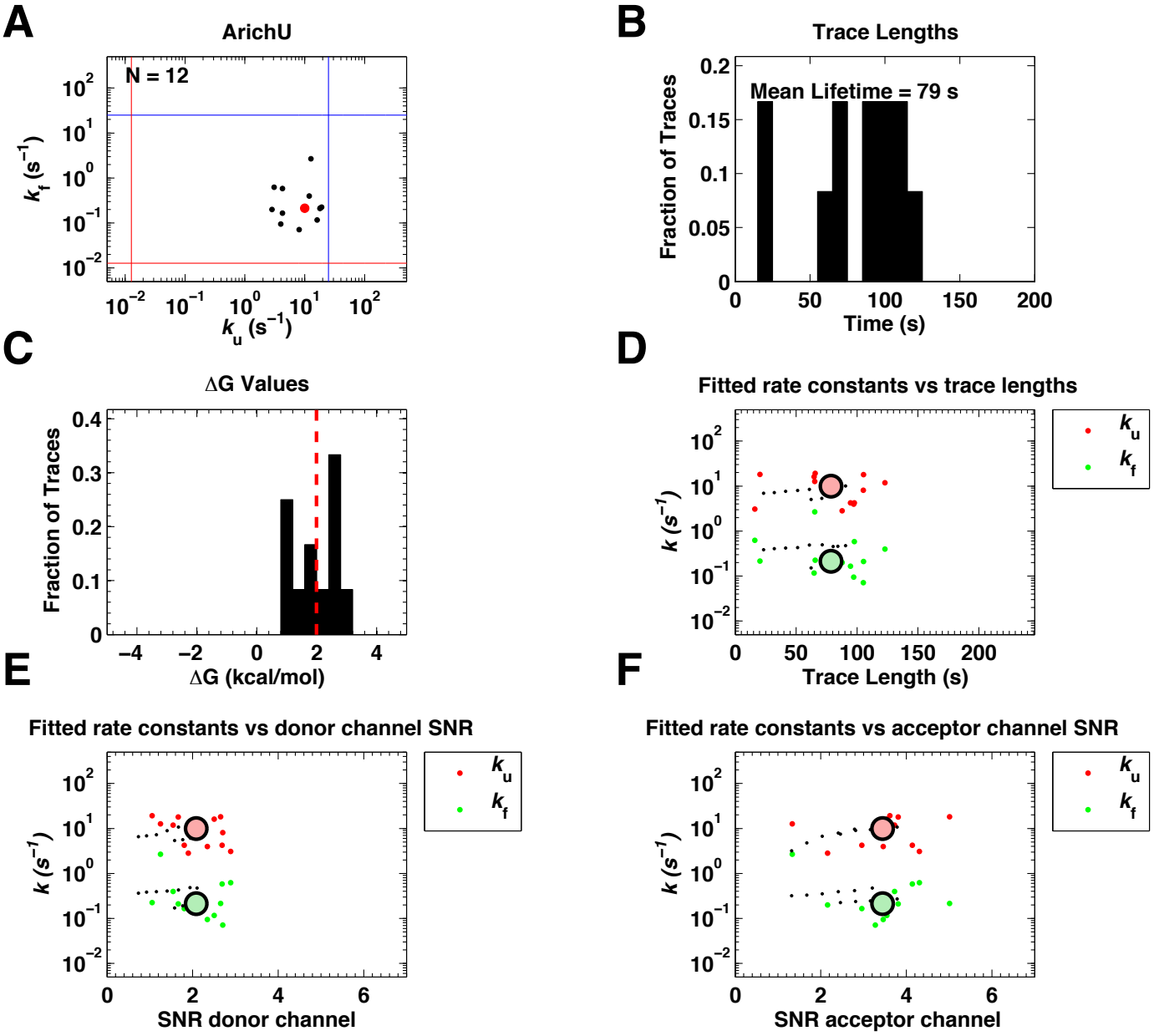


Figure S32-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

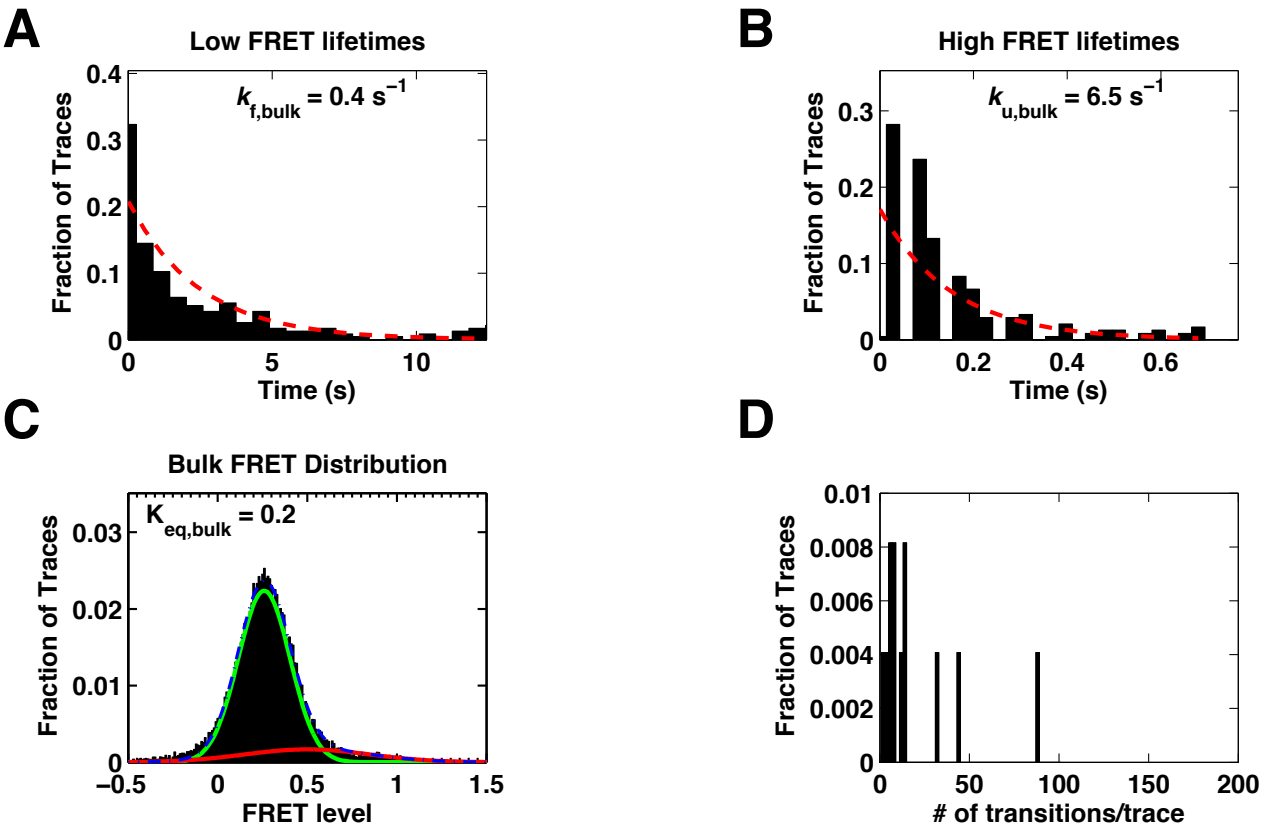


Figure S32-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

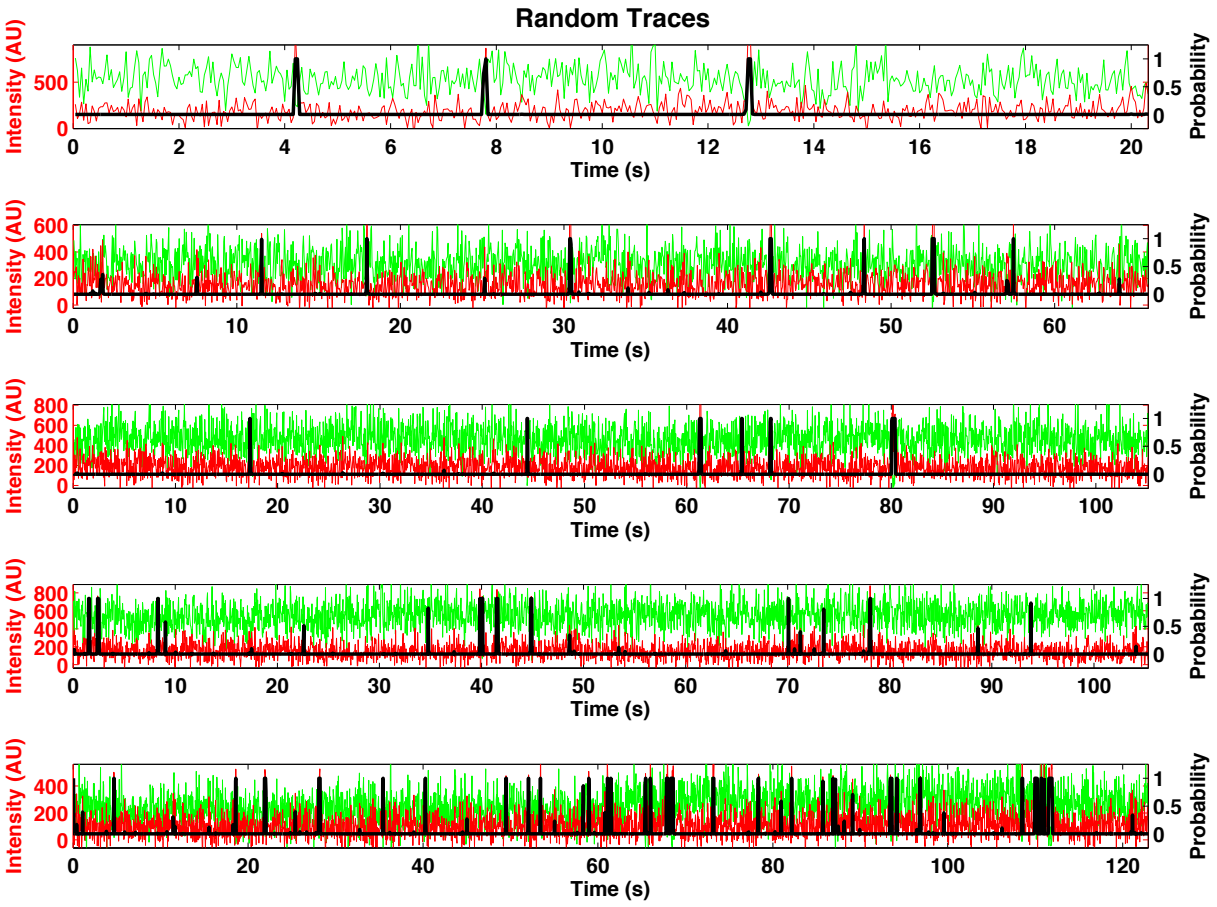


Figure S32-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S33-1. Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	3.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	30

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S33-2. Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.4	0.3 - 0.7	2.5
	$k_u(s^{-1})$	6.7	3.5 - 9.2	3.4
	K_{eq}	0.1	0.0 - 0.2	5.4
	SNR green	2.0	1.8 - 2.3	0.7
	SNR red	3.5	3.1 - 4.0	1.2
	ΔG(kcal/mol)	2.0	1.1 - 1.9	1.0
Fits from Cumulative Data[2]	Lifetime (s)	36.4	26.2 - 54.0	36.4
	$k_{f,\ bulk}(s^{-1})$	0.4	0.4 - 0.3	0.3
	$k_{u,\ bulk}(s^{-1})$	8.0	8.9 - 7.1	3.9
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	1.1	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

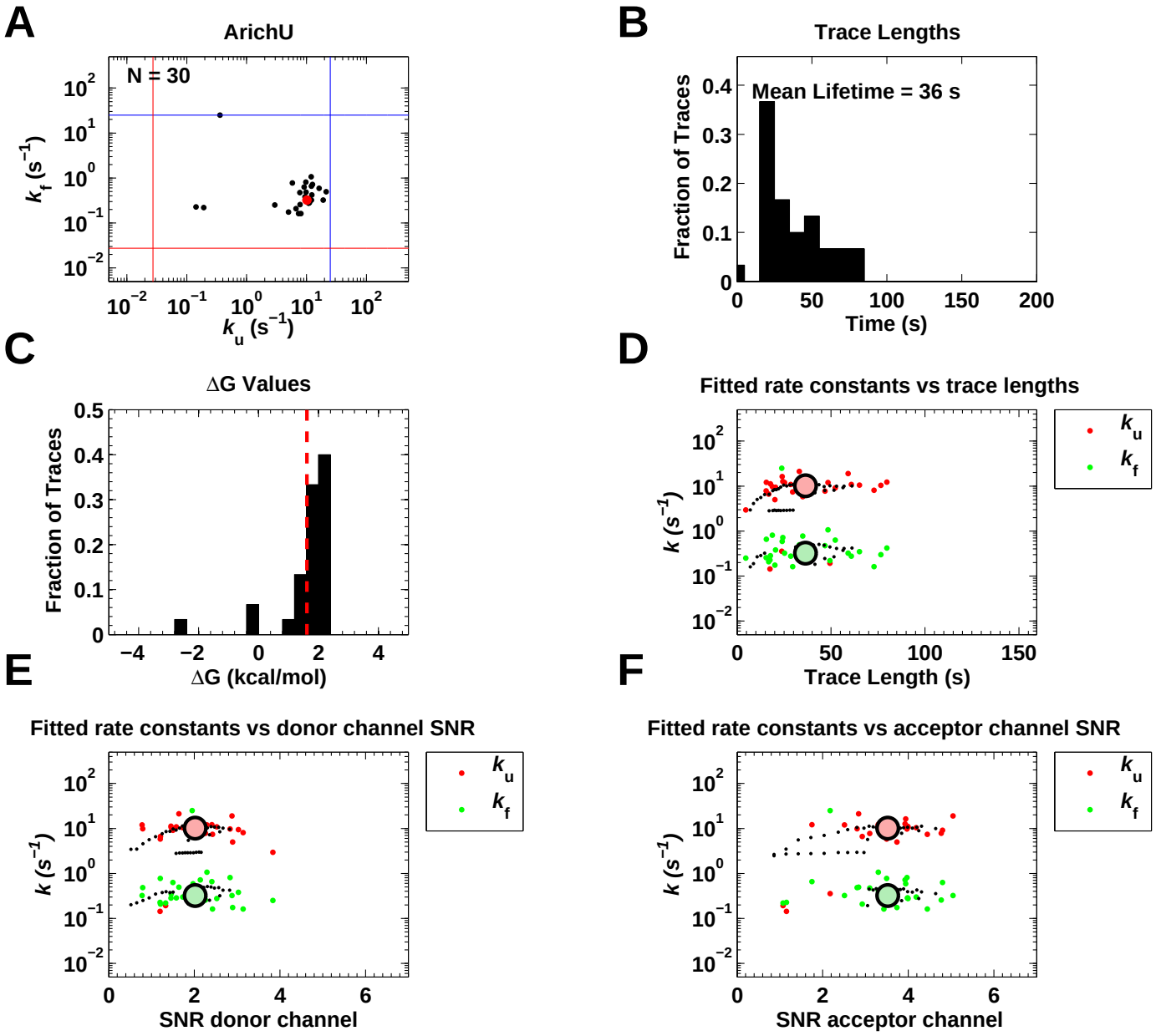


Figure S33-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

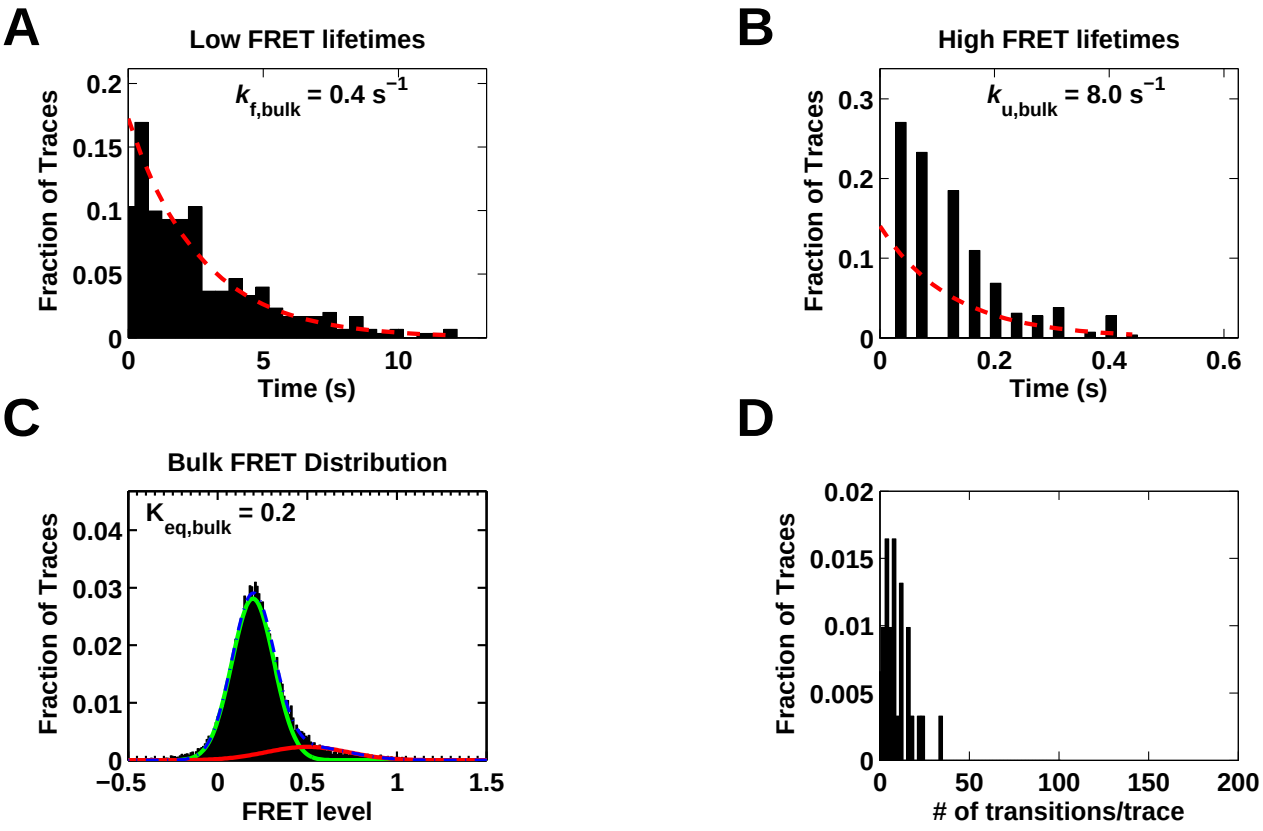


Figure S33-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

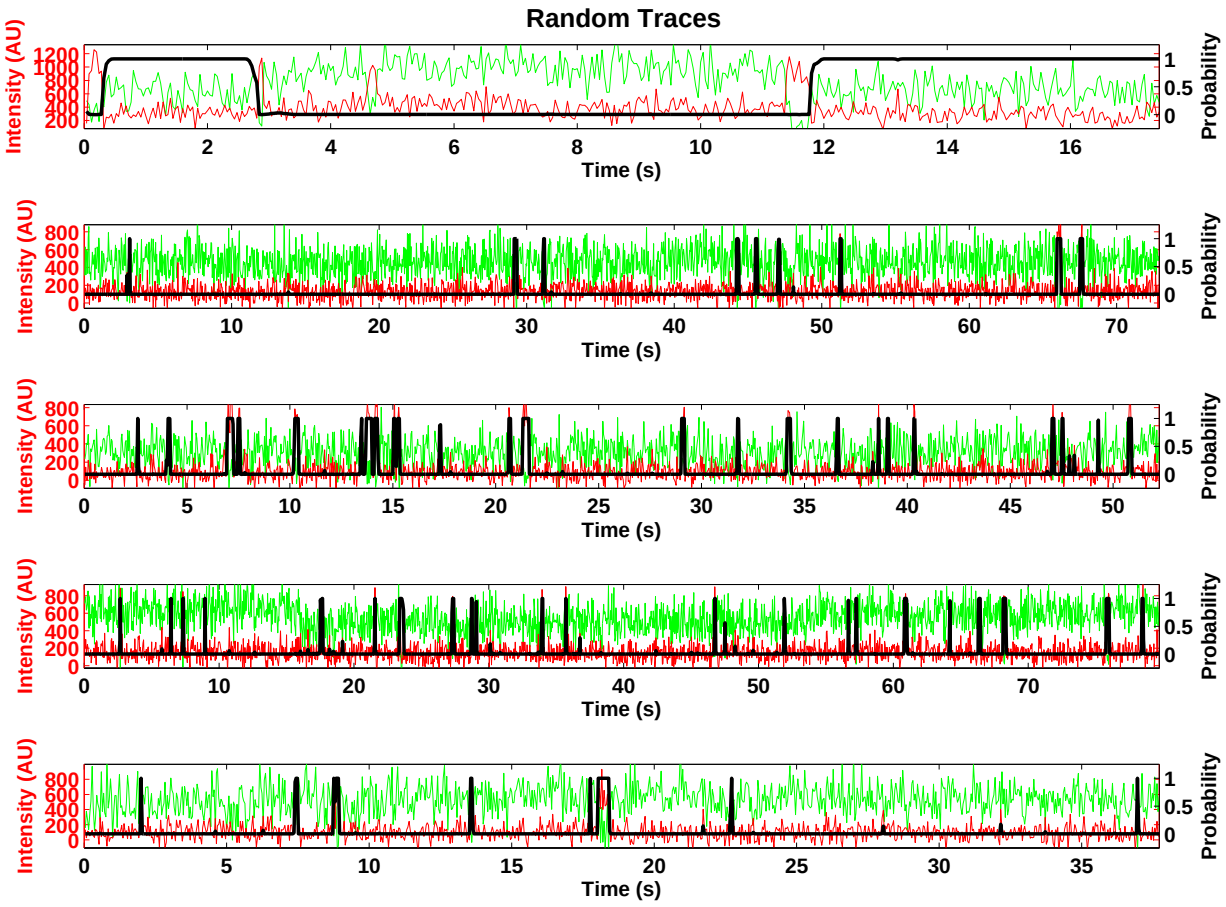


Figure 3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S34-1.Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	4.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	46

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S34-2. Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.6	0.5 - 0.7	1.8
	$k_u(s^{-1})$	8.3	6.8 - 9.6	1.8
	K_{eq}	0.1	0.1 - 0.1	2.3
	SNR green	2.4	2.1 - 2.5	0.7
	SNR red	3.6	3.3 - 3.8	0.9
	ΔG(kcal/mol)	1.6	1.3 - 1.6	0.5
Fits from Cumulative Data[2]	Lifetime (s)	38.6	29.5 - 52.7	38.6
	$k_{f,\ bulk}(s^{-1})$	0.7	0.7 - 0.6	0.6
	$k_{u,\ bulk}(s^{-1})$	7.0	7.5 - 6.6	5.7
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.8	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

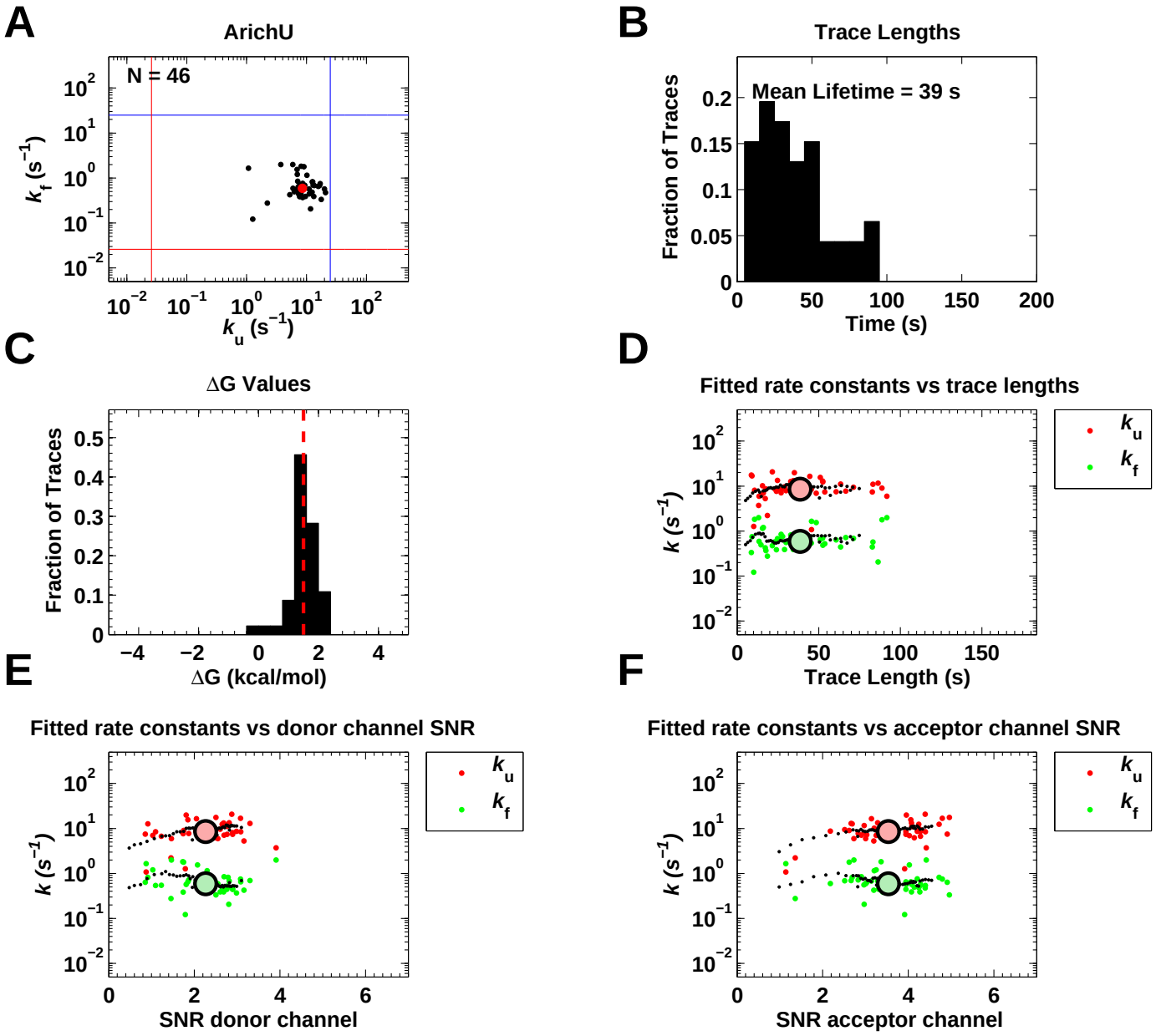


Figure S34-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

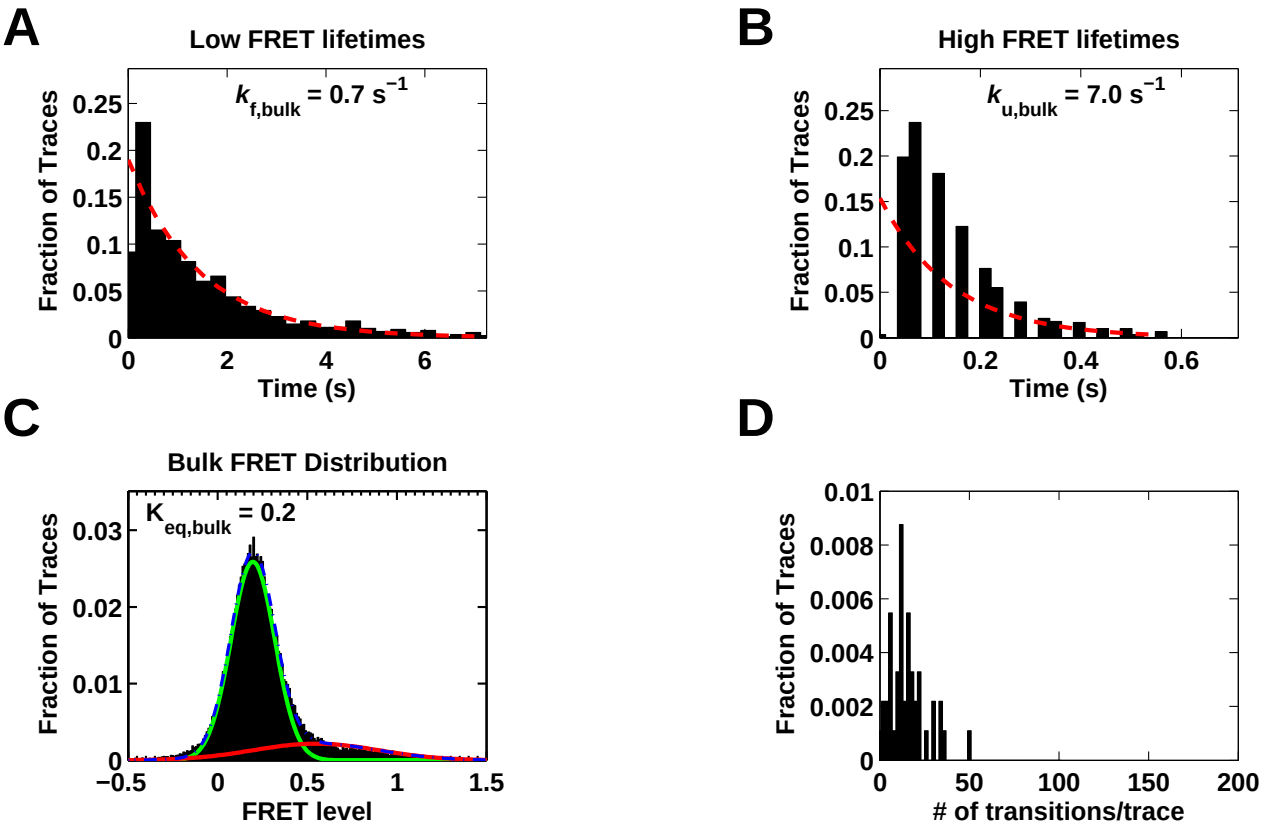


Figure S34-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

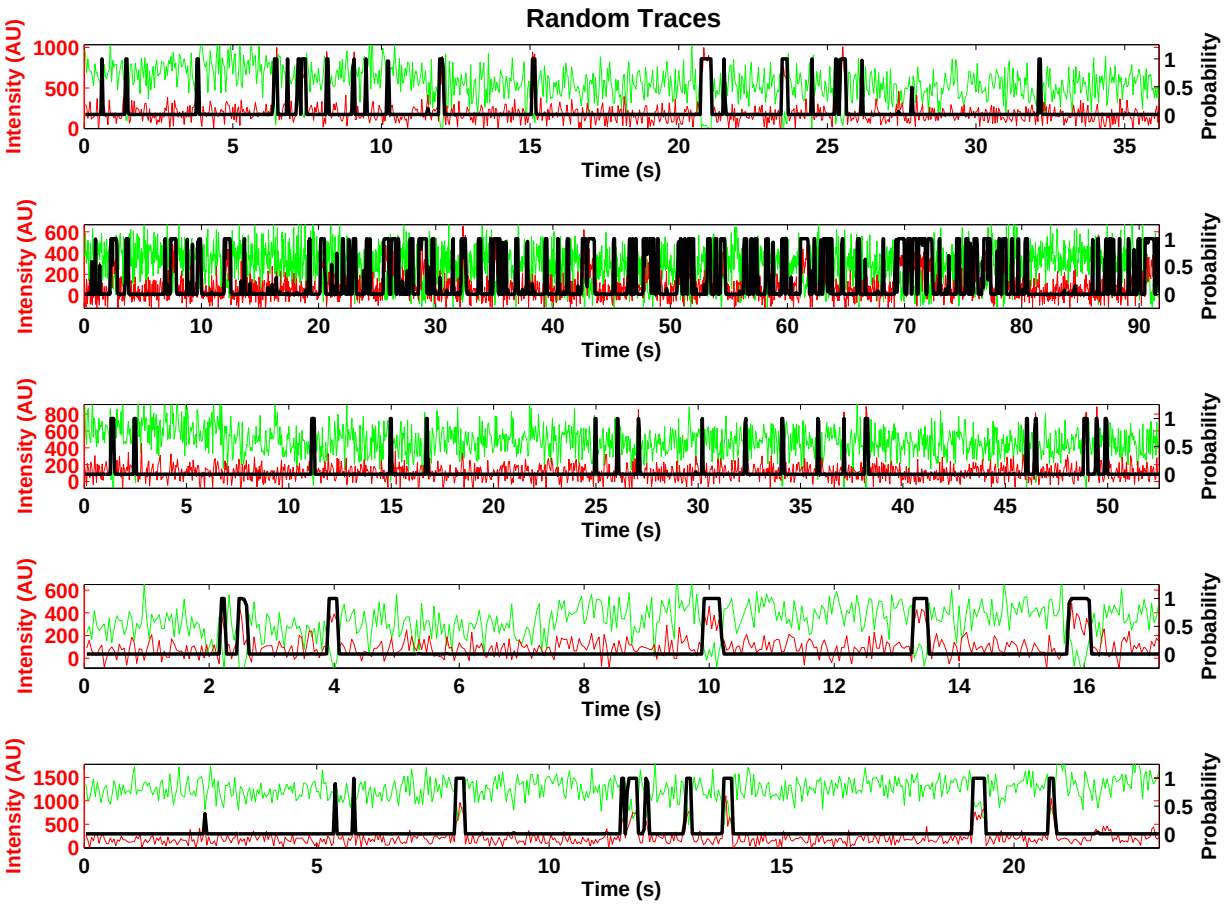


Figure S34-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S35-1.Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	43

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S35-2.Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.8	0.6 - 1.0	2.0
	$k_u(s^{-1})$	5.2	3.9 - 6.6	2.4
	K_{eq}	0.2	0.1 - 0.2	2.5
	SNR green	3.2	2.8 - 3.4	1.0
	SNR red	4.7	3.9 - 4.7	1.4
	ΔG(kcal/mol)	1.3	0.9 - 1.2	0.5
Fits from Cumulative Data[2]	Lifetime (s)	59.5	45.1 - 82.2	59.5
	$k_{f,\ bulk}(s^{-1})$	0.9	0.9 - 0.8	0.8
	$k_{u,\ bulk}(s^{-1})$	6.6	6.9 - 6.2	4.1
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

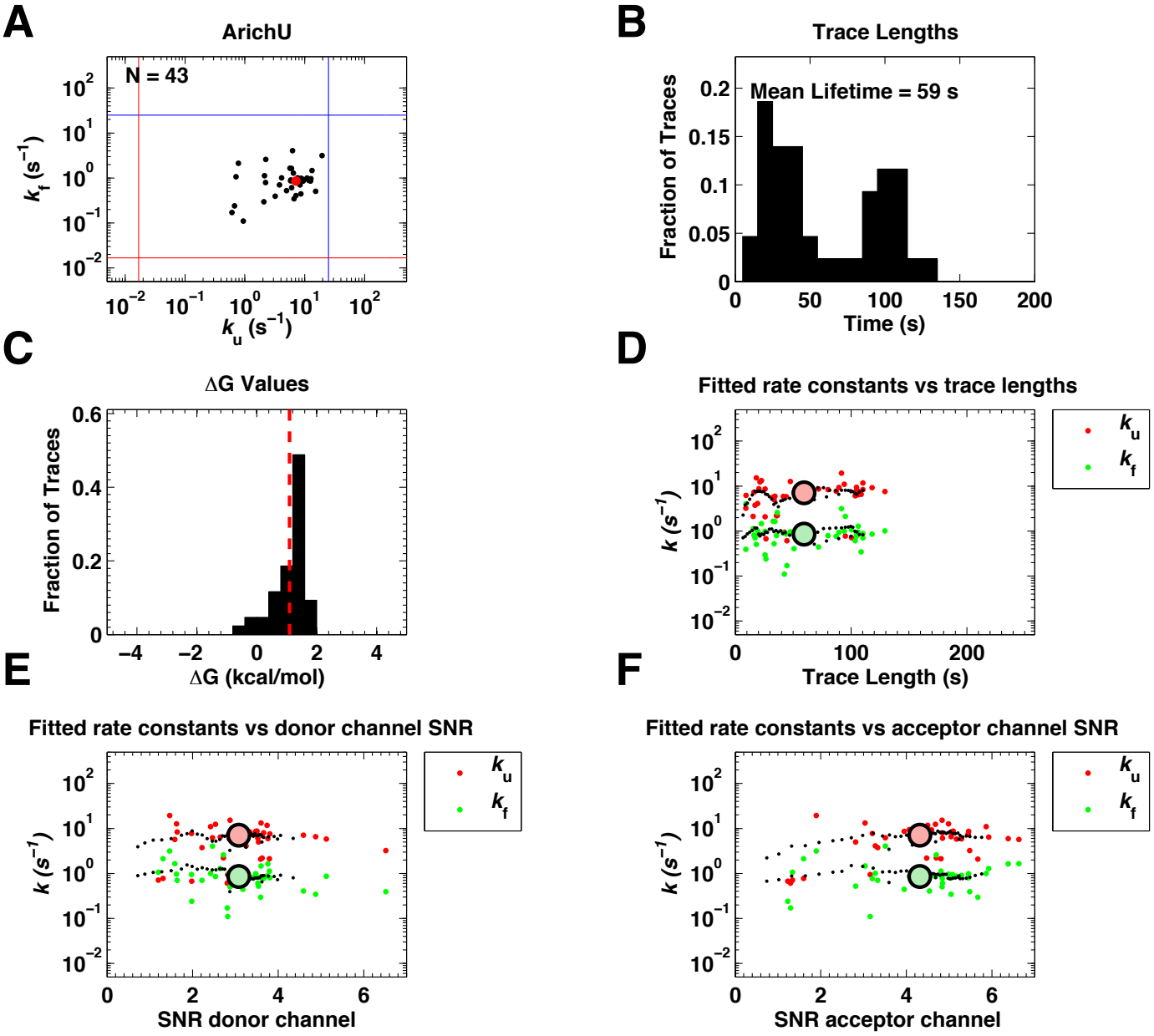


Figure S35-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

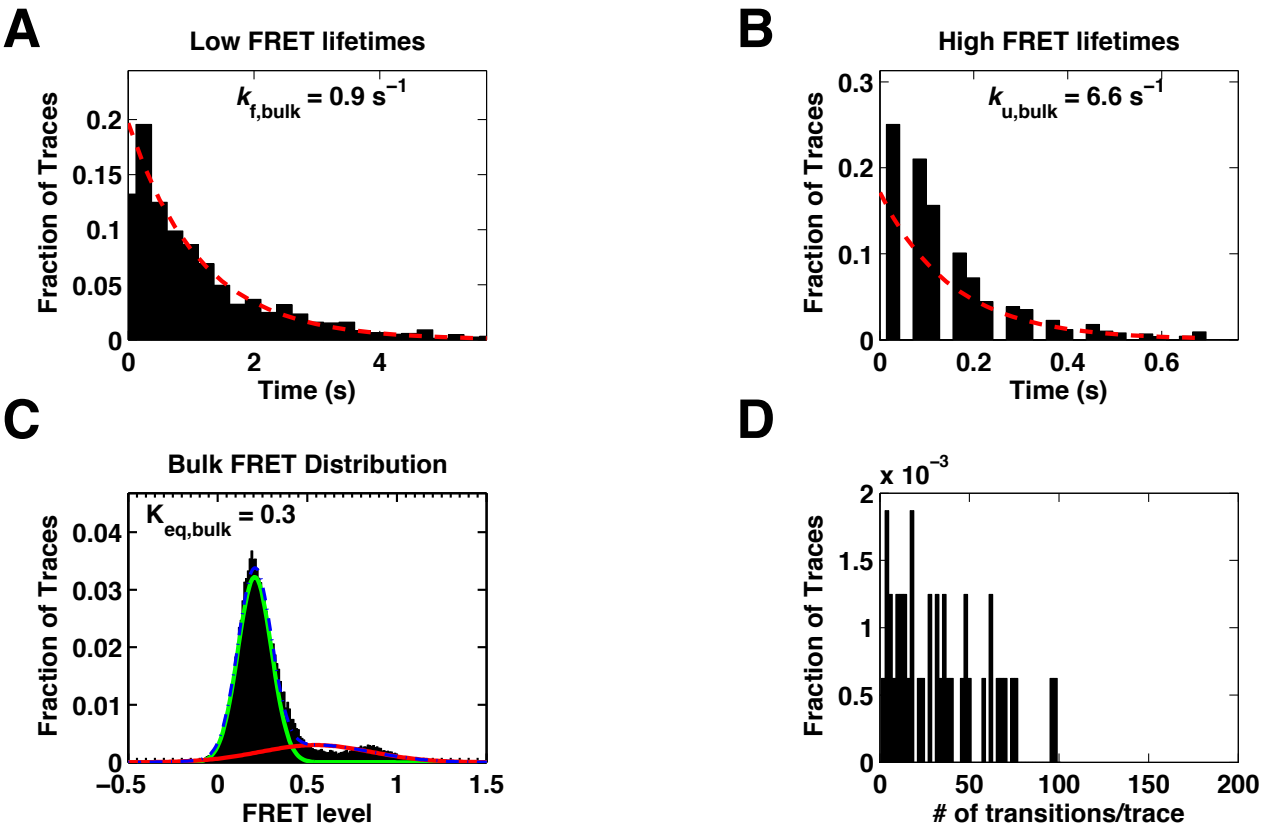


Figure S35-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

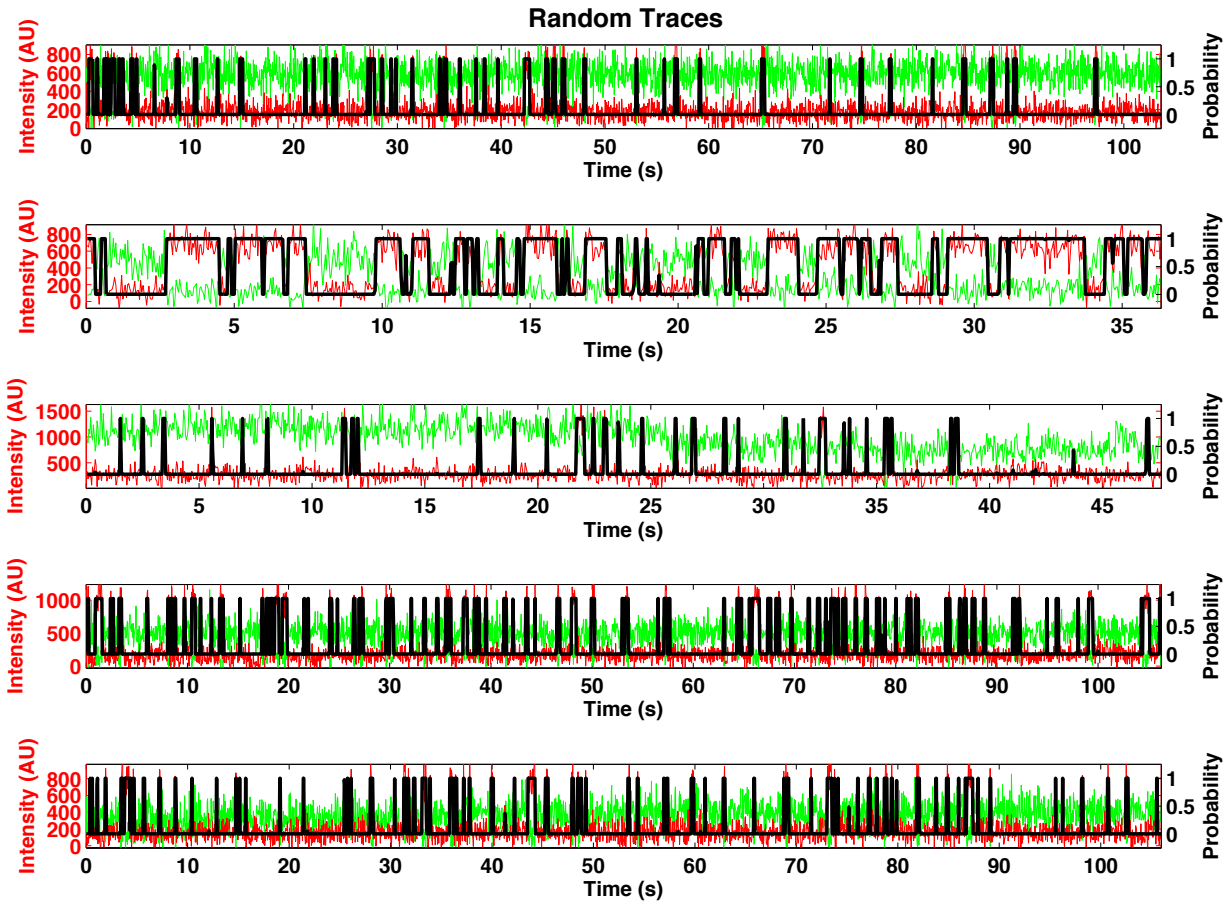


Figure S25-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S36-1.Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	10.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	53

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S36-2. Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	1.8	1.4 - 2.1	2.2
	$k_u(s^{-1})$	4.9	3.6 - 5.9	2.4
	K_{eq}	0.4	0.3 - 0.5	3.6
	SNR green	2.6	2.4 - 2.8	0.8
	SNR red	3.6	3.3 - 3.8	0.9
	ΔG(kcal/mol)	0.6	0.4 - 0.8	0.8
Fits from Cumulative Data[2]	Lifetime (s)	95.4	74.1 - 127.3	95.4
	$k_{f,\ bulk}(s^{-1})$	1.9	1.9 - 1.8	1.6
	$k_{u,\ bulk}(s^{-1})$	5.1	5.2 - 5.0	4.1
	$K_{eq,bulk}$	0.5	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.5	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

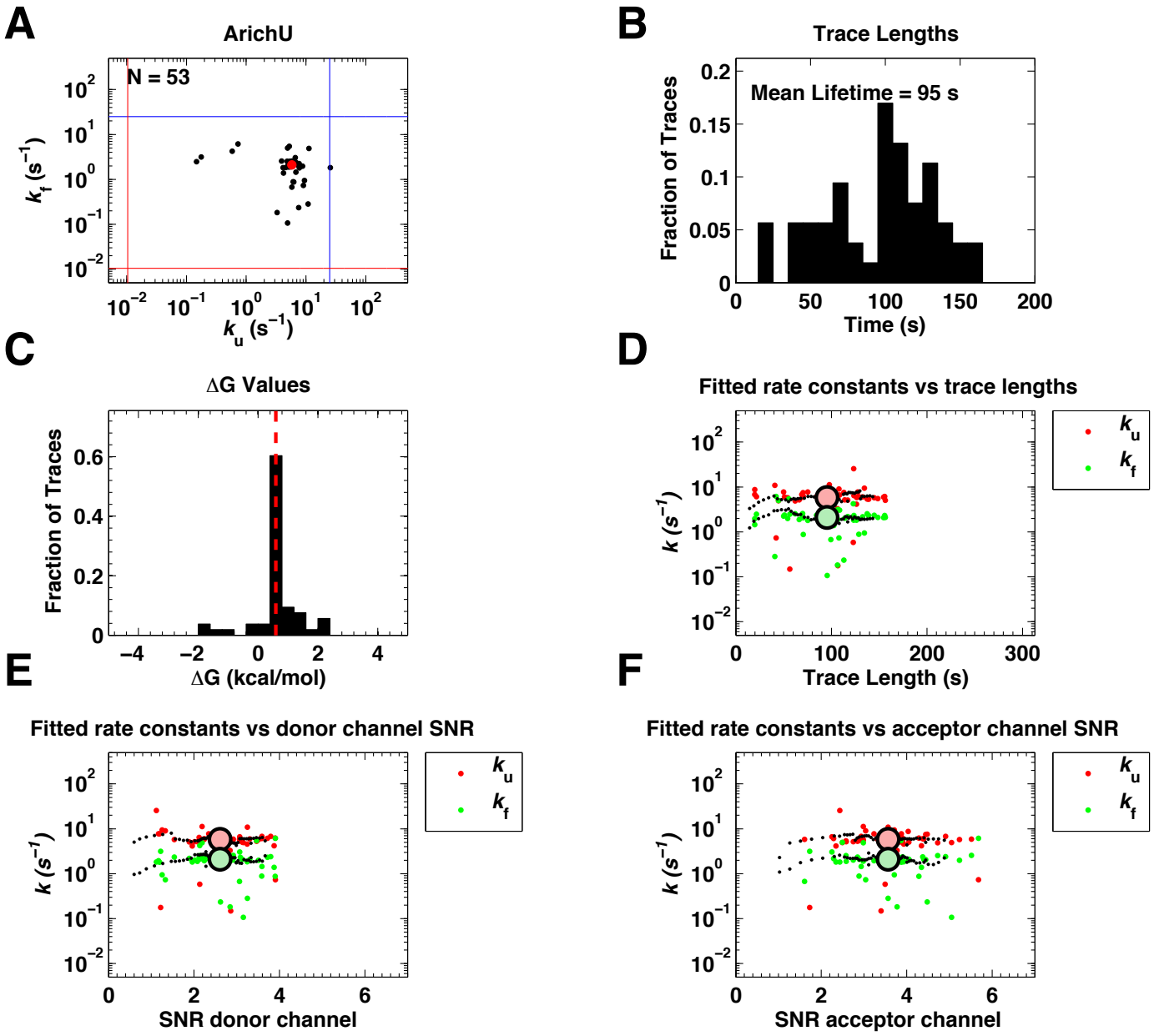


Figure S36-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

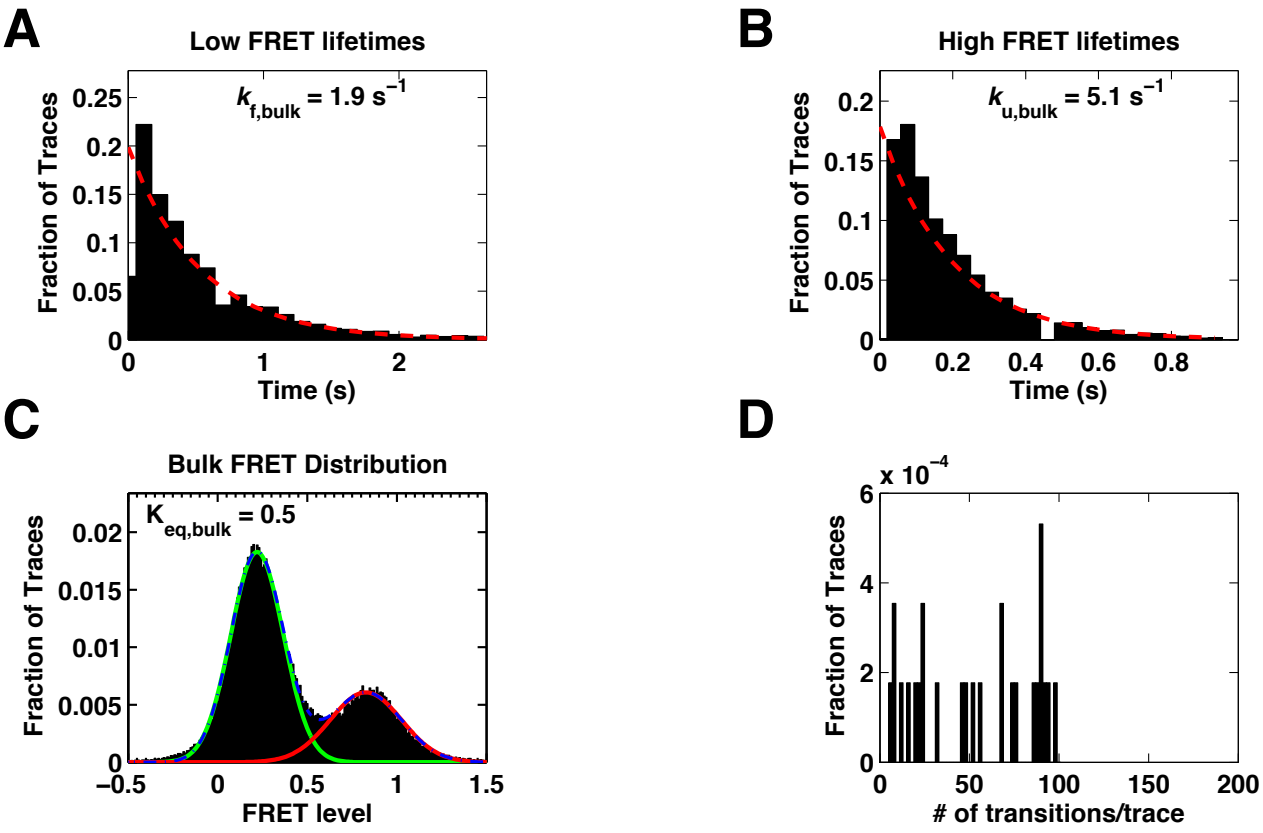


Figure S36-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

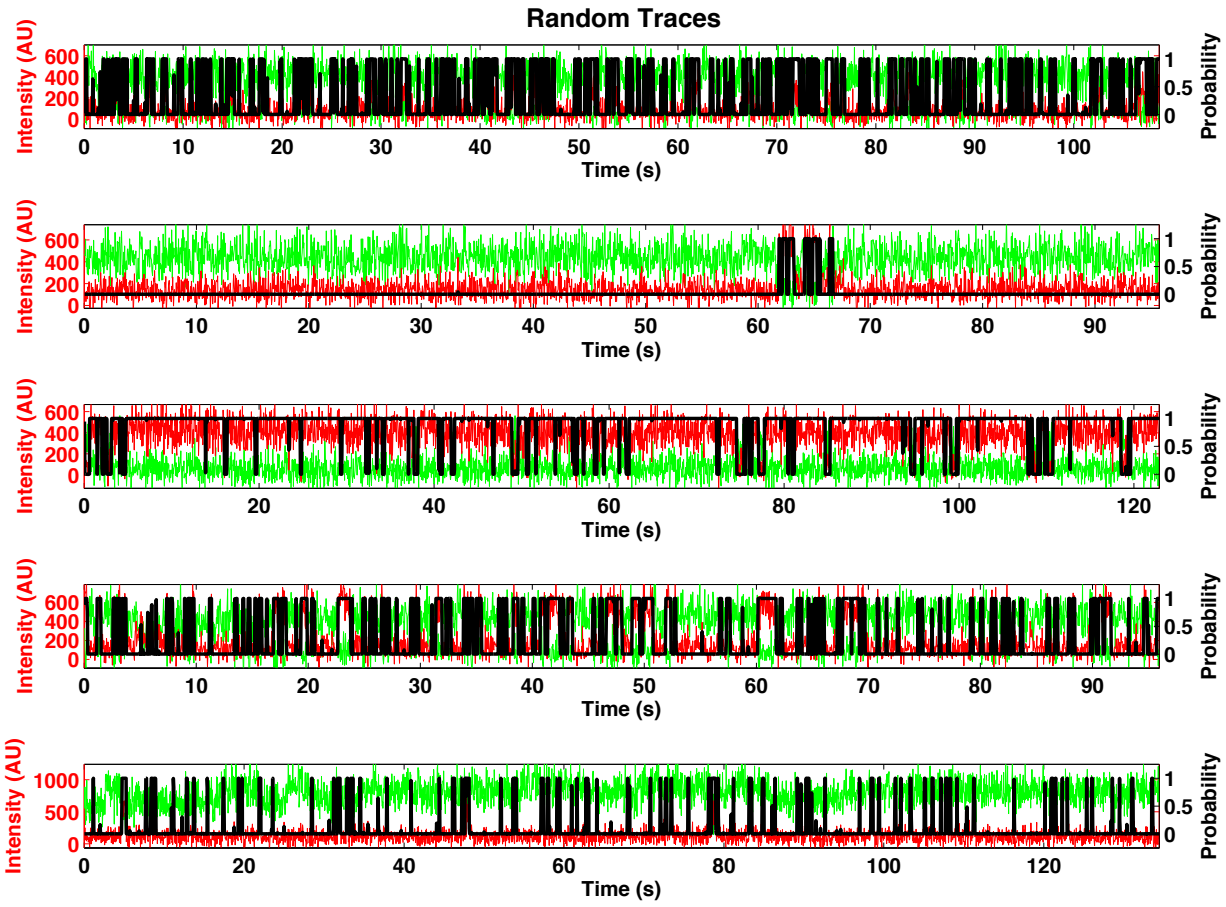


Figure S36-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S37-1.Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	20.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	62

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S37-2.Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	4.0	3.5 - 4.5	1.6
	$k_u(s^{-1})$	2.4	1.8 - 2.8	2.4
	K_{eq}	1.7	1.3 - 2.1	2.6
	SNR green	2.2	2.3 - 2.9	1.2
	SNR red	3.7	3.5 - 4.1	1.3
	ΔG(kcal/mol)	-0.3	-0.4 - -0.2	0.6
Fits from Cumulative Data[2]	Lifetime (s)	65.1	51.5 - 84.9	65.1
	$k_{f,\ bulk}(s^{-1})$	3.8	3.9 - 3.7	3.3
	$k_{u,\ bulk}(s^{-1})$	2.6	2.6 - 2.5	2.3
	$K_{eq,bulk}$	1.6	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.3	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

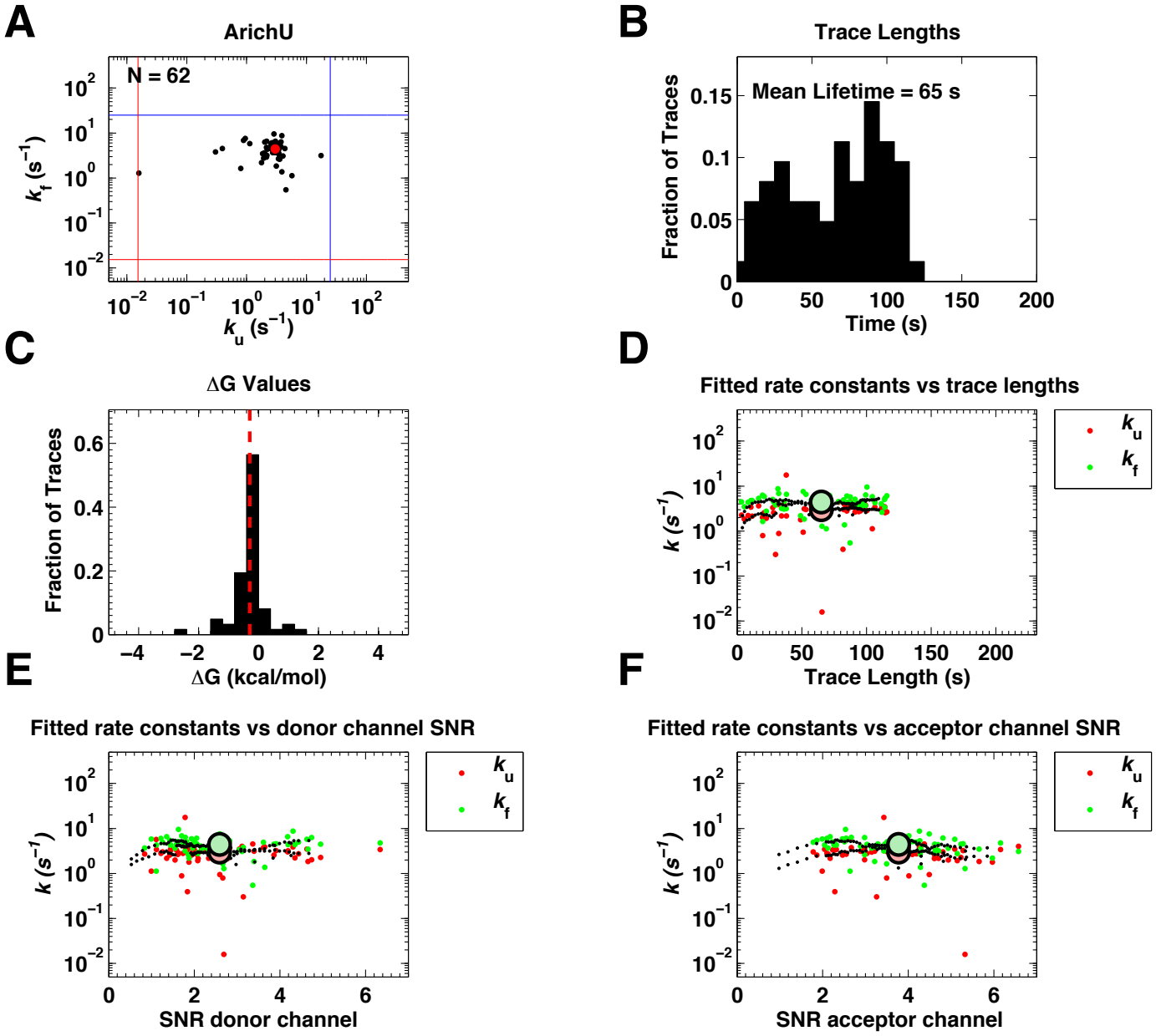


Figure S37-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

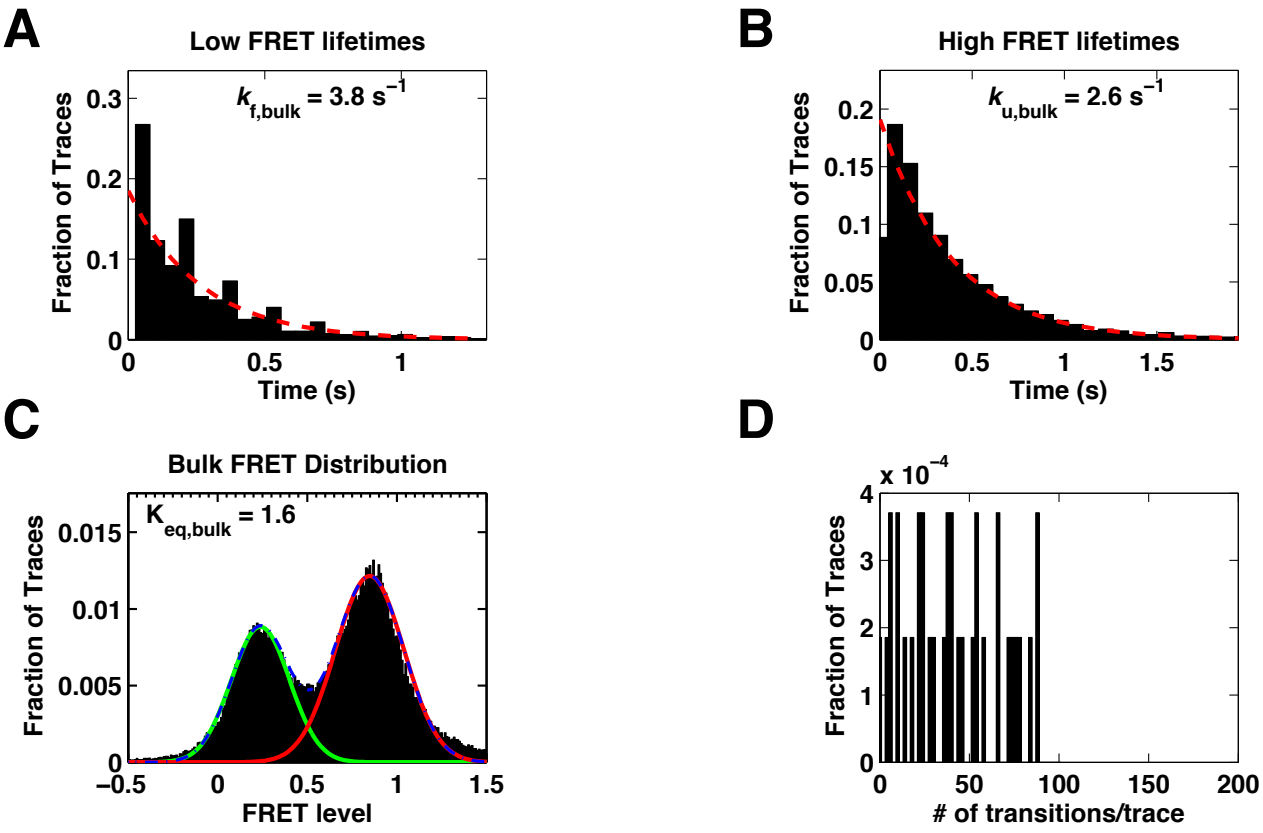


Figure S37-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

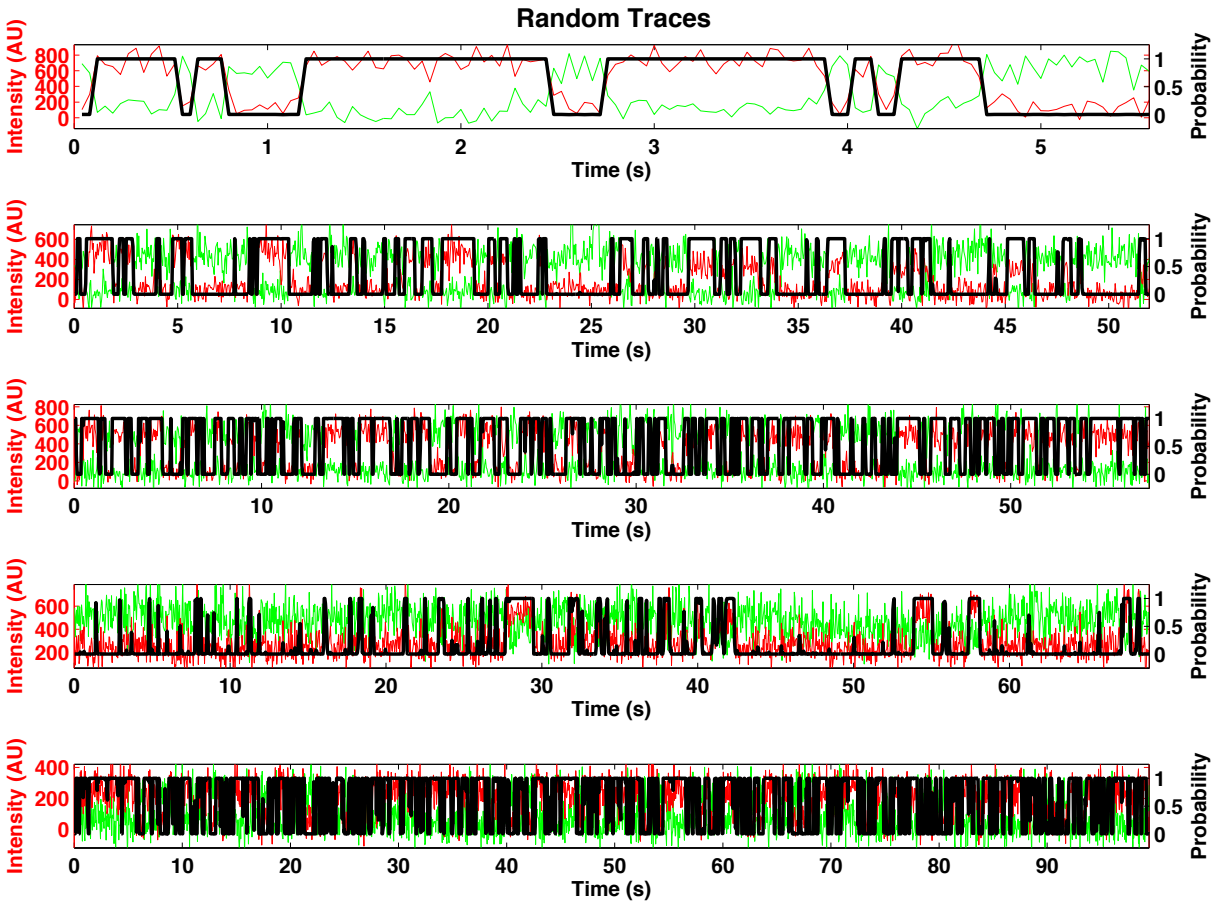


Figure S37-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S38-1.Variant and Conditions

Variant:	ArichU
$MgCl_2$ (mM)	50.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	50

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S38-2. Folding parameters of smFRET the variant ArichU inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	5.0	4.2 - 5.6	1.7
	$k_u(s^{-1})$	1.5	1.2 - 1.7	1.8
	K_{eq}	3.4	2.8 - 4.2	2.1
	SNR green	1.8	1.8 - 2.2	0.7
	SNR red	2.9	2.7 - 3.1	0.7
	ΔG(kcal/mol)	-0.8	-0.9 - -0.6	0.4
Fits from Cumulative Data[2]	Lifetime (s)	93.2	72.0 - 125.6	93.2
	$k_{f,\ bulk}(s^{-1})$	4.8	5.0 - 4.7	3.9
	$k_{u,\ bulk}(s^{-1})$	1.5	1.5 - 1.4	1.3
	$K_{eq,bulk}$	3.9	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.8	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

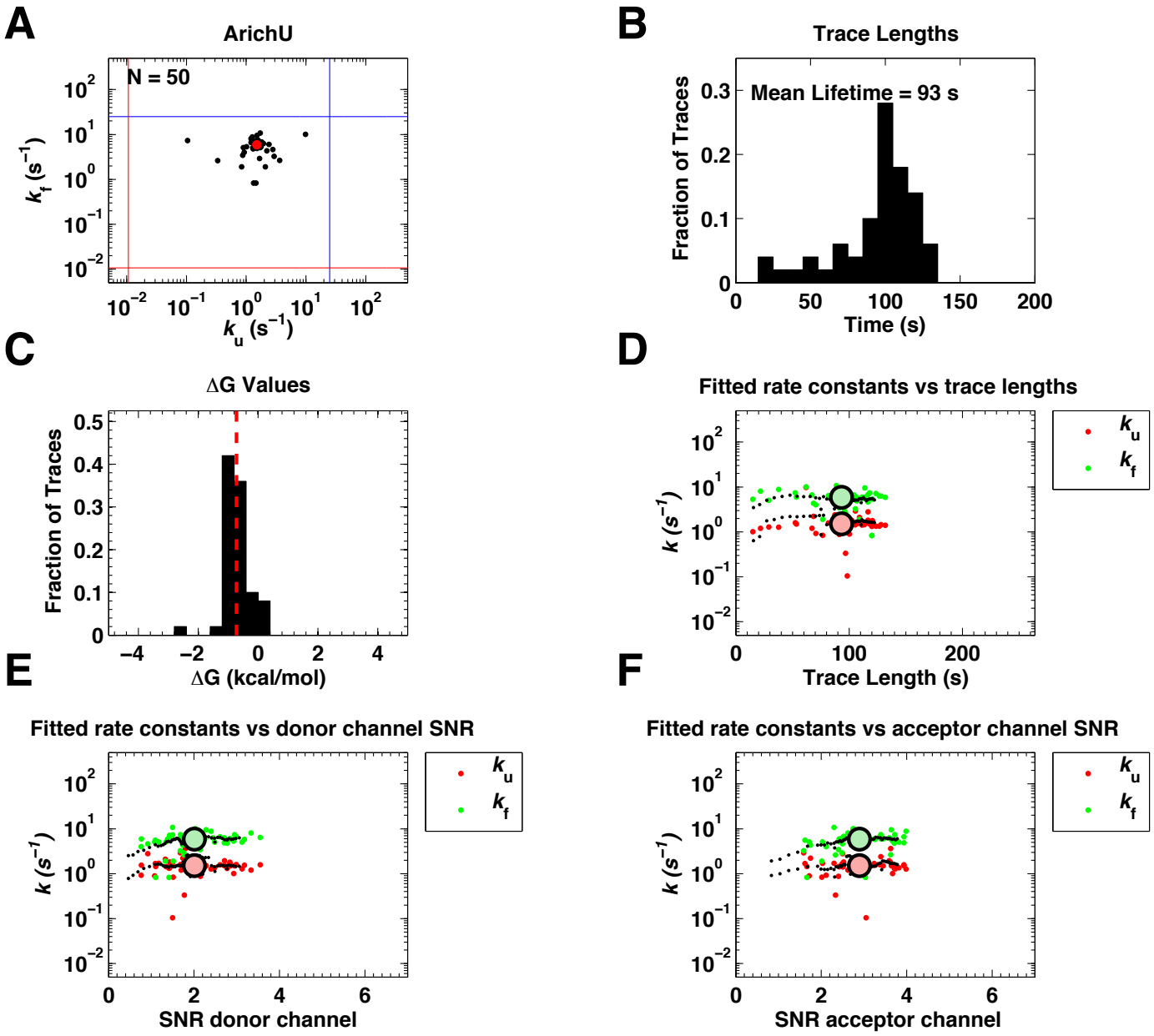


Figure S38-1. smFRET data assessment for ArichU. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

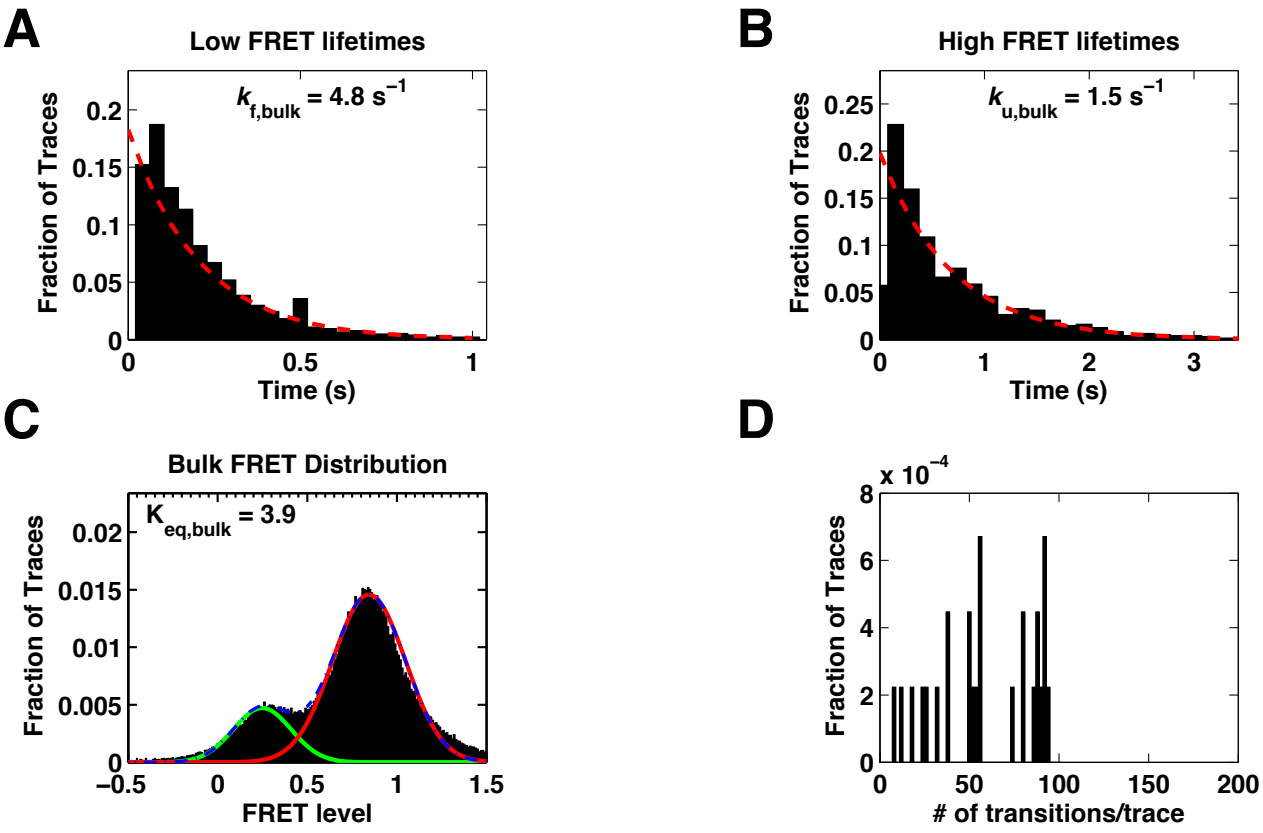


Figure S38-2. smFRET data assesment of aggregate data for ArichU. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

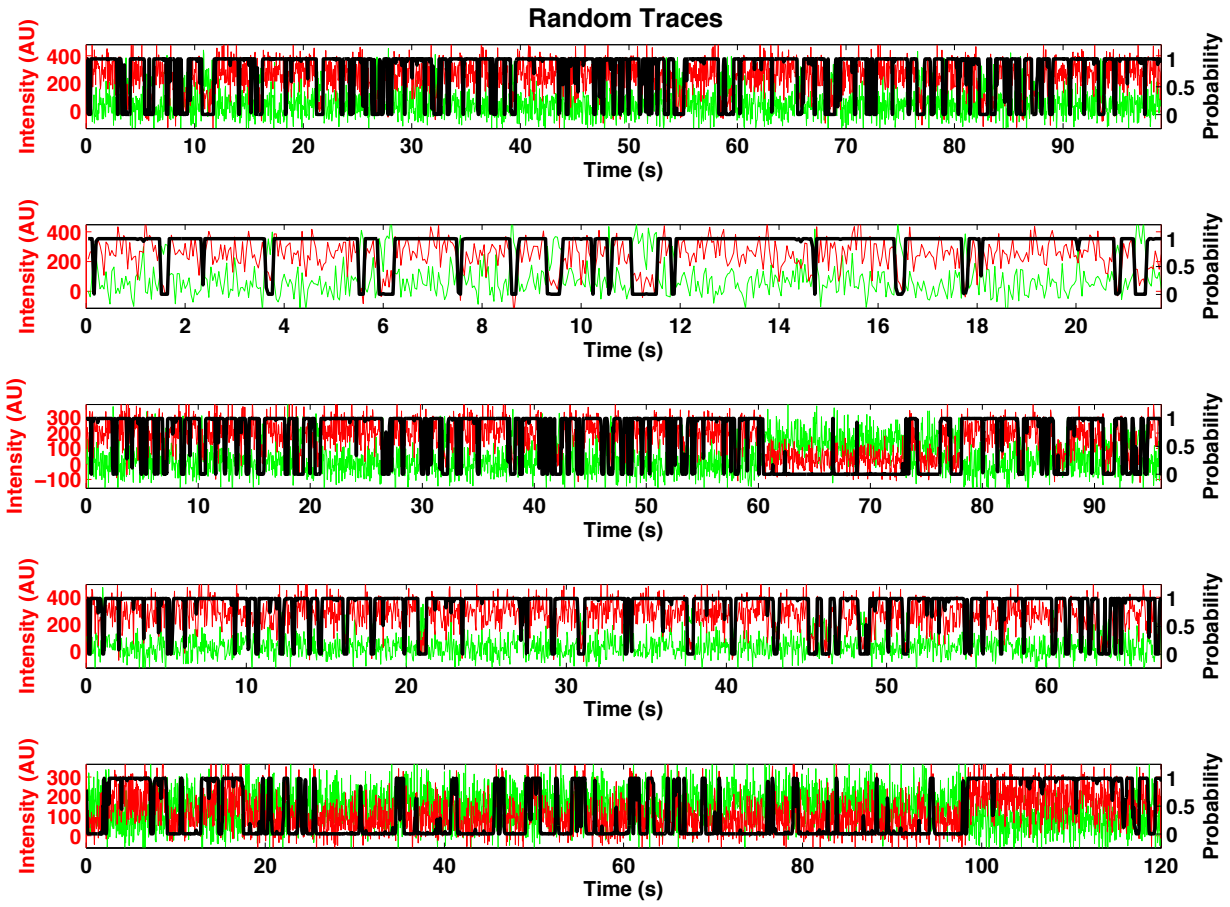


Figure S38-3. Randomly selected FRET traces of ArichU. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S39-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ Extended TL Cy3b-Atto674N
$MgCl_2$ (mM)	0.1
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	130

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S39-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ Extended TL Cy3b-Atto674N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	3.2	3.0 - 3.4	1.5
	$k_u(s^{-1})$	7.2	6.9 - 7.6	1.3
	K_{eq}	0.4	0.4 - 0.5	1.6
	SNR green	2.1	2.0 - 2.1	0.3
	SNR red	0.9	0.9 - 0.9	0.1
	ΔG(kcal/mol)	0.5	0.4 - 0.5	0.3
Fits from Cumulative Data[2]	Lifetime (s)	14.3	12.1 - 17.1	14.3
	$k_{f,\ bulk}(s^{-1})$	3.2	3.3 - 3.1	2.9
	$k_{u,\ bulk}(s^{-1})$	6.8	7.1 - 6.6	6.6
	$K_{eq,bulk}$	0.4	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.5	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

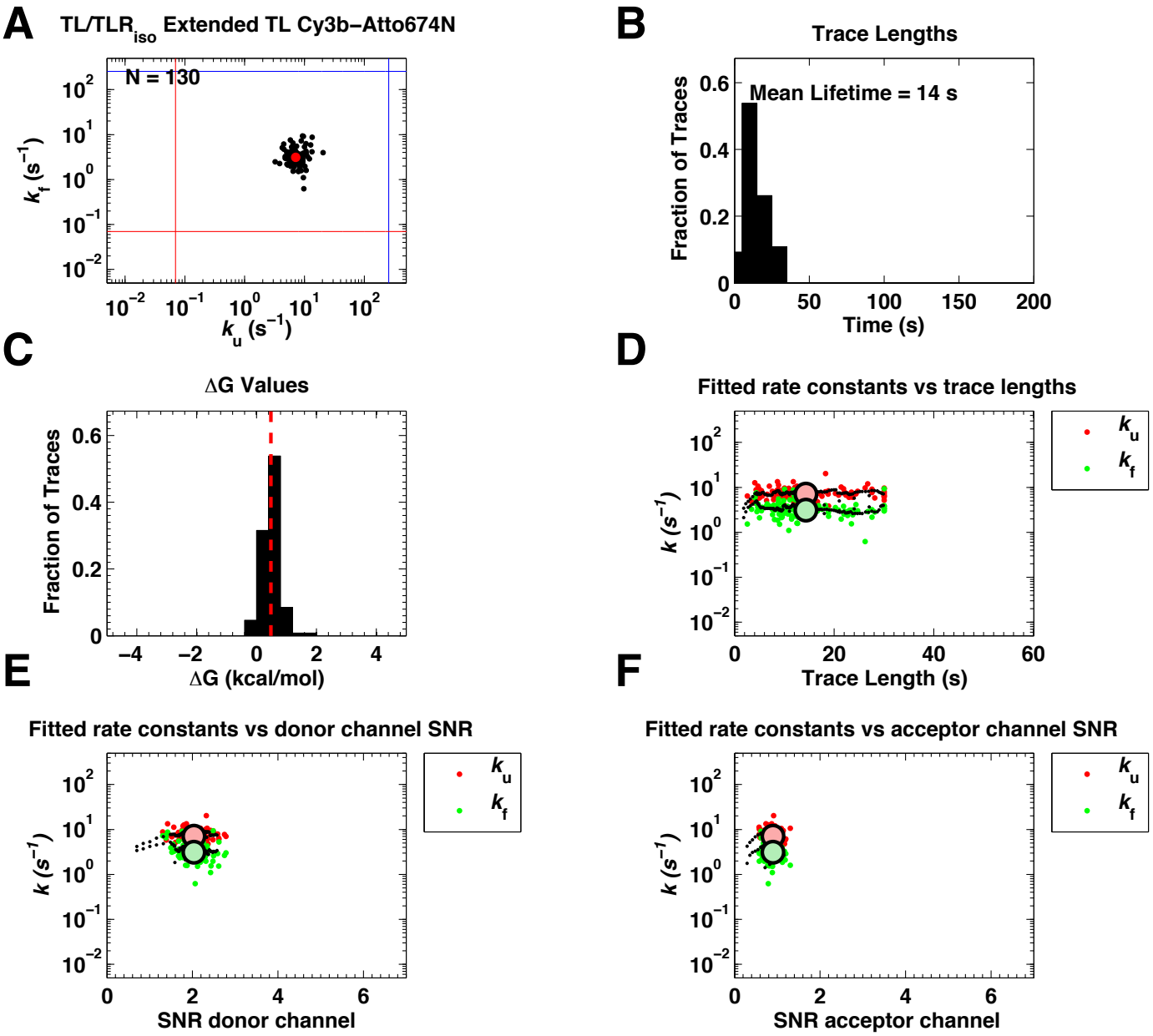


Figure S39-1. smFRET data assessment for TL/TLR$_{iso}$ Extended TL Cy3b-Atto674N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

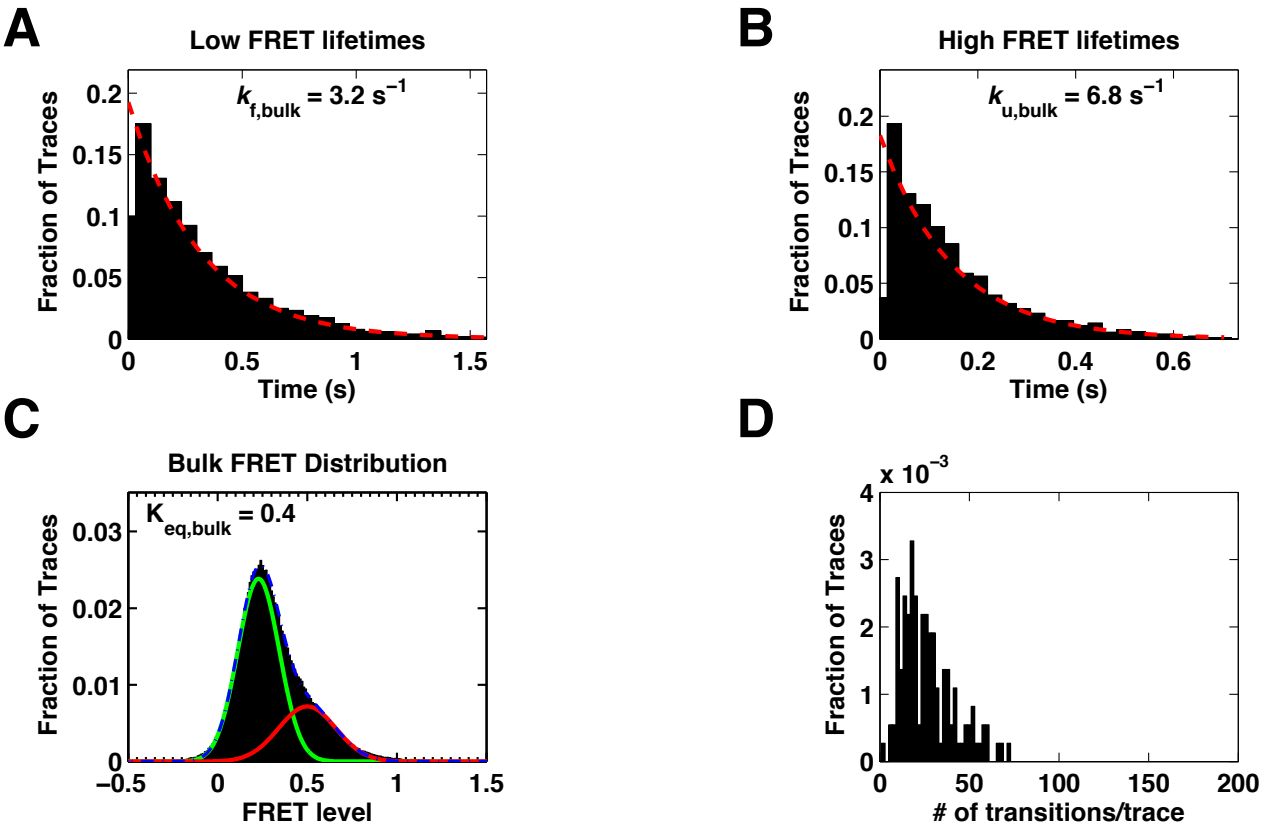


Figure S39-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ Extended TL Cy3b-Atto674N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

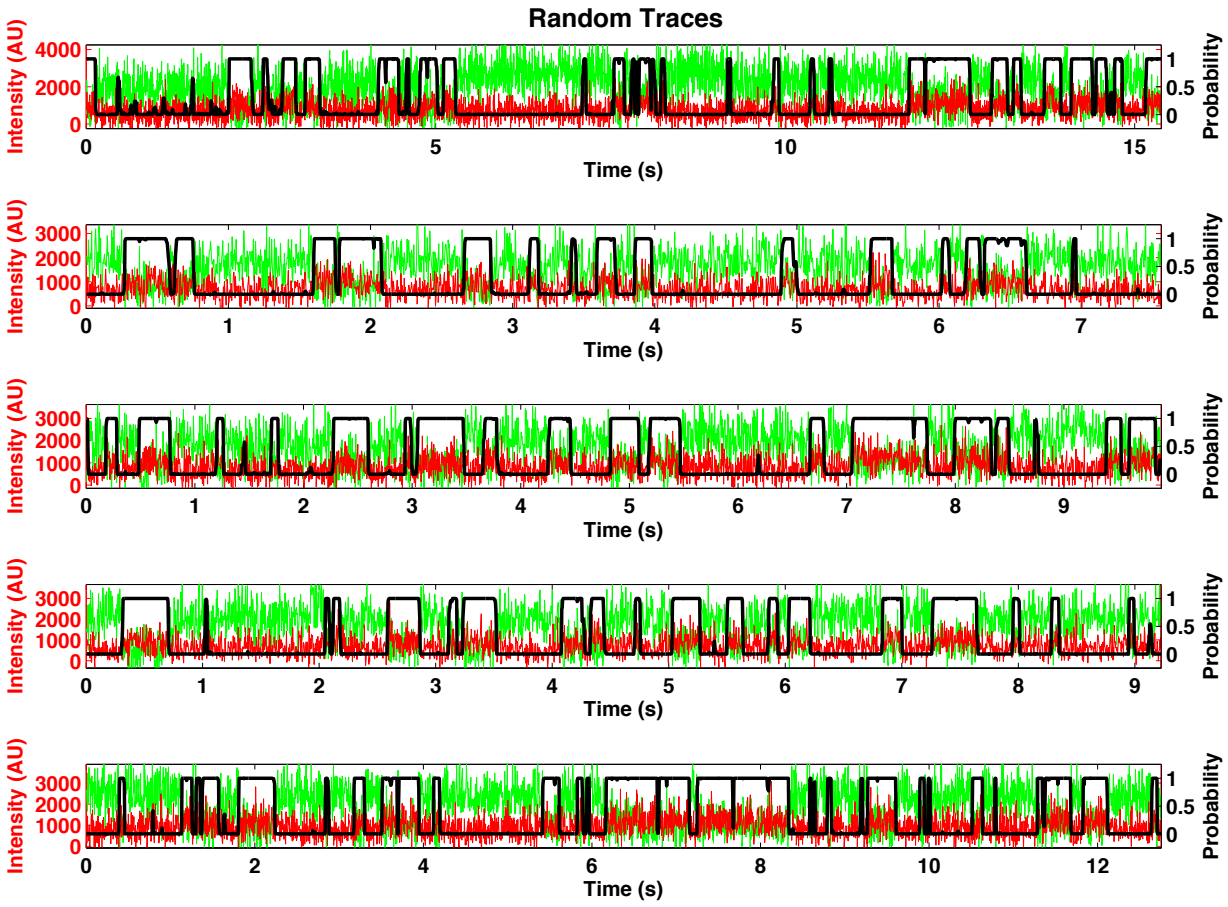


Figure S39-3. Randomly selected FRET traces of TL/TLR$_{iso}$ Extended TL Cy3b-Atto674N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S40-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N
$MgCl_2$ (mM)	1.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	253
SNR Threshold[2]	0.50
Number of Traces	86

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S40-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	5.8	5.4 - 6.3	1.4
	$k_u(s^{-1})$	6.3	6.0 - 6.7	1.3
	K_{eq}	0.9	0.8 - 1.0	1.6
	SNR green	2.2	2.1 - 2.3	0.4
	SNR red	1.1	1.0 - 1.1	0.2
	ΔG(kcal/mol)	0.1	-0.0 - 0.1	0.3
Fits from Cumulative Data[2]	Lifetime (s)	11.8	9.7 - 14.8	11.8
	$k_{f,\ bulk}(s^{-1})$	5.8	6.0 - 5.5	5.4
	$k_{u,\ bulk}(s^{-1})$	6.0	6.2 - 5.8	5.7
	$K_{eq,bulk}$	1.0	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

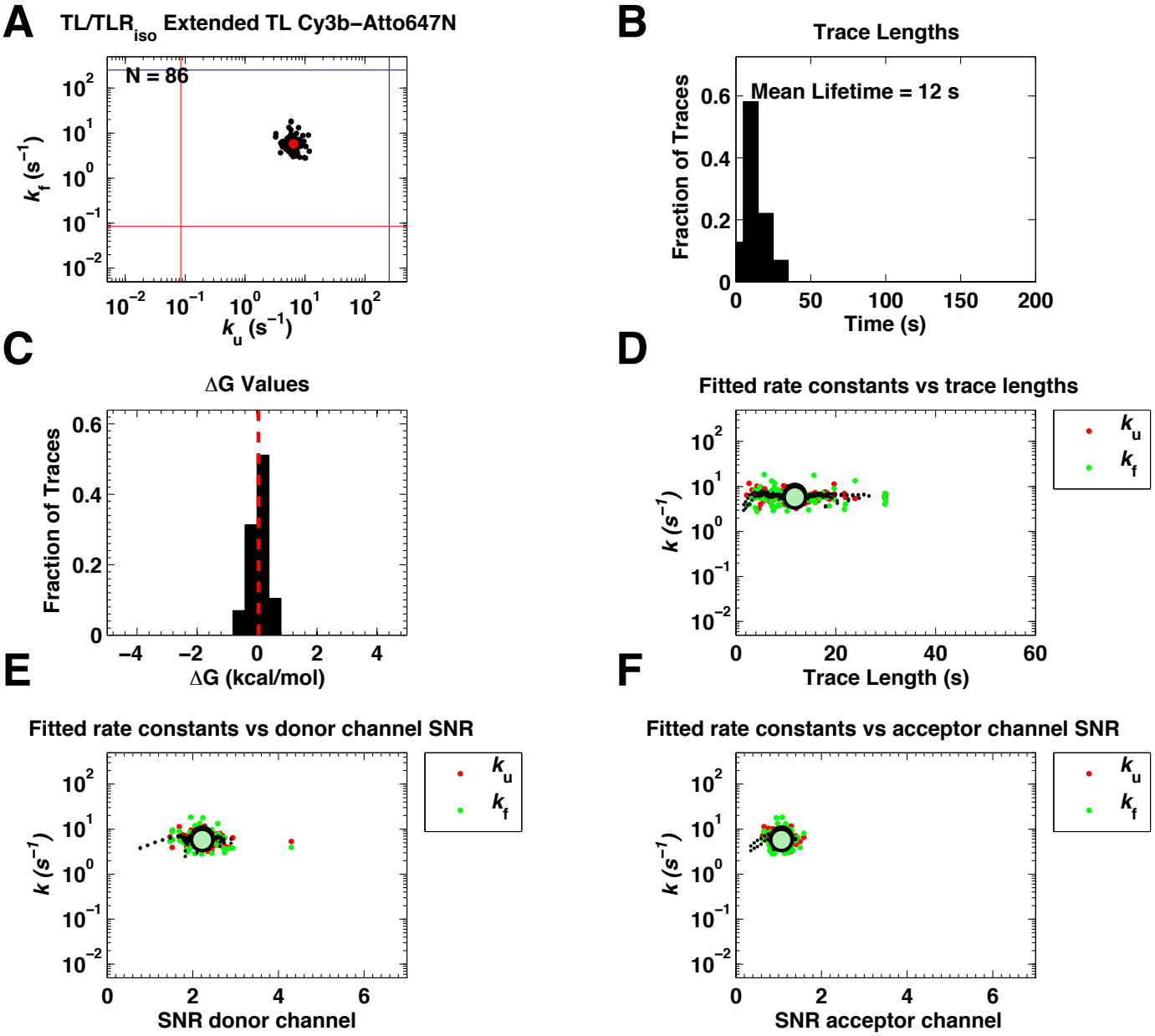


Figure S40-1. smFRET data assessment for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

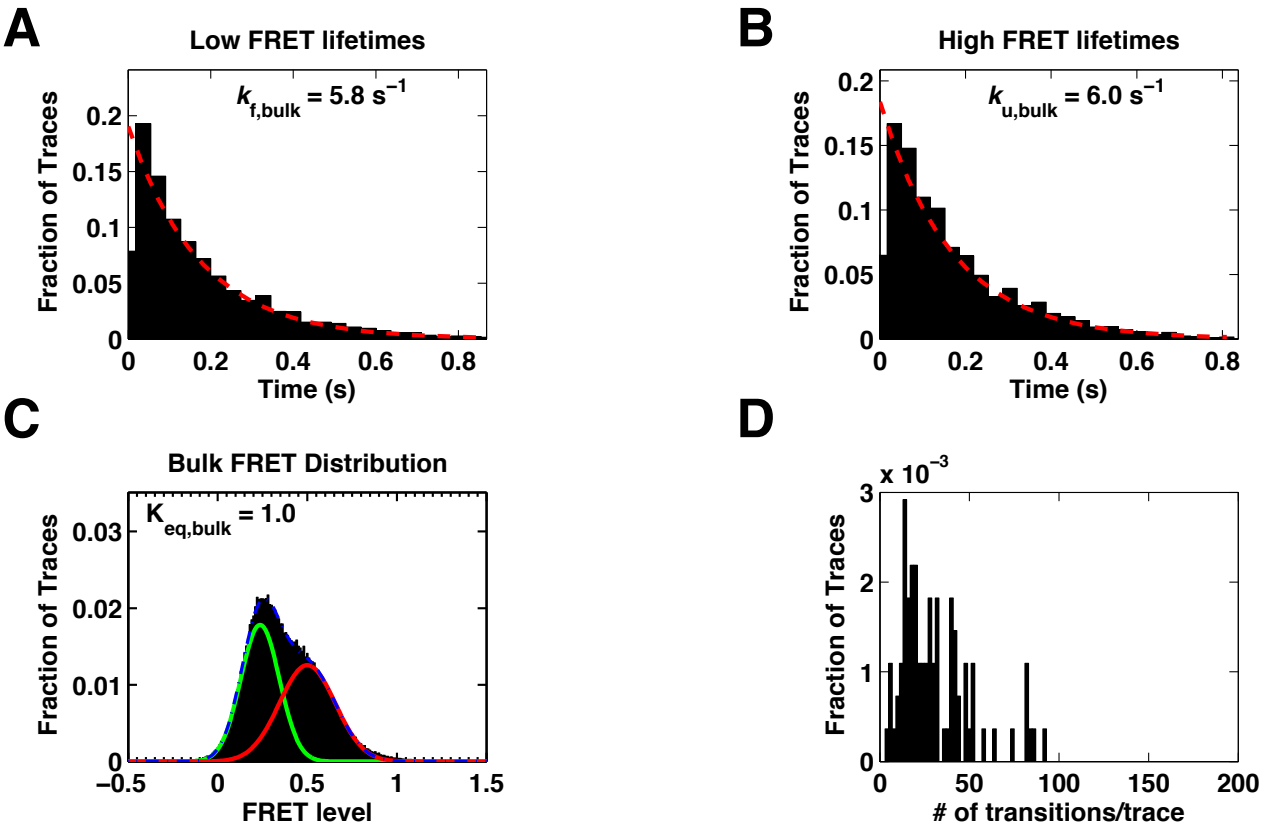


Figure S40-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

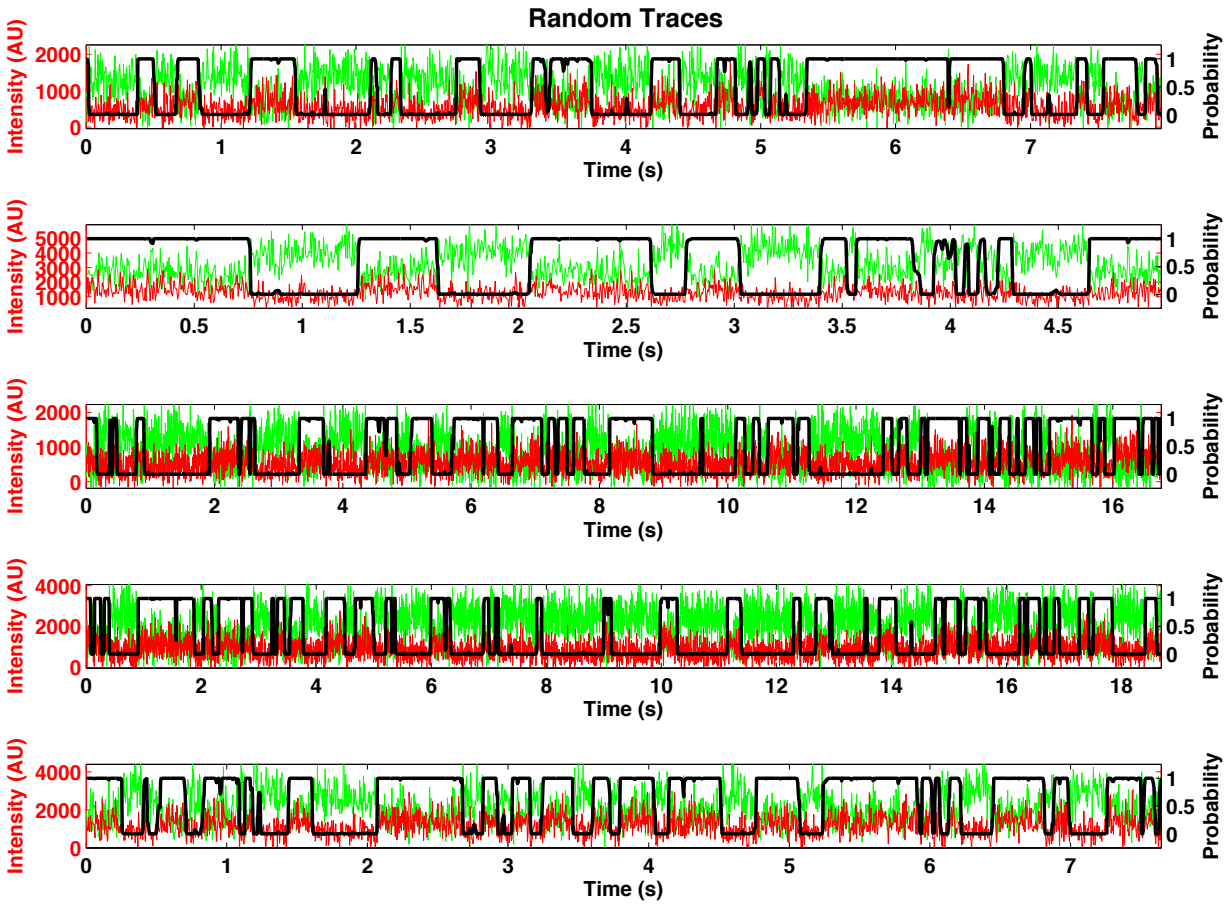


Figure S40-3. Randomly selected FRET traces of TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S41-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N
$MgCl_2$ (mM)	2.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	98

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S41-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	13.0	11.9 - 14.3	1.6
	$k_u(s^{-1})$	5.8	5.5 - 6.2	1.4
	K_{eq}	2.3	2.0 - 2.5	1.6
	SNR green	2.4	2.4 - 2.6	0.5
	SNR red	1.2	1.1 - 1.2	0.3
	ΔG(kcal/mol)	-0.5	-0.5 - -0.4	0.3
Fits from Cumulative Data[2]	Lifetime (s)	10.4	8.6 - 12.8	10.4
	$k_{f,\ bulk}(s^{-1})$	13.1	13.5 - 12.6	11.7
	$k_{u,\ bulk}(s^{-1})$	5.7	5.9 - 5.5	5.4
	$K_{eq,bulk}$	3.1	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

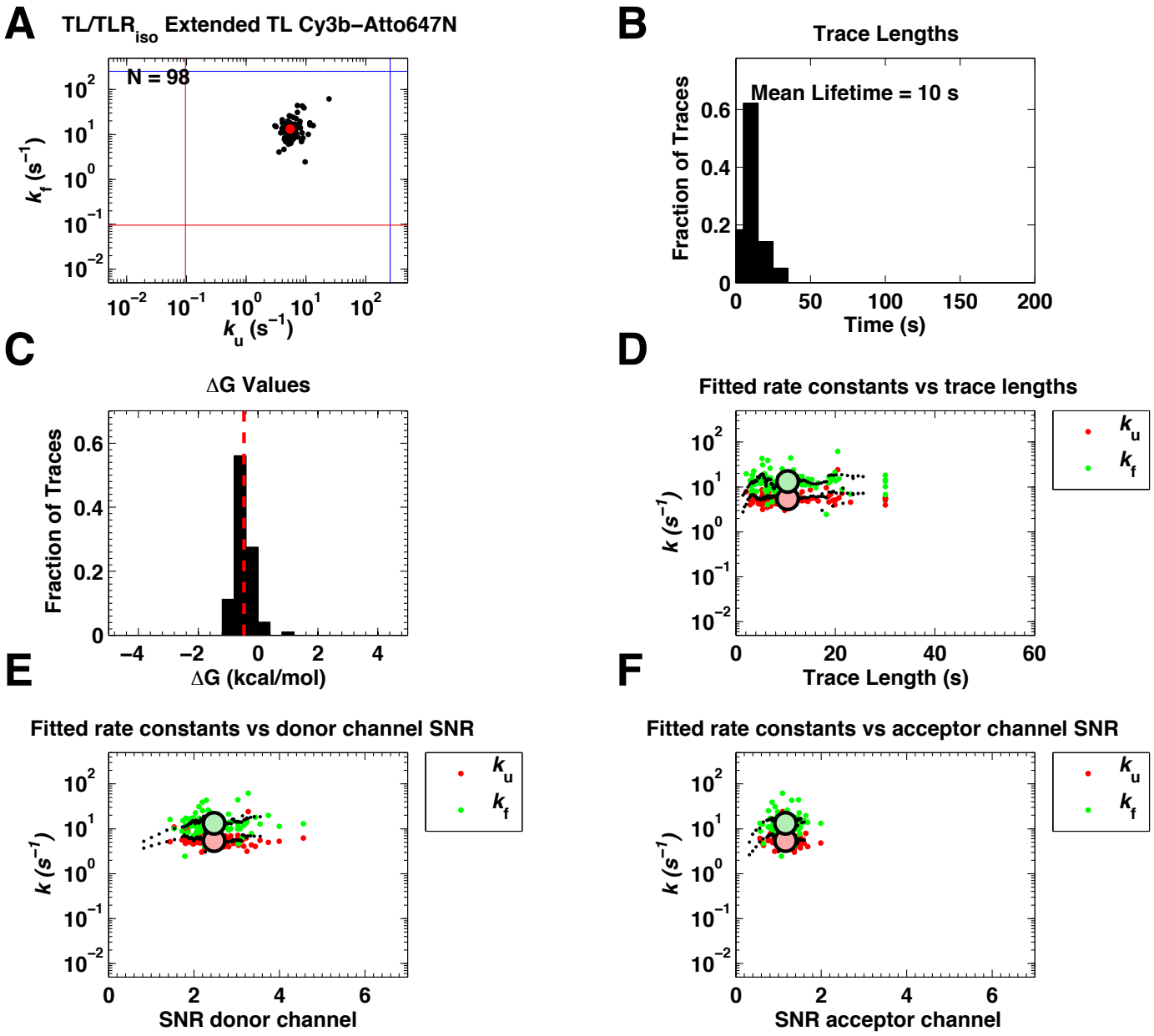


Figure S41-1. smFRET data assessment for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

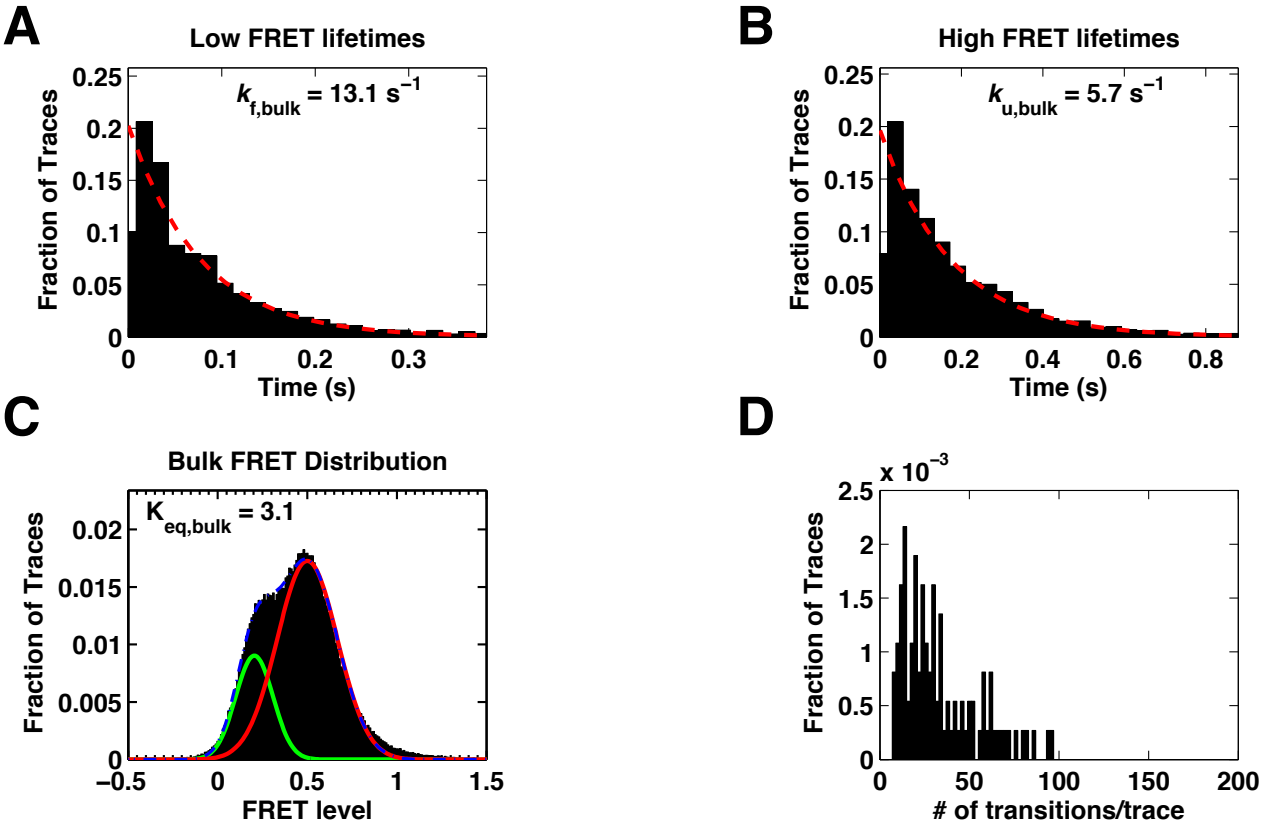


Figure S41-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

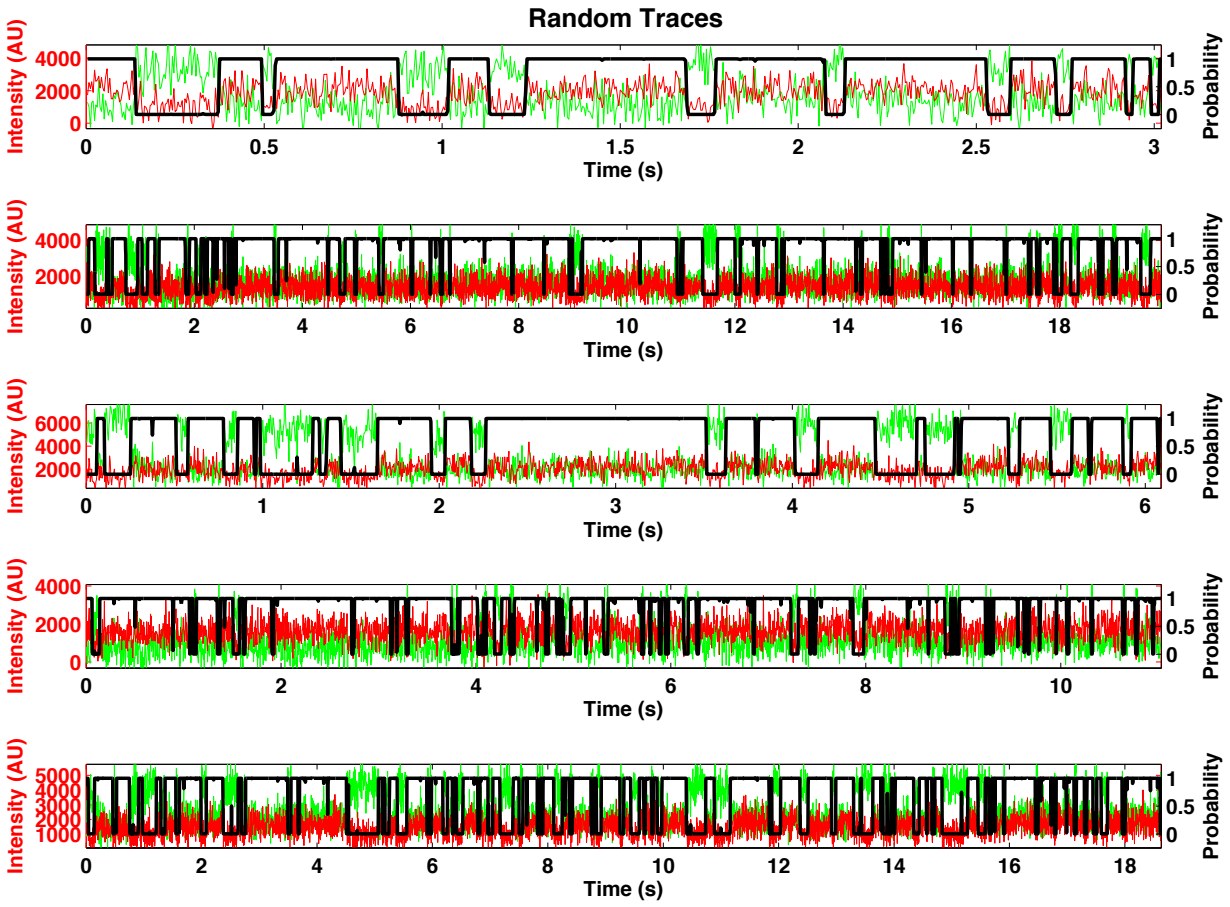


Figure S41-3. Randomly selected FRET traces of TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S42-1. Variant and Conditions

Variant:	TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N
$MgCl_2$ (mM)	10.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	140

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S42-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	24.4	22.5 - 26.1	1.6
	$k_u(s^{-1})$	3.4	3.2 - 3.6	1.5
	K_{eq}	7.3	6.5 - 8.0	1.9
	SNR green	2.7	2.7 - 2.9	0.6
	SNR red	1.3	1.2 - 1.3	0.4
	ΔG(kcal/mol)	-1.2	-1.2 - -1.1	0.4
Fits from Cumulative Data[2]	Lifetime (s)	9.1	7.8 - 10.9	9.1
	$k_{f,\ bulk}(s^{-1})$	22.8	23.6 - 22.0	19.2
	$k_{u,\ bulk}(s^{-1})$	3.2	3.3 - 3.1	3.0
	$K_{eq,bulk}$	11.4	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.4	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

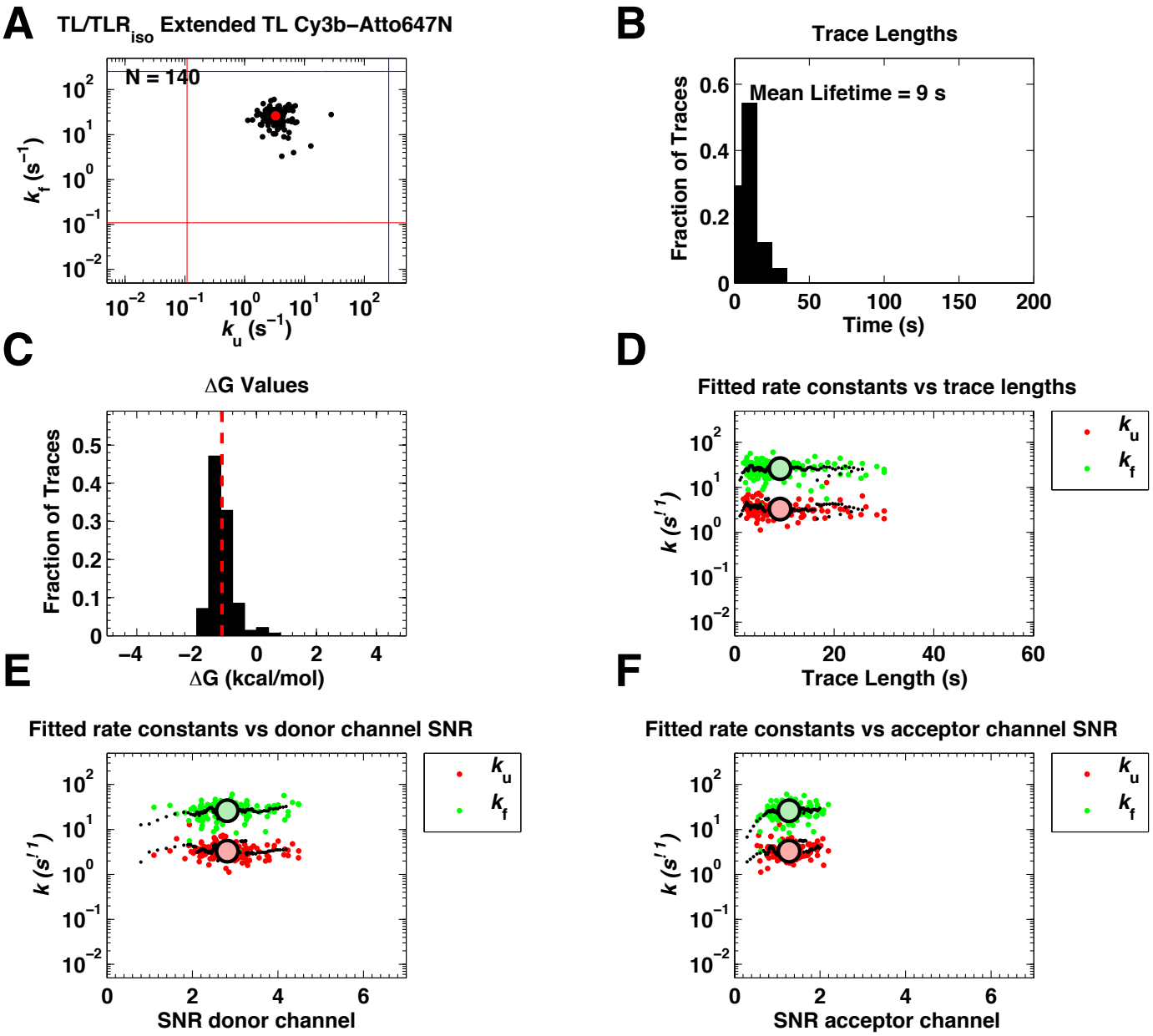


Figure S42-1. smFRET data assessment for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N . (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

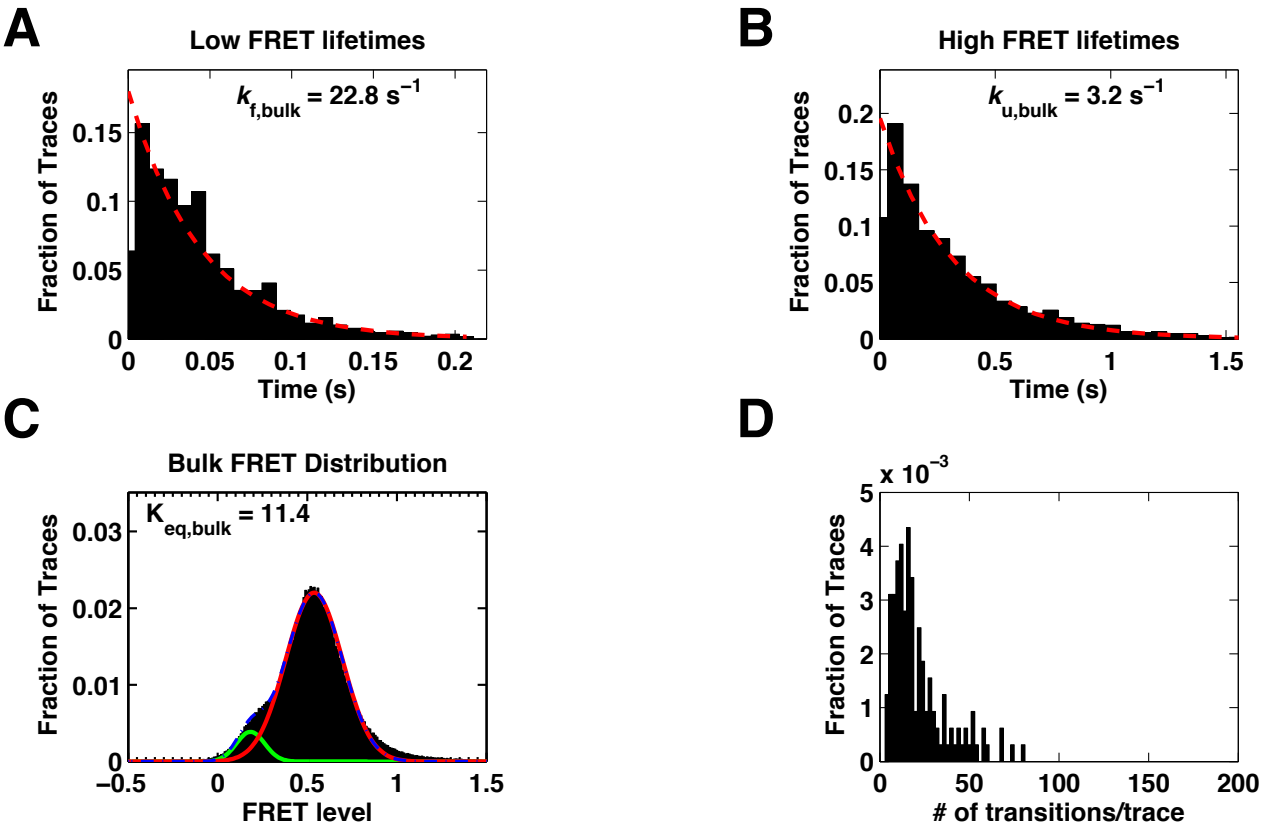


Figure S42-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N . (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

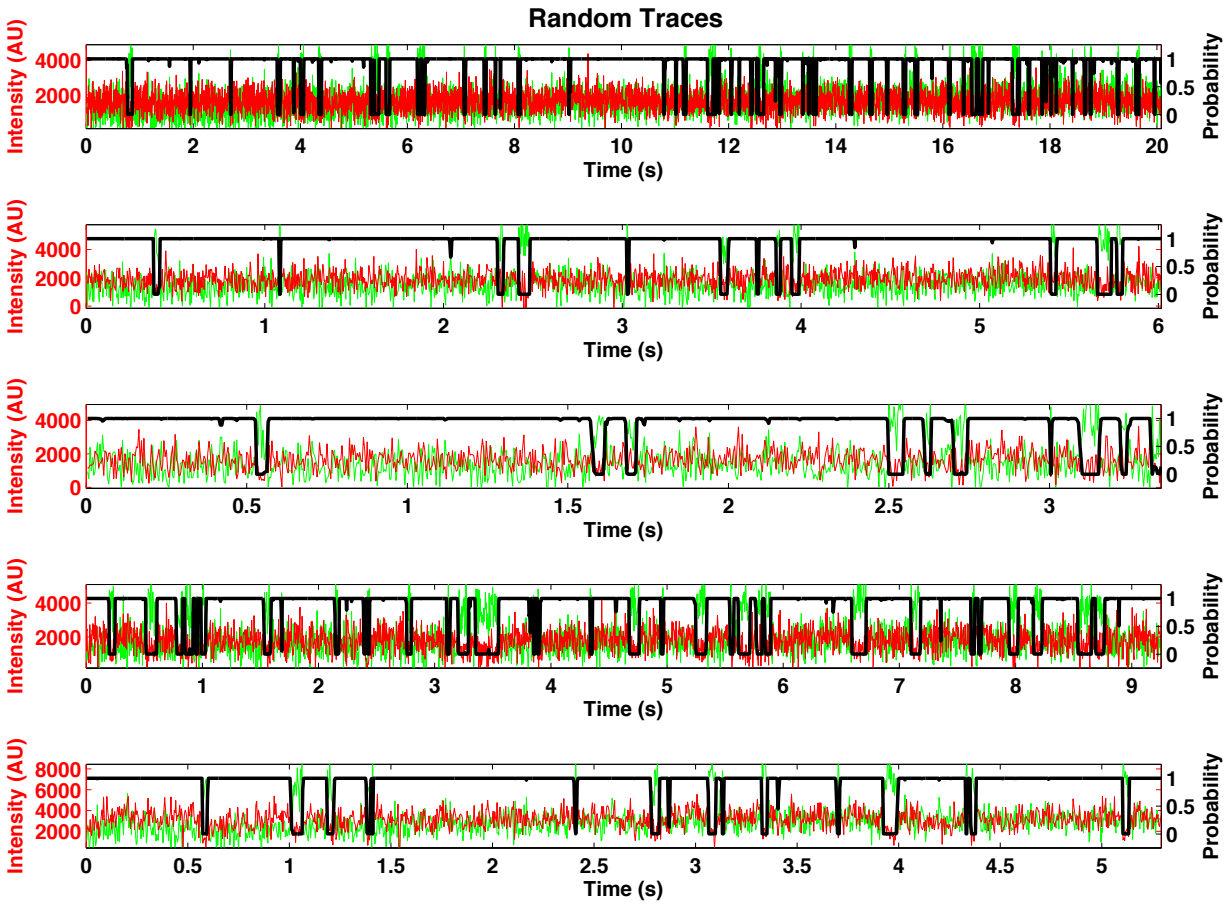


Figure S42-3. Randomly selected FRET traces of TL/TLR$_{iso}$ Extended TL Cy3b-Atto647N . The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S43-1.Variant and Conditions

Variant:	TLTLR$_{iso}$ T14
$MgCl_2$ (mM)	0.1
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	128

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S43-2.Folding parameters of smFRET the variant TLTLR$_{iso}$ T14 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	2.8	2.7 - 3.0	1.4
	$k_u(s^{-1})$	18.3	17.3 - 19.3	1.4
	K_{eq}	0.2	0.1 - 0.2	1.4
	SNR green	1.5	1.4 - 1.5	0.2
	SNR red	1.6	1.5 - 1.6	0.3
	ΔG(kcal/mol)	1.1	1.1 - 1.1	0.2
Fits from Cumulative Data[2]	Lifetime (s)	7.7	6.5 - 9.2	7.7
	$k_{f,\ bulk}(s^{-1})$	2.5	2.6 - 2.4	2.4
	$k_{u,\ bulk}(s^{-1})$	15.8	16.5 - 15.1	15.3
	$K_{eq,bulk}$	0.1	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	1.3	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

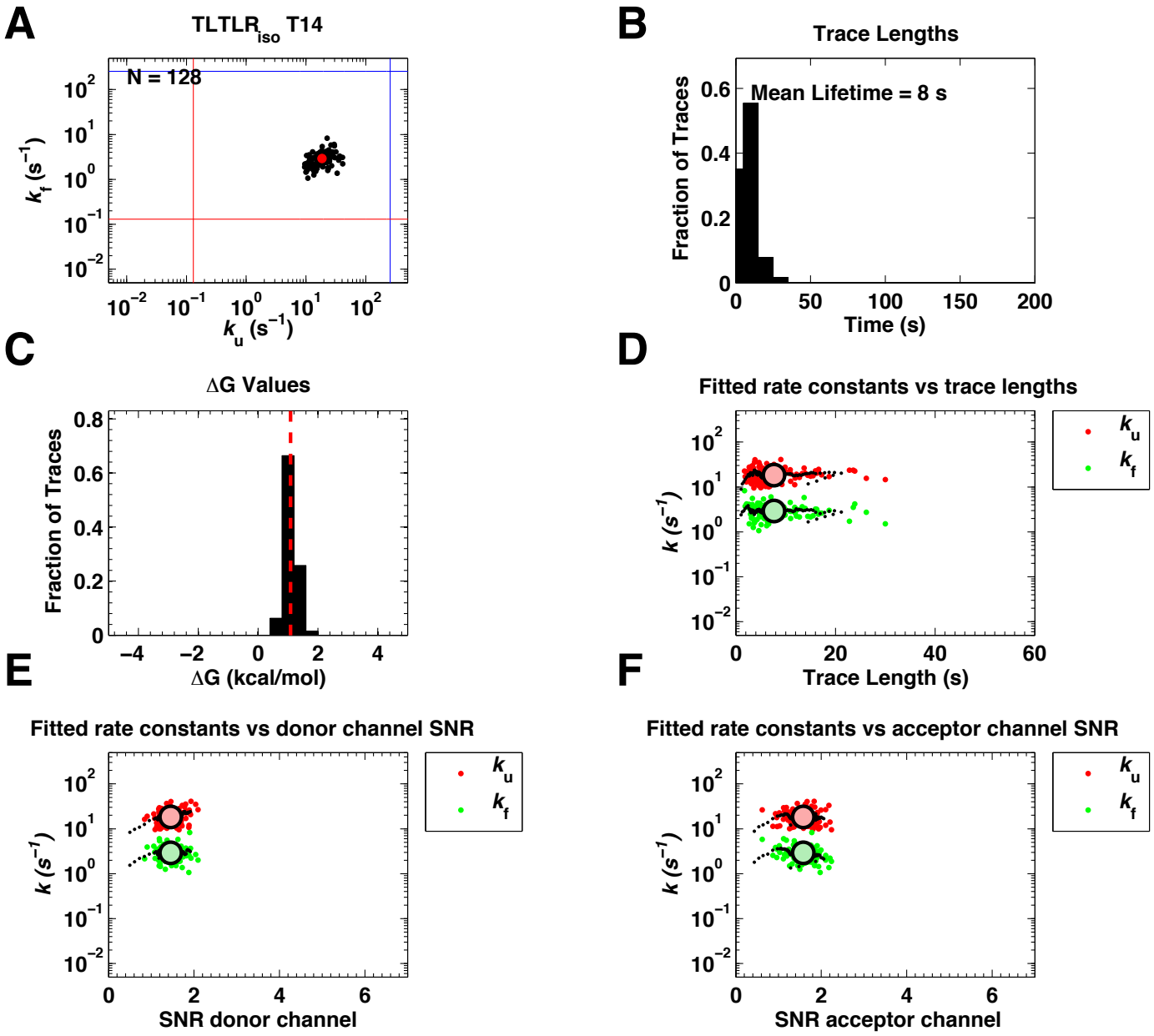


Figure S43-1. smFRET data assessment for TLTLR$_{iso}$ T14. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

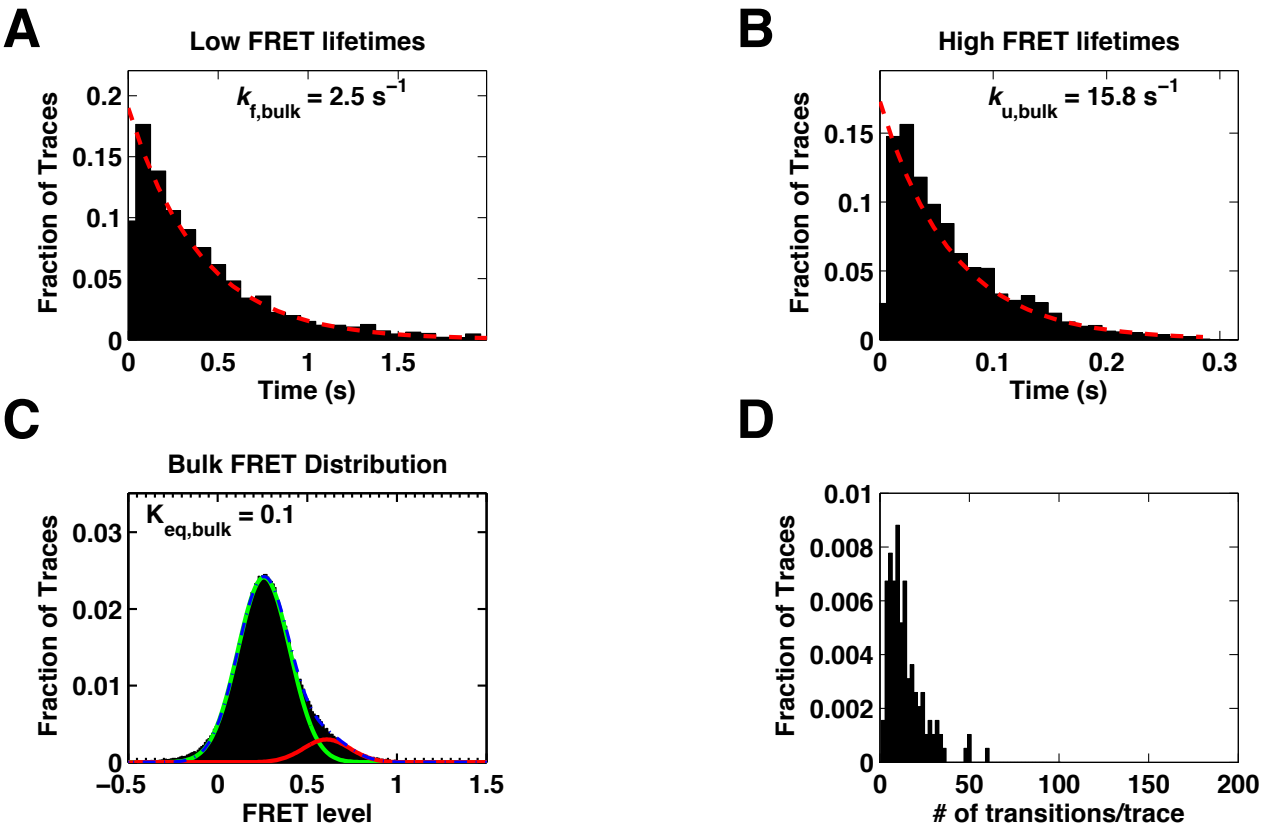


Figure S43-2. smFRET data assesment of aggregate data for TLTLR$_{iso}$ T14. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

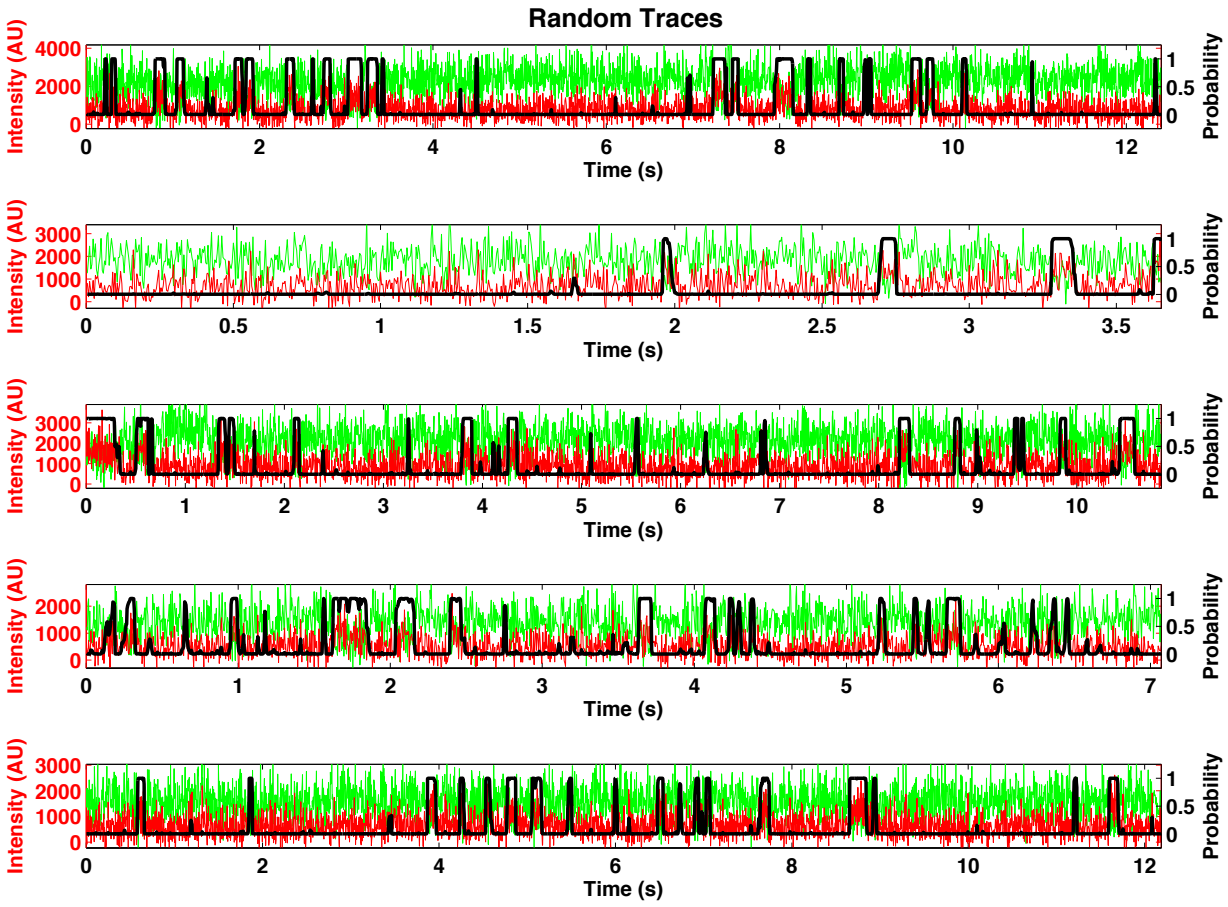


Figure S43-3. Randomly selected FRET traces of TLTLR$_{iso}$ T14. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S44-1.Variant and Conditions

Variant:	TL/TLR$_{iso}$ T14
$MgCl_2$ (mM)	1.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	252
SNR Threshold[2]	0.50
Number of Traces	120

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S44-2.Folding parameters of smFRET the variant TL/TLR$_{iso}$ T14 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	5.9	5.6 - 6.3	1.4
	$k_u(s^{-1})$	16.9	16.1 - 17.9	1.3
	K_{eq}	0.4	0.3 - 0.4	1.5
	SNR green	1.4	1.4 - 1.5	0.3
	SNR red	1.7	1.6 - 1.7	0.4
	ΔG(kcal/mol)	0.6	0.6 - 0.7	0.2
Fits from Cumulative Data[2]	Lifetime (s)	5.5	4.6 - 6.6	5.5
	$k_{f,\ bulk}(s^{-1})$	5.5	5.7 - 5.3	5.3
	$k_{u,\ bulk}(s^{-1})$	15.5	16.1 - 14.9	15.0
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

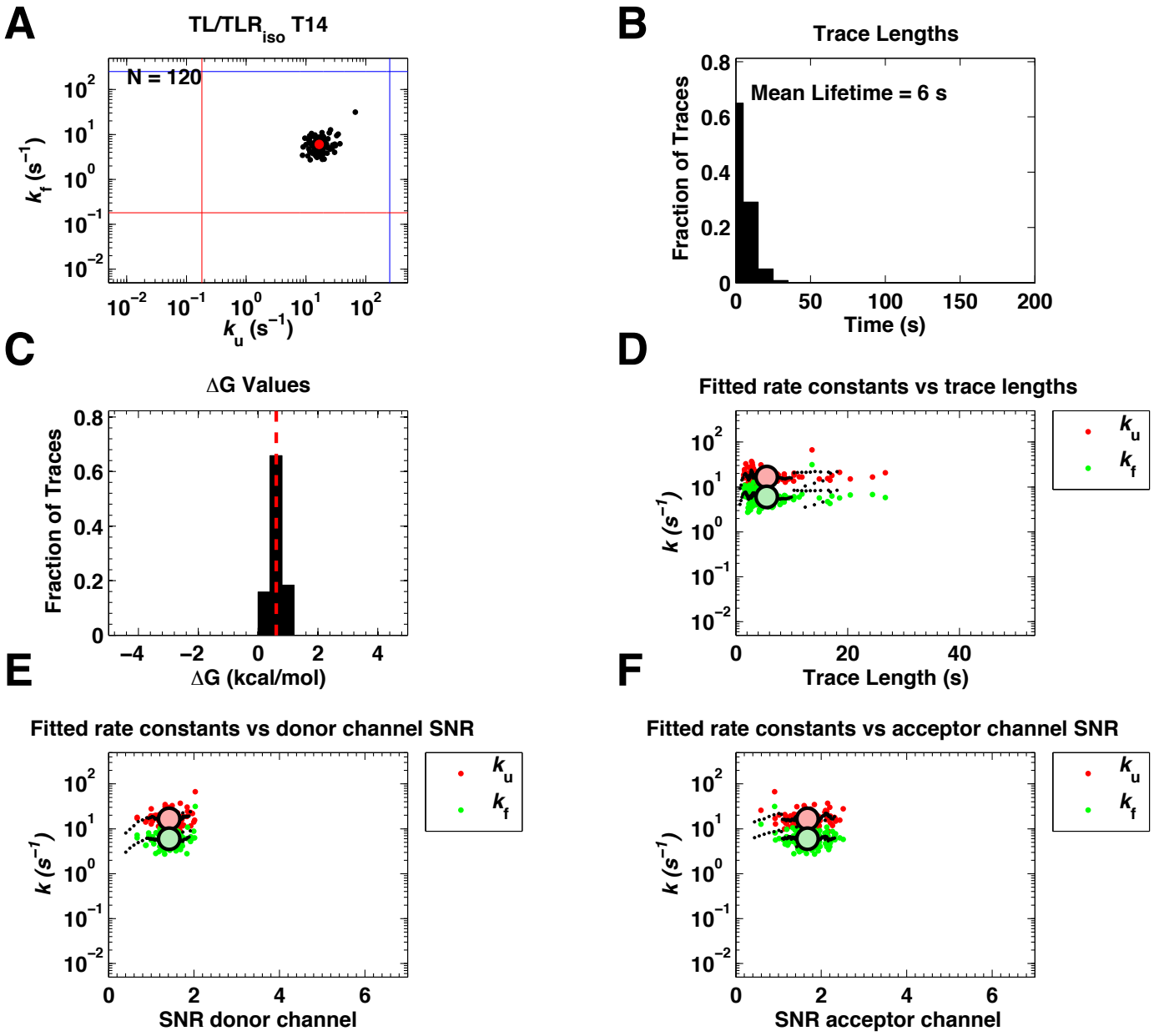


Figure S44-1. smFRET data assessment for TL/TLR$_{iso}$ T14. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

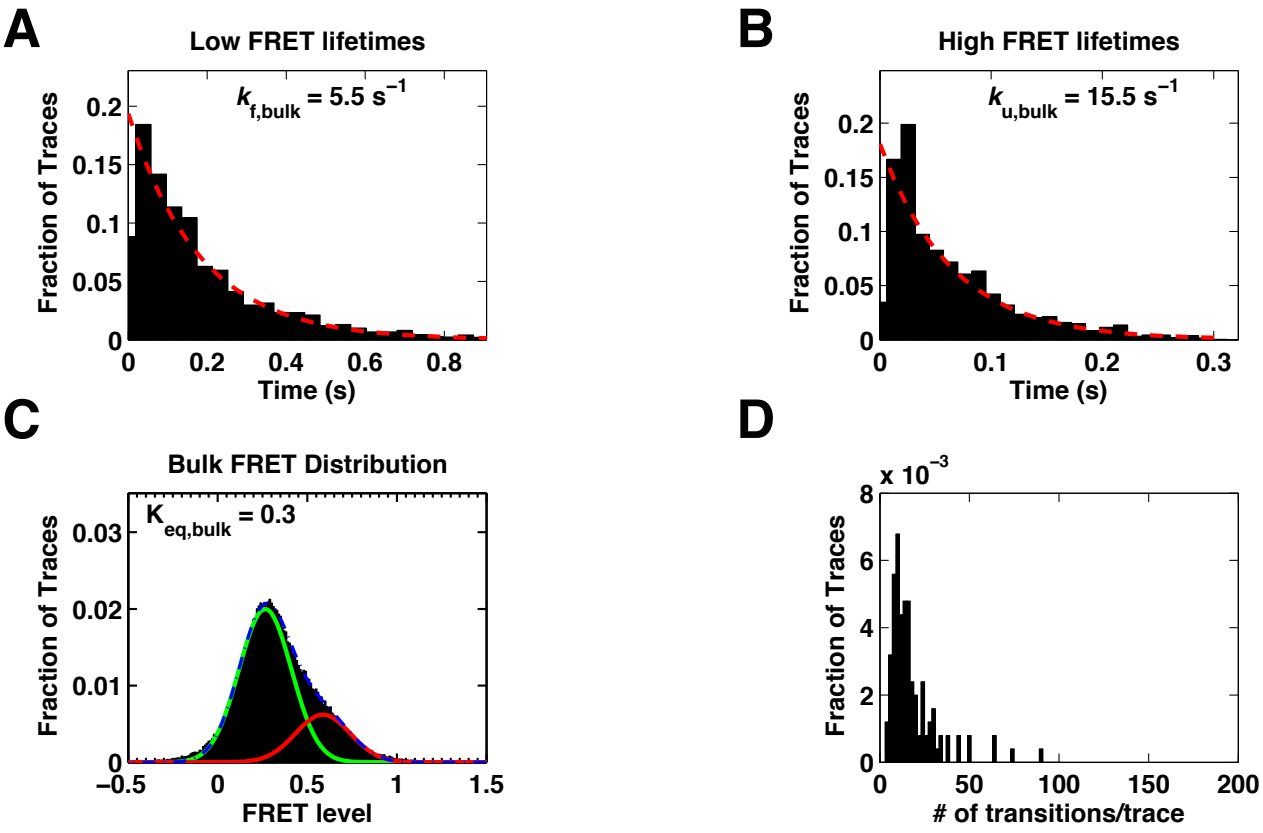


Figure S44-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ T14. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

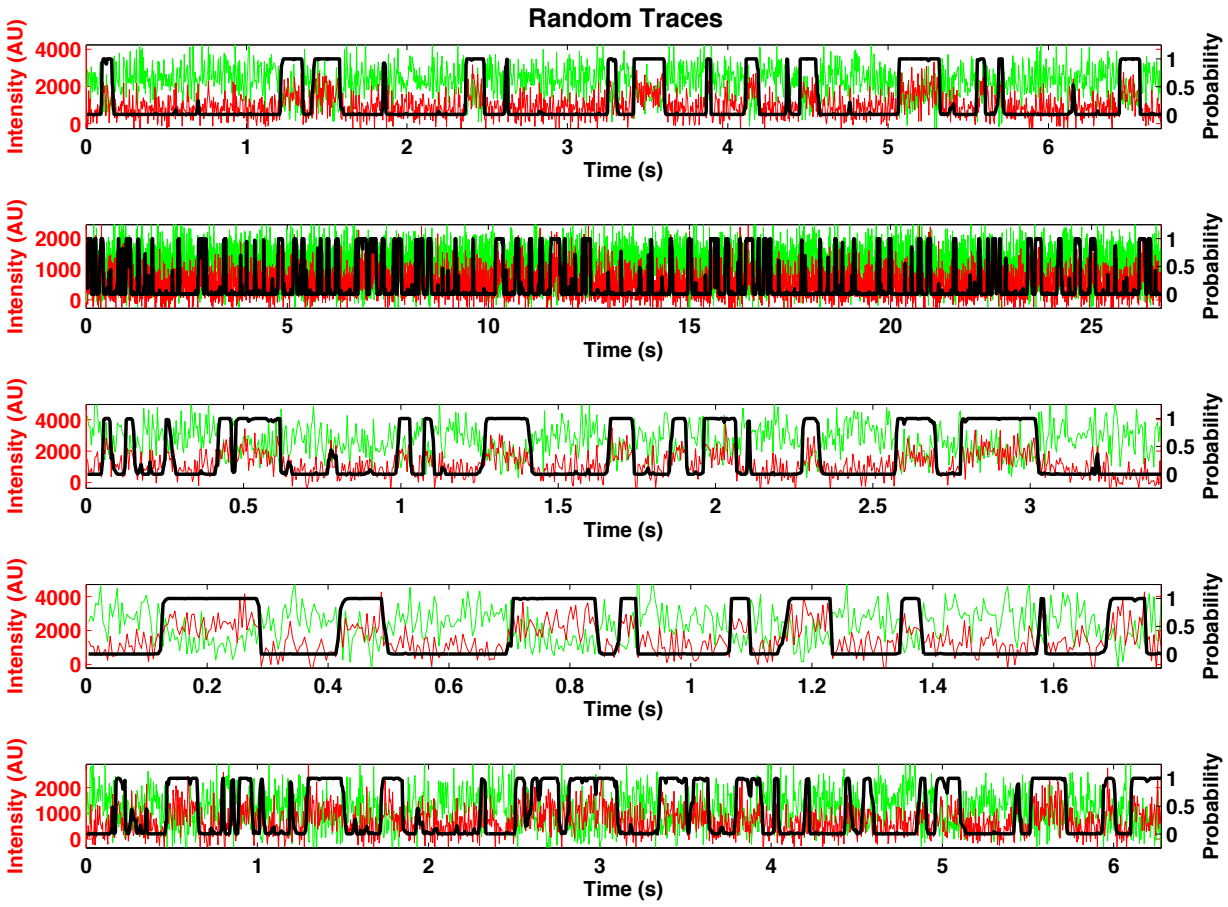


Figure S44-3. Randomly selected FRET traces of TL/TLR$_{iso}$ T14. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S45-1.Variant and Conditions

Variant:	TL/TLR$_{iso}$ T14
$MgCl_2$ (mM)	2.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	257
SNR Threshold[2]	0.50
Number of Traces	91

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S45-2. Folding parameters of smFRET the variant TL/TLR$_{iso}$ T14 inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	29.1	26.5 - 30.5	1.4
	$k_u(s^{-1})$	12.1	11.6 - 12.7	1.3
	K_{eq}	2.4	2.1 - 2.6	1.5
	SNR green	1.5	1.4 - 1.5	0.3
	SNR red	1.7	1.6 - 1.7	0.3
	ΔG(kcal/mol)	-0.5	-0.5 - -0.4	0.2
Fits from Cumulative Data[2]	Lifetime (s)	5.1	4.2 - 6.3	5.1
	$k_{f,\ bulk}(s^{-1})$	25.1	26.0 - 24.2	23.0
	$k_{u,\ bulk}(s^{-1})$	10.0	10.3 - 9.6	9.6
	$K_{eq,bulk}$	4.1	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.8	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

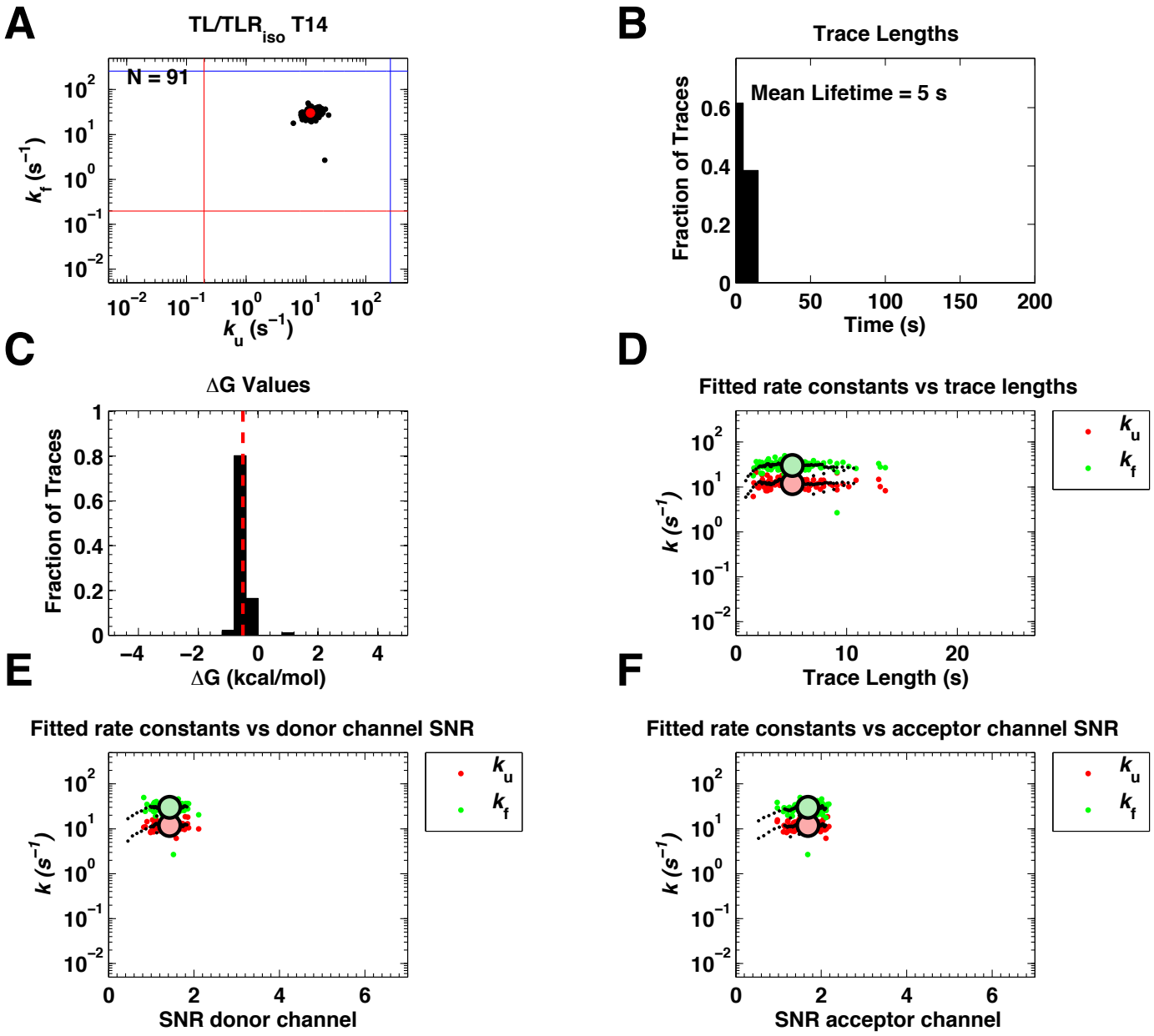


Figure S45-1. smFRET data assessment for TL/TLR$_{iso}$ T14. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

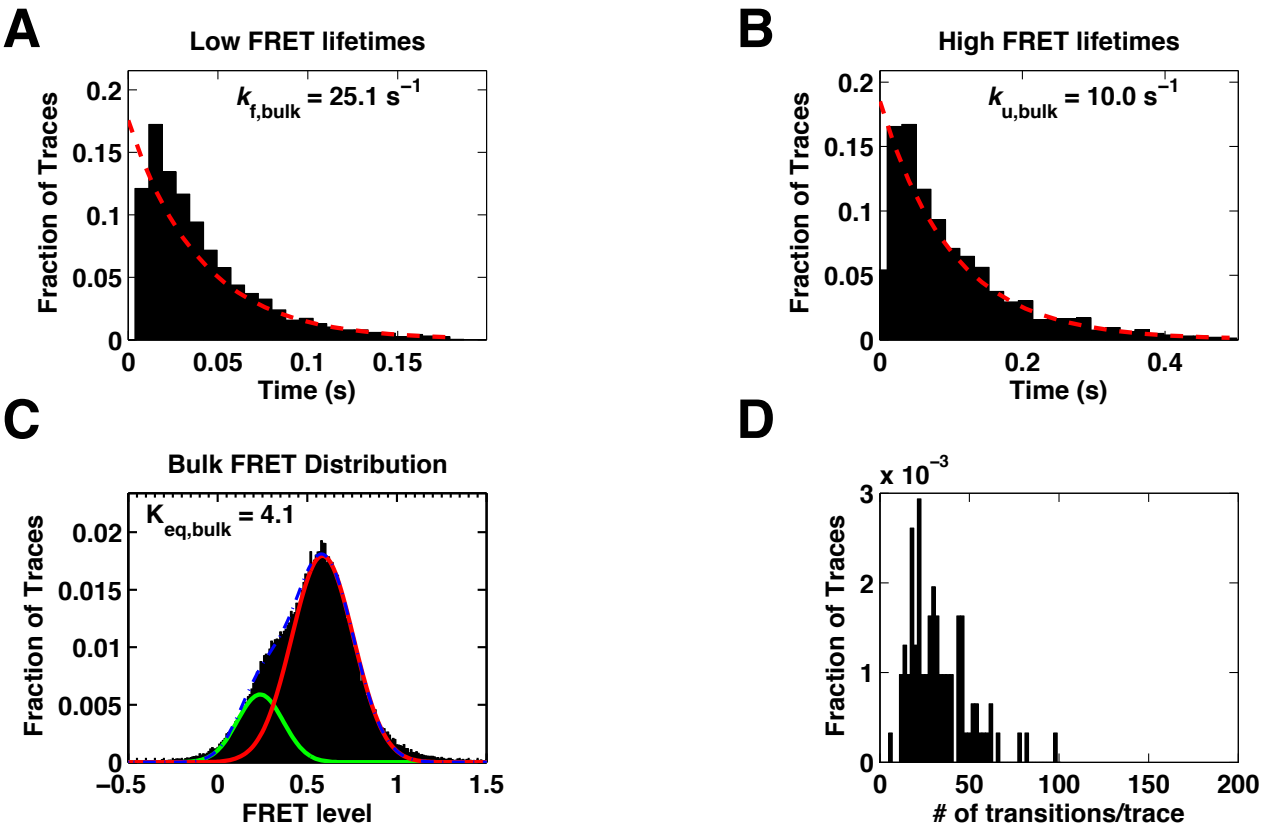


Figure S45-2. smFRET data assesment of aggregate data for TL/TLR$_{iso}$ T14. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

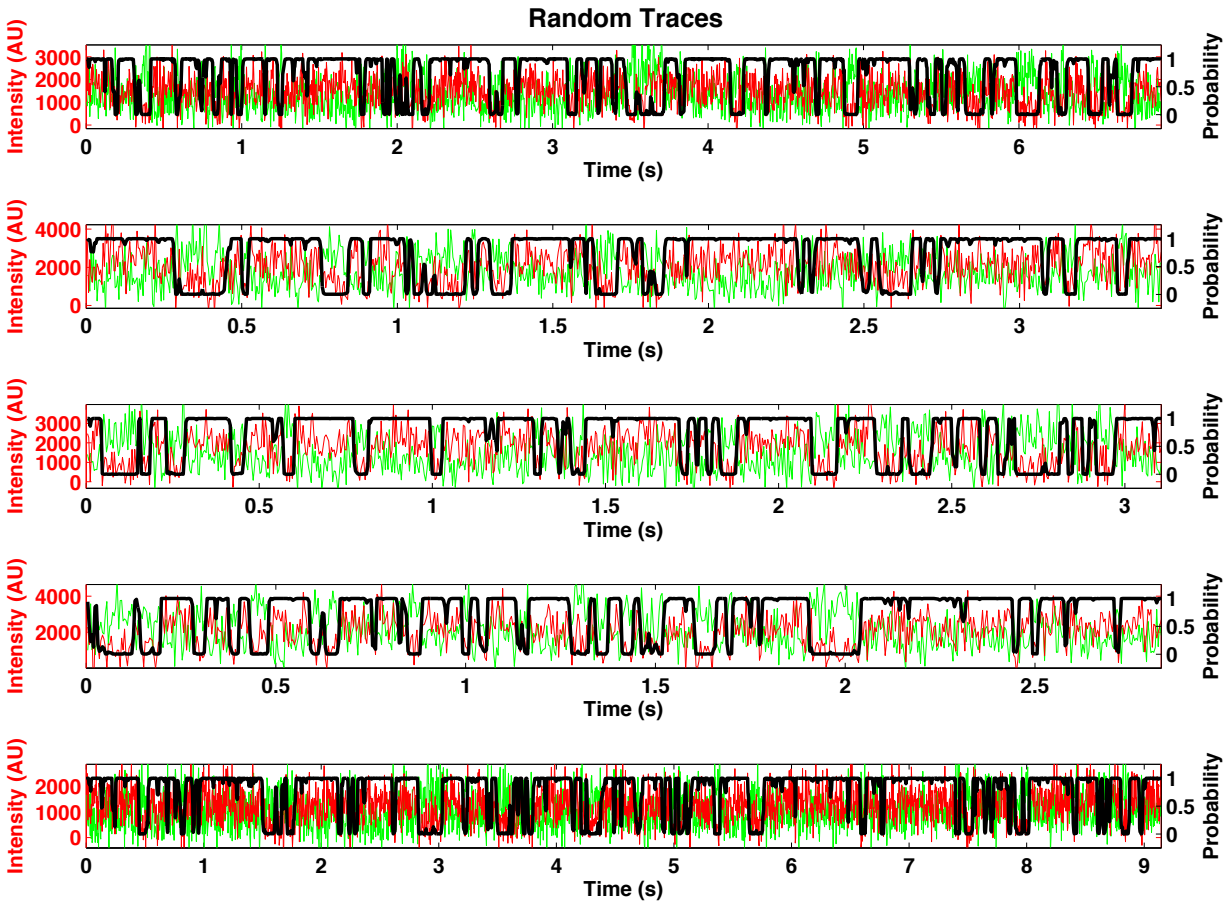


Figure S45-3. Randomly selected FRET traces of TL/TLR$_{iso}$ T14. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S46-1.Variant and Conditions

Variant:	MC/MCR$_{iso}$
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	288
SNR Threshold[2]	0.50
Number of Traces	60

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S46-2.Folding parameters of smFRET the variant MC/MCR$_{iso}$ inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	11.2	10.0 - 12.7	1.6
	$k_u(s^{-1})$	45.2	39.5 - 50.4	1.6
	K_{eq}	0.2	0.2 - 0.3	2.0
	SNR green	1.9	1.9 - 2.1	0.5
	SNR red	0.7	0.7 - 0.8	0.2
	ΔG(kcal/mol)	0.9	0.7 - 0.9	0.4
Fits from Cumulative Data[2]	Lifetime (s)	11.5	9.1 - 15.1	11.5
	$k_{f,\ bulk}(s^{-1})$	10.5	10.8 - 10.2	7.9
	$k_{u,\ bulk}(s^{-1})$	38.3	39.4 - 37.1	32.3
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.8	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

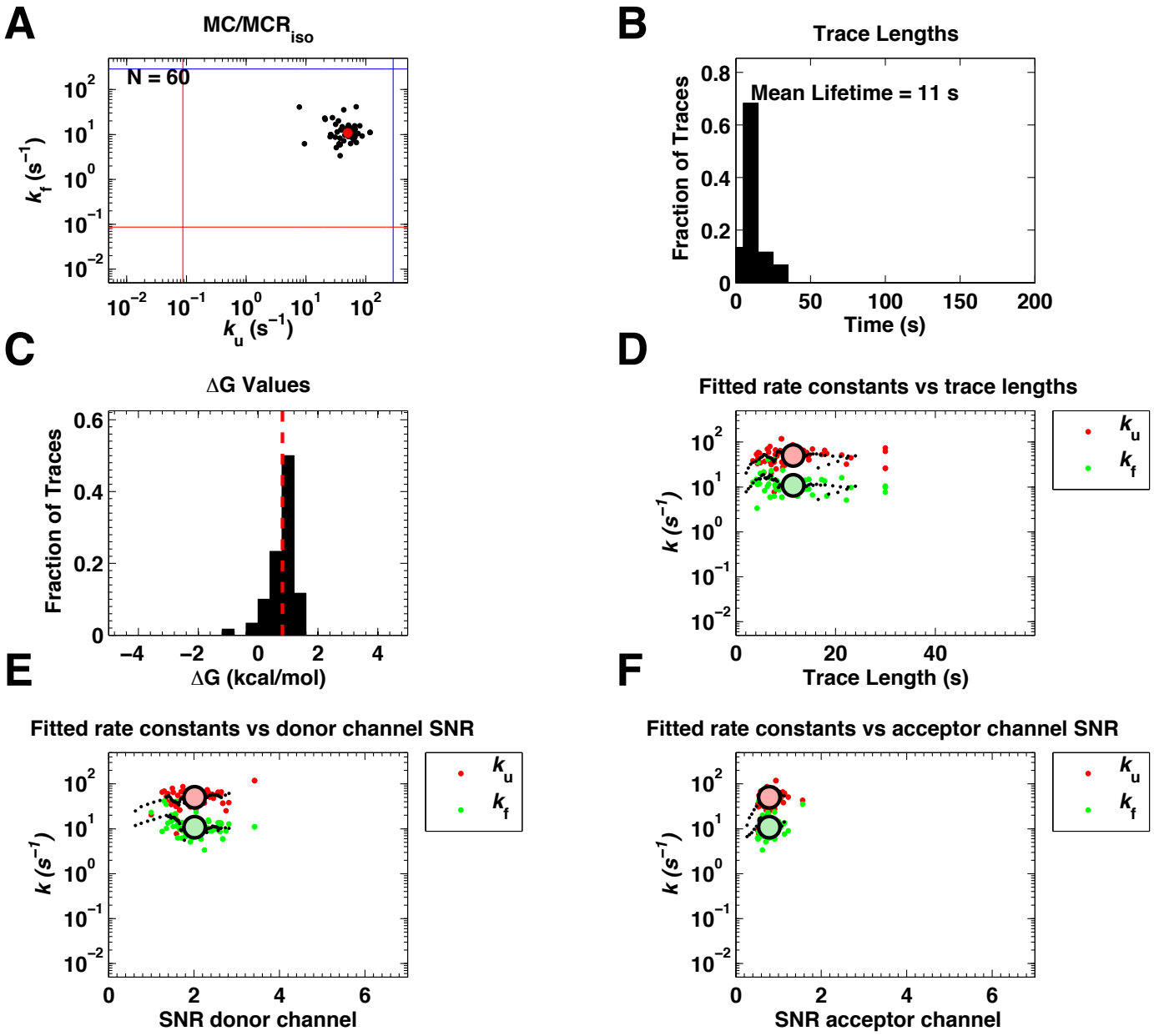


Figure S46-1. smFRET data assessment for MC/MCR$_{iso}$. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

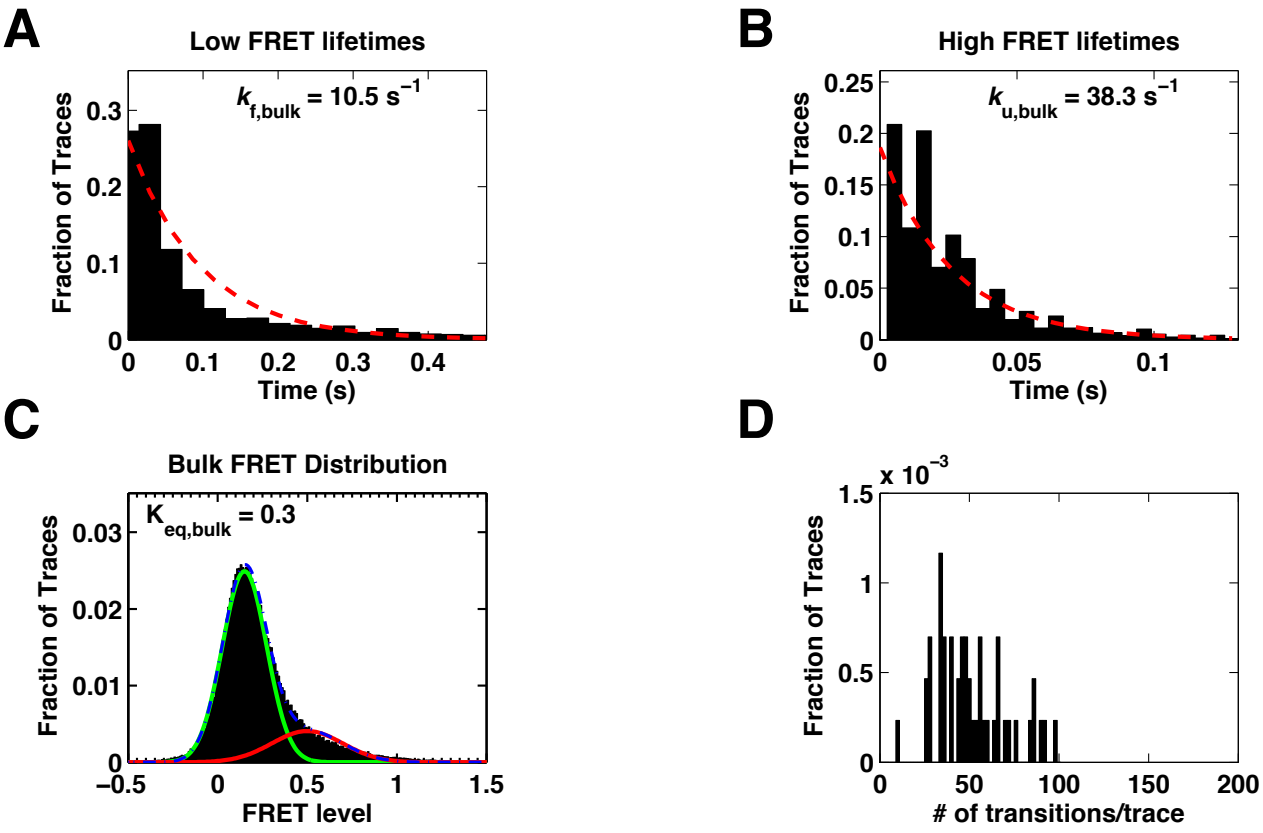


Figure S46-2. smFRET data assesment of aggregate data for MC/MCR$_{iso}$. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

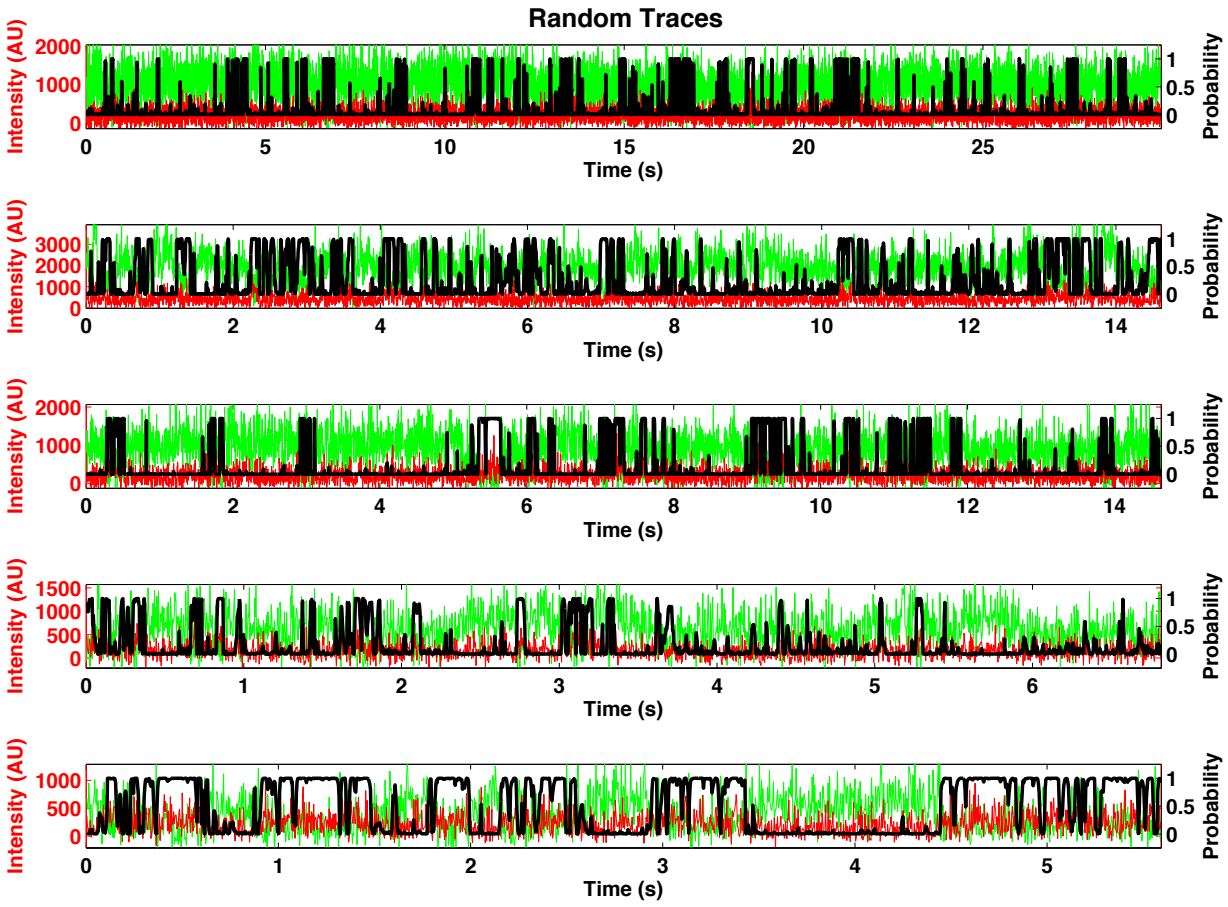


Figure S46-3. Randomly selected FRET traces of MC/MCR$_{iso}$. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S47-1.Variant and Conditions

Variant:	MC/MCR$_{iso}$
$MgCl_2$ (mM)	10.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	256
SNR Threshold[2]	0.50
Number of Traces	55

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S47-2.Folding parameters of smFRET the variant MC/MCR$_{iso}$ inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	9.9	9.0 - 11.6	1.6
	$k_u(s^{-1})$	37.4	33.3 - 42.6	1.6
	K_{eq}	0.3	0.2 - 0.3	1.8
	SNR green	2.4	2.1 - 2.4	0.5
	SNR red	1.0	1.0 - 1.2	0.3
	ΔG(kcal/mol)	0.7	0.7 - 0.9	0.3
Fits from Cumulative Data[2]	Lifetime (s)	8.8	6.8 - 11.6	8.8
	$k_{f,\ bulk}(s^{-1})$	12.9	13.4 - 12.5	8.5
	$k_{u,\ bulk}(s^{-1})$	33.7	34.9 - 32.5	30.7
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.6	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

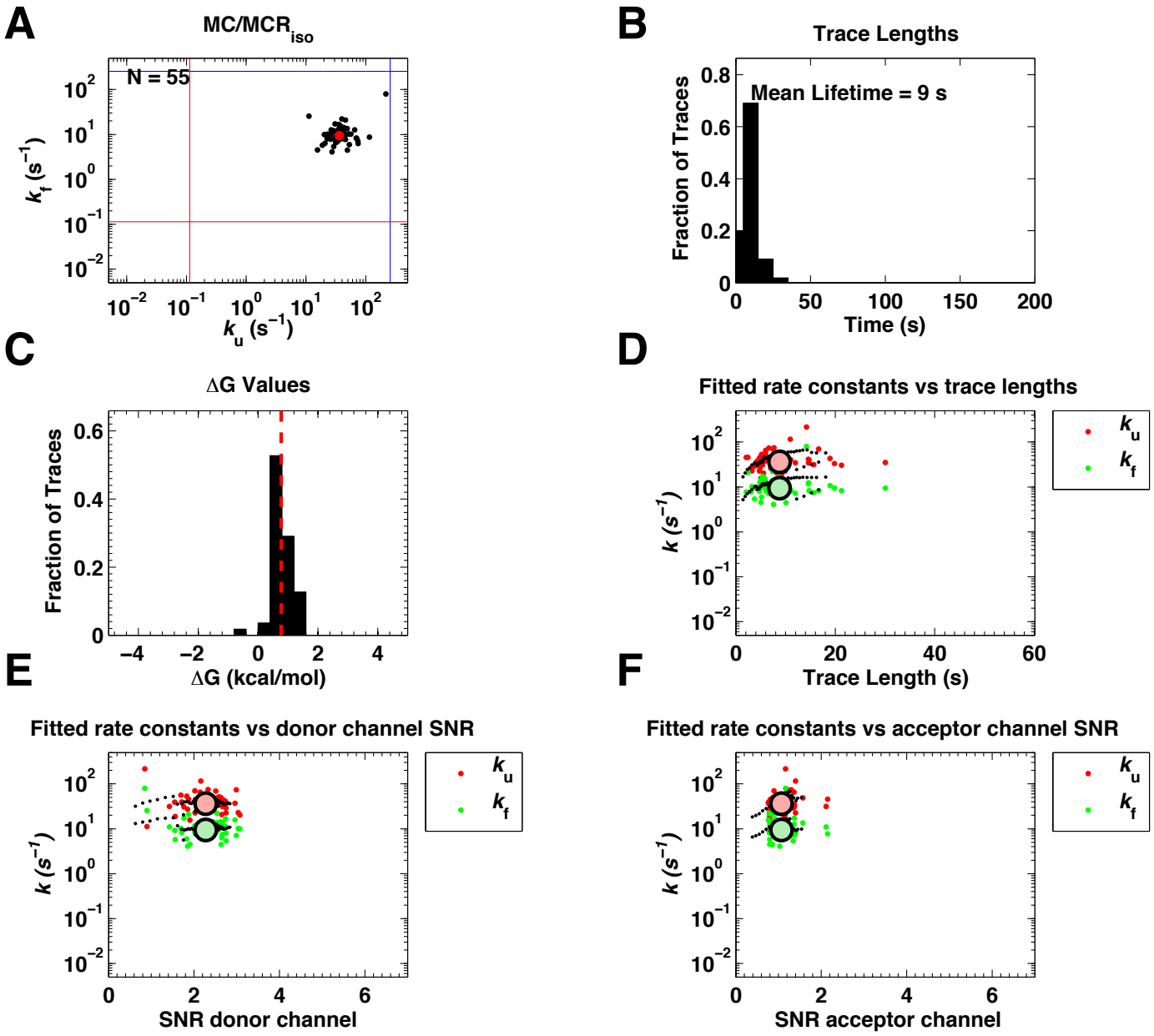


Figure S47-1. smFRET data assessment for MC/MCR$_{iso}$. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

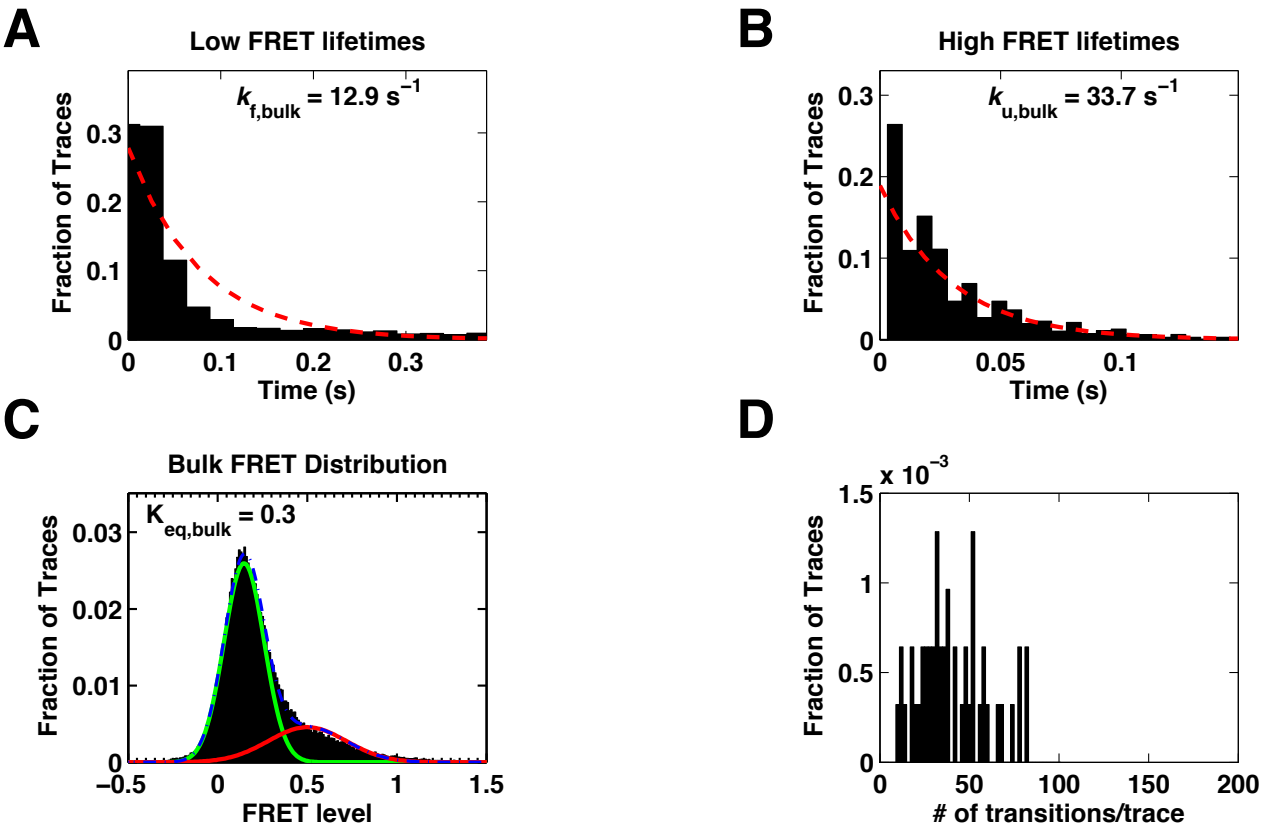


Figure S47-2. smFRET data assesment of aggregate data for MC/MCR$_{iso}$. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

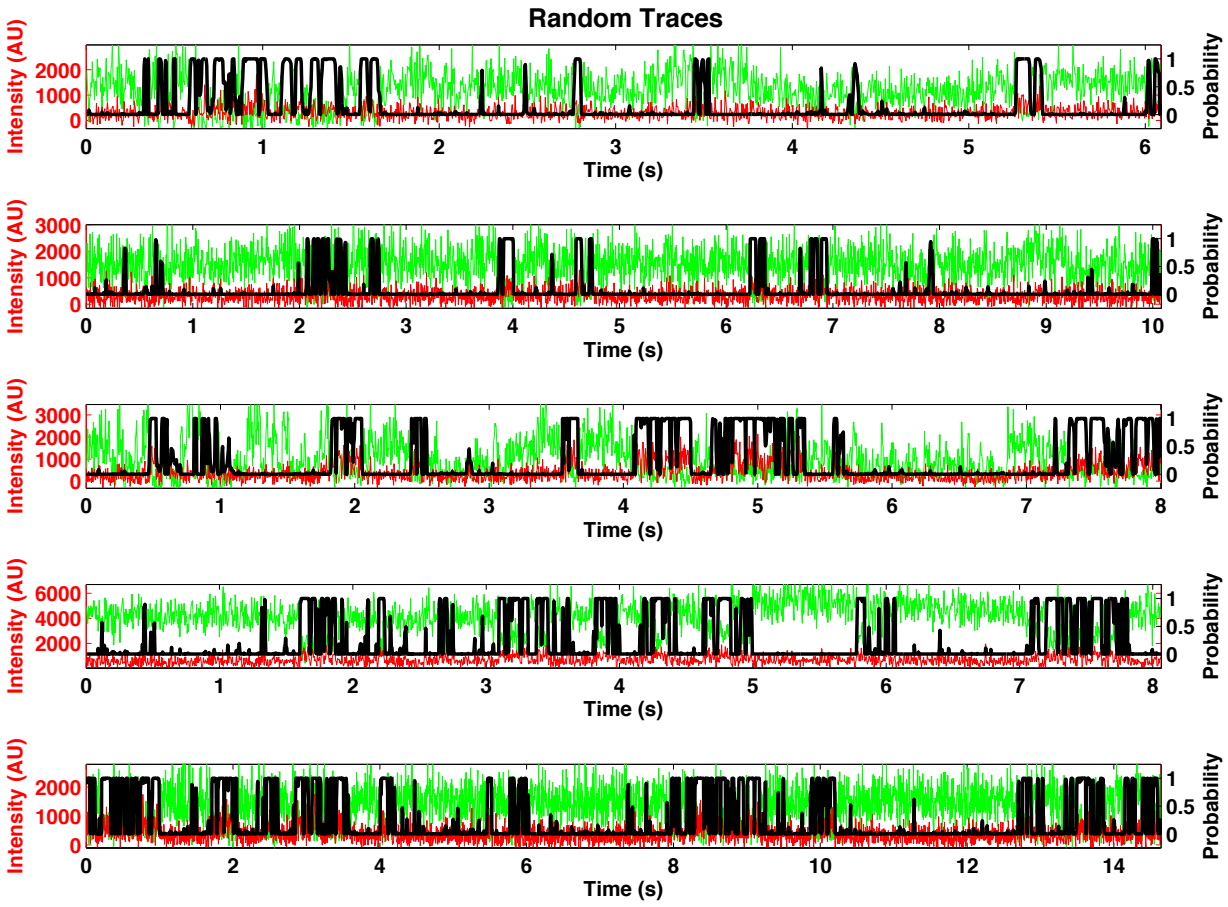


Figure S47-3. Randomly selected FRET traces of MC/MCR$_{iso}$. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S48-1.Variant and Conditions

Variant:	MC/MCR$_{iso}$
$MgCl_2$ (mM)	30.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	111

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S48-1.Folding parameters of smFRET the variant MC/MCR$_{iso}$ inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	12.9	12.0 - 13.8	1.5
	$k_u(s^{-1})$	20.2	19.1 - 21.5	1.4
	K_{eq}	0.6	0.6 - 0.7	1.6
	SNR green	2.9	2.8 - 3.0	0.7
	SNR red	1.4	1.4 - 1.6	0.4
	ΔG(kcal/mol)	0.2	0.2 - 0.3	0.3
Fits from Cumulative Data[2]	Lifetime (s)	9.0	7.5 - 10.9	9.0
	$k_{f,\ bulk}(s^{-1})$	14.1	14.4 - 13.7	11.0
	$k_{u,\ bulk}(s^{-1})$	17.1	17.6 - 16.7	16.1
	$K_{eq,bulk}$	1.1	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.1	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

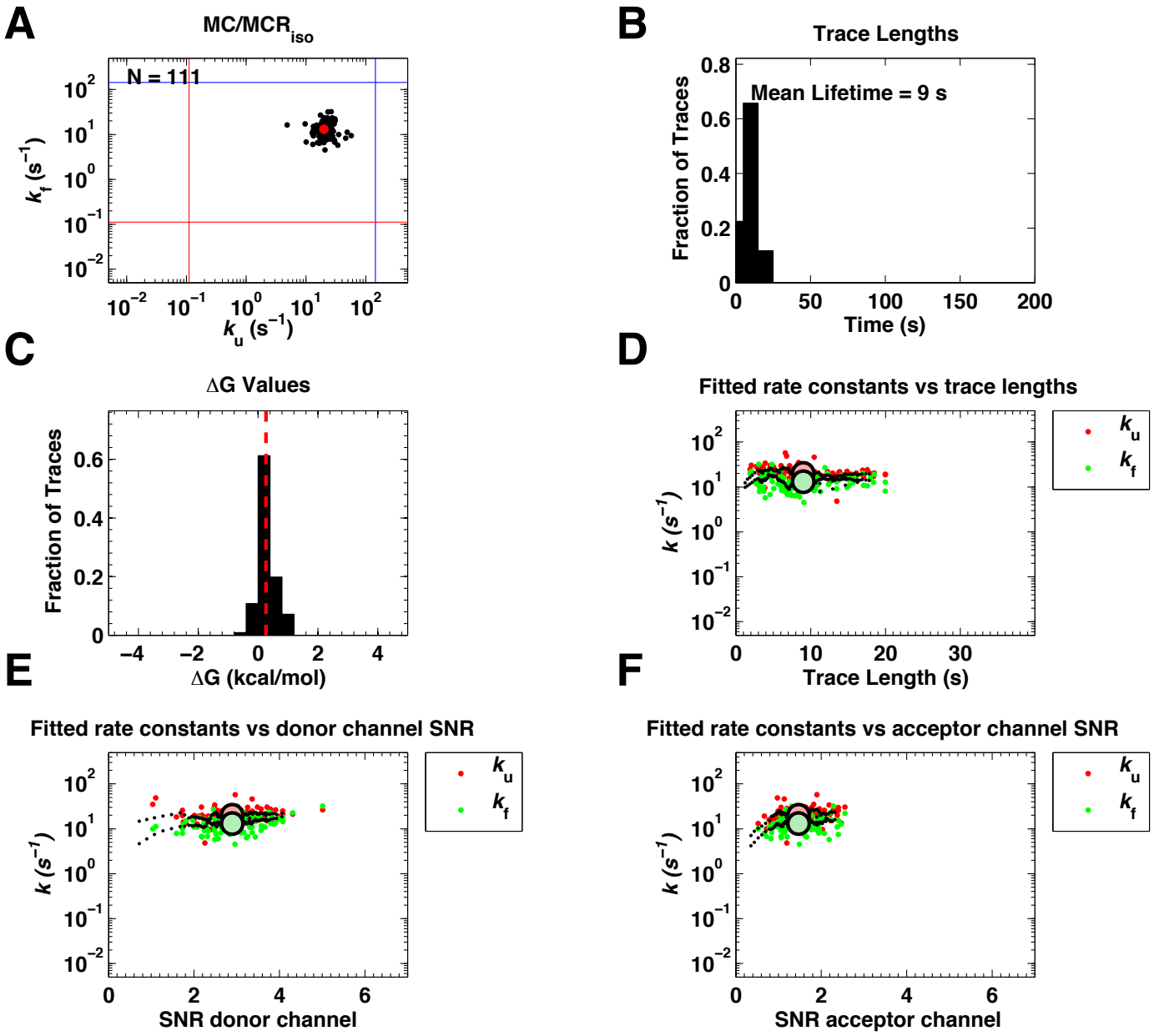


Figure S48-1. smFRET data assessment for MC/MCR$_{iso}$. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

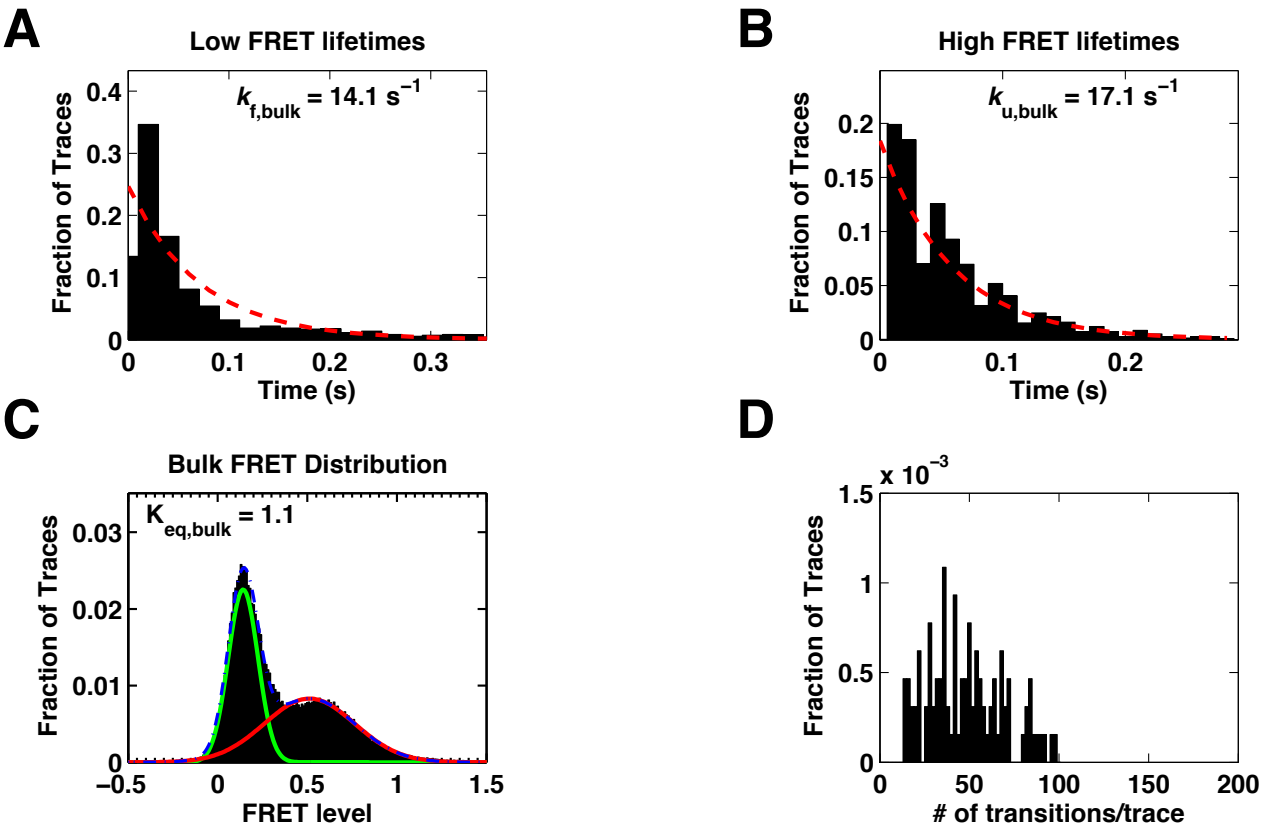


Figure S48-2. smFRET data assesment of aggregate data for MC/MCR$_{iso}$. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

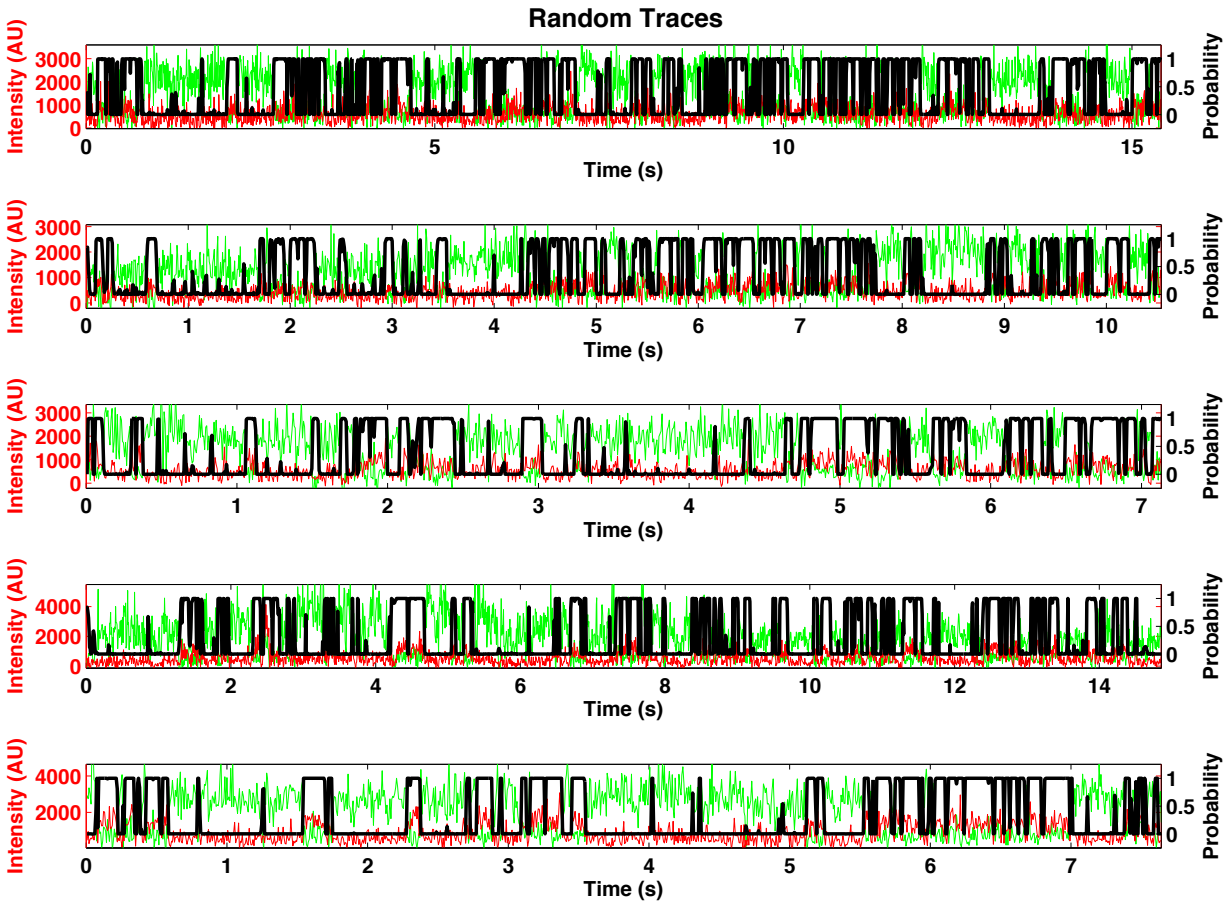


Figure S48-3. Randomly selected FRET traces of MC/MCR$_{iso}$. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S49-1. Variant and Conditions

Variant:	MC/MCR$_{iso}$
$MgCl_2$ (mM)	40.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.50
Number of Traces	182

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S49-2. Folding parameters of smFRET the variant MC/MCR$_{iso}$ inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	15.0	13.7 - 16.0	1.6
	$k_u(s^{-1})$	19.5	17.8 - 20.7	1.6
	K_{eq}	0.8	0.7 - 0.8	1.7
	SNR green	2.7	2.6 - 2.9	0.8
	SNR red	1.4	1.4 - 1.5	0.4
	ΔG(kcal/mol)	0.1	0.1 - 0.2	0.3
Fits from Cumulative Data[2]	Lifetime (s)	7.5	6.5 - 8.7	7.5
	$k_{f,\ bulk}(s^{-1})$	16.0	16.4 - 15.7	12.7
	$k_{u,\ bulk}(s^{-1})$	17.4	17.8 - 17.1	16.6
	$K_{eq,bulk}$	1.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.1	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

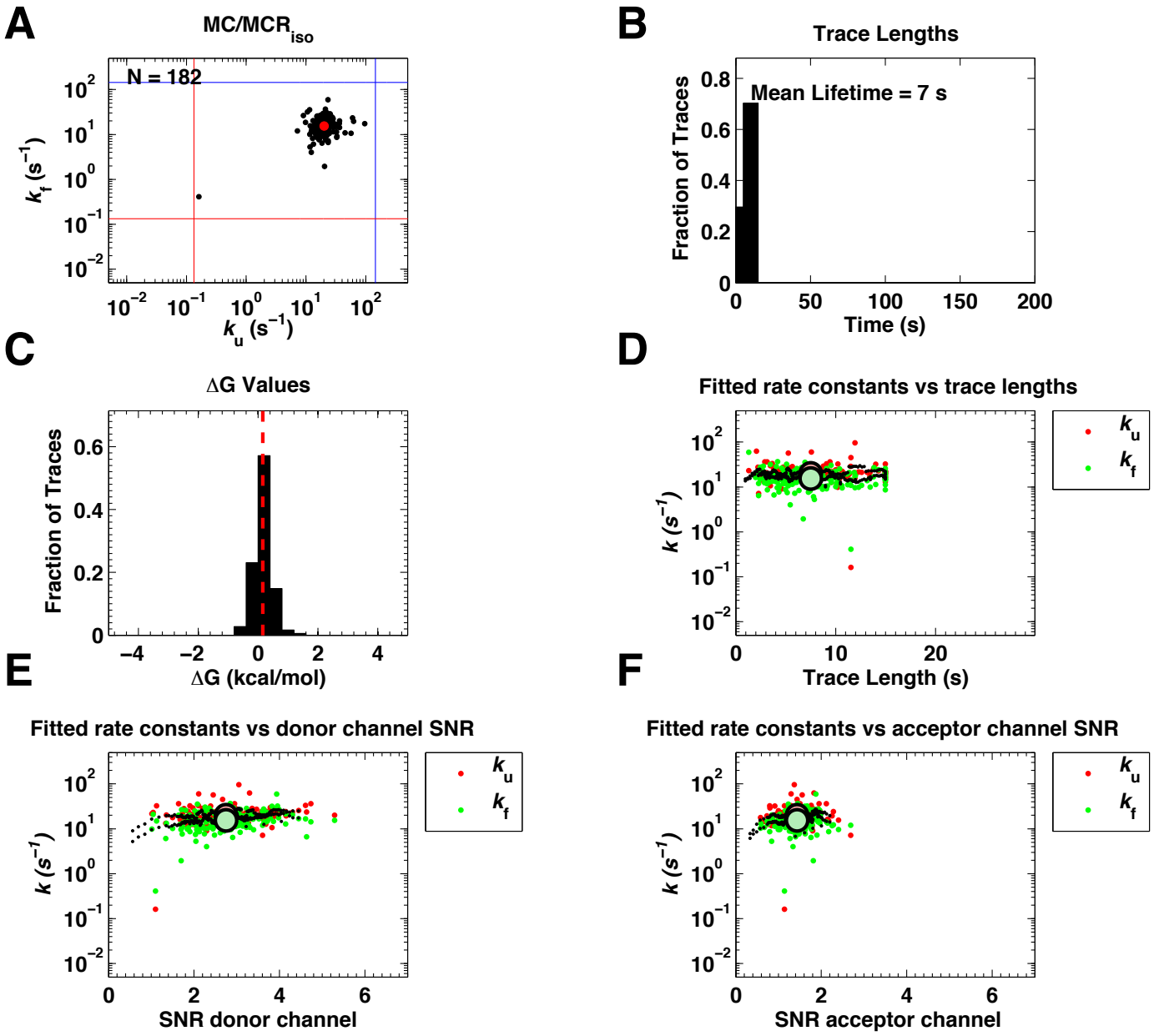


Figure S49-1. smFRET data assessment for MC/MCR$_{iso}$. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

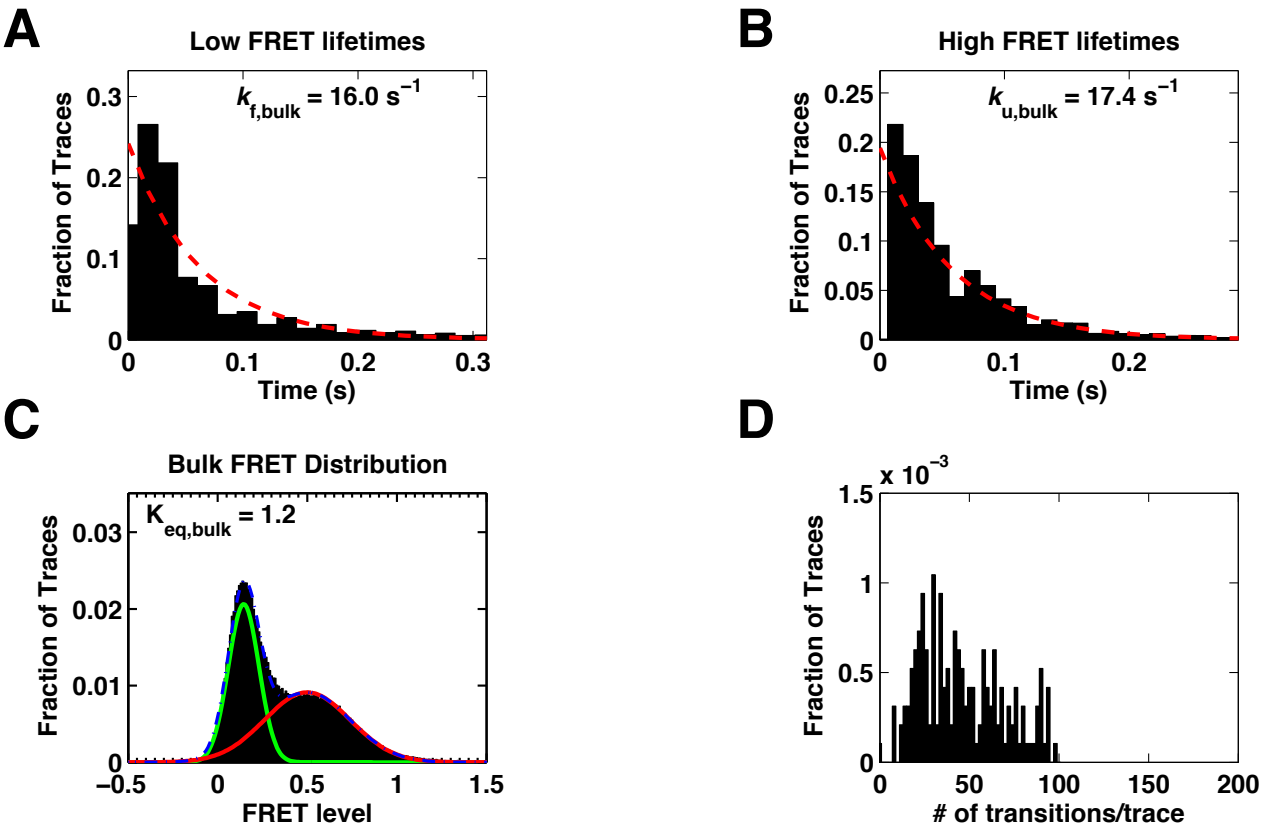


Figure S49-2. smFRET data assesment of aggregate data for MC/MCR$_{iso}$. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

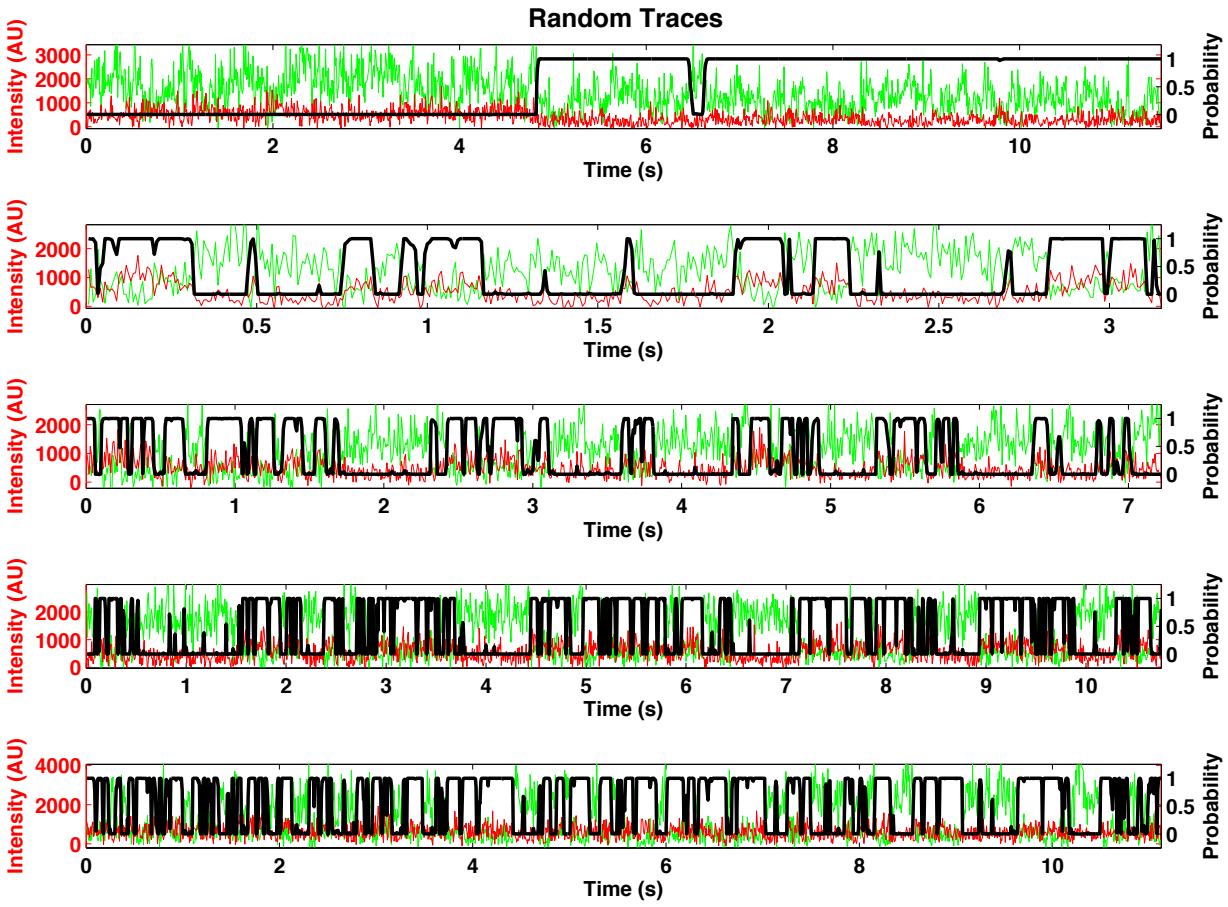


Figure S49-3. Randomly selected FRET traces of MC/MCR$_{iso}$. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S50-1.Variant and Conditions

Variant:	MC/MCR$_{iso}$
$MgCl_2$ (mM)	50.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	257
SNR Threshold[2]	0.50
Number of Traces	96

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S50-2. Folding parameters of smFRET the variant MC/MCR$_{iso}$ inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	18.4	17.1 - 19.7	1.4
	$k_u(s^{-1})$	22.3	20.9 - 24.2	1.4
	K_{eq}	0.8	0.7 - 0.9	1.6
	SNR green	2.4	2.4 - 2.6	0.7
	SNR red	1.2	1.2 - 1.3	0.4
	ΔG(kcal/mol)	0.1	0.1 - 0.2	0.3
Fits from Cumulative Data[2]	Lifetime (s)	7.6	6.3 - 9.4	7.6
	$k_{f,\ bulk}(s^{-1})$	18.9	19.4 - 18.5	15.2
	$k_{u,\ bulk}(s^{-1})$	19.3	19.8 - 18.8	18.3
	$K_{eq,bulk}$	1.6	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.3	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

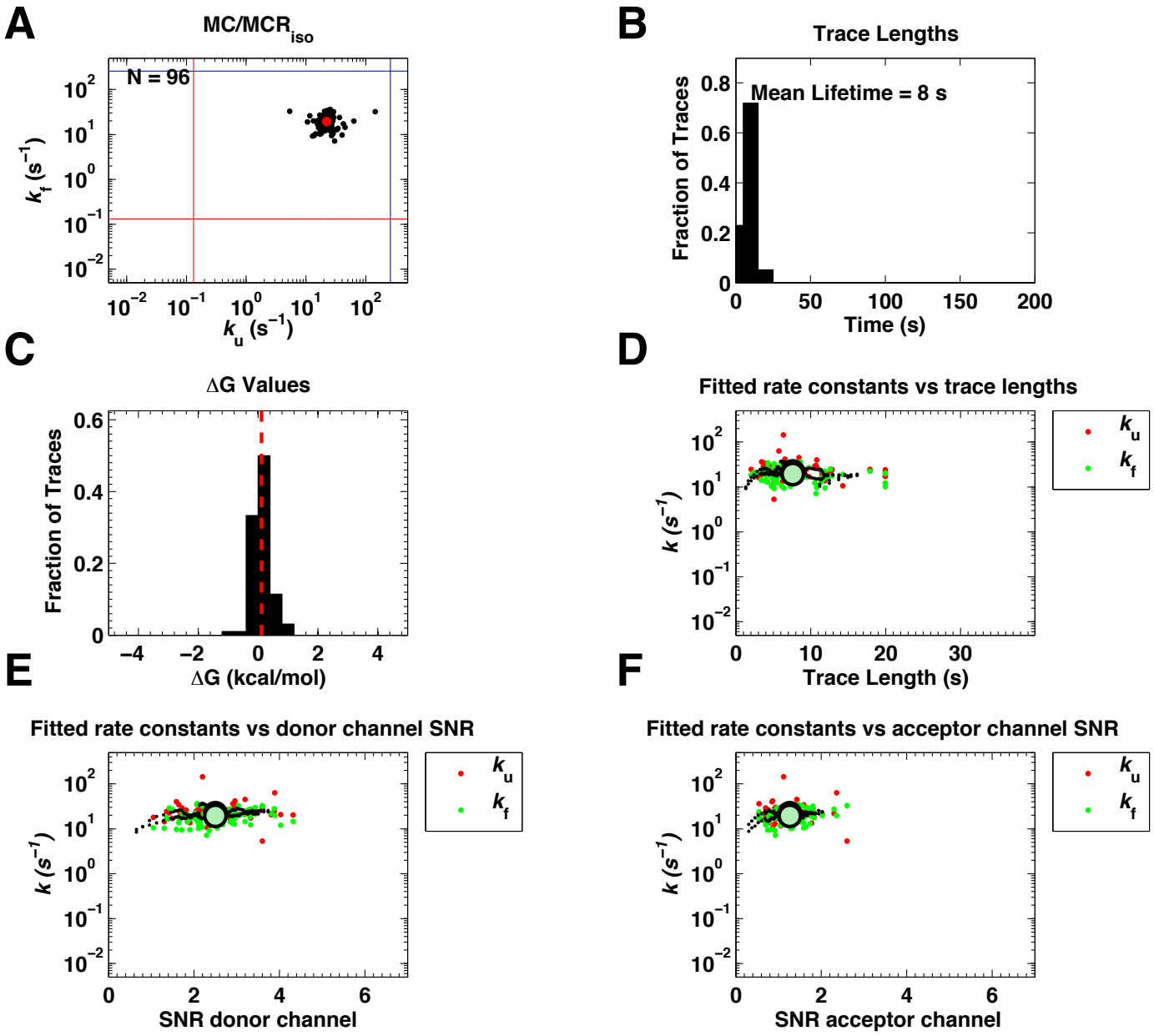


Figure S50-1. smFRET data assessment for MC/MCR$_{iso}$. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

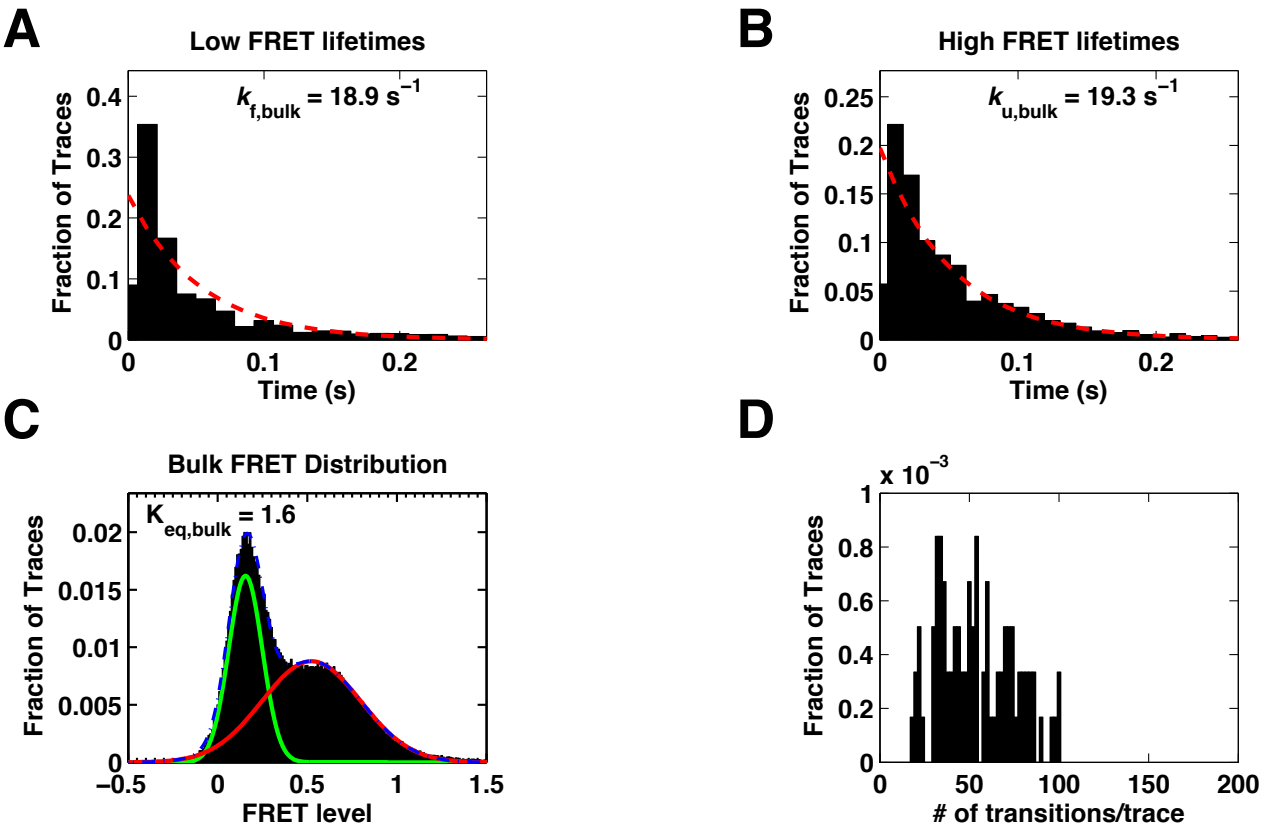


Figure S50-2. smFRET data assesment of aggregate data for MC/MCR$_{iso}$. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

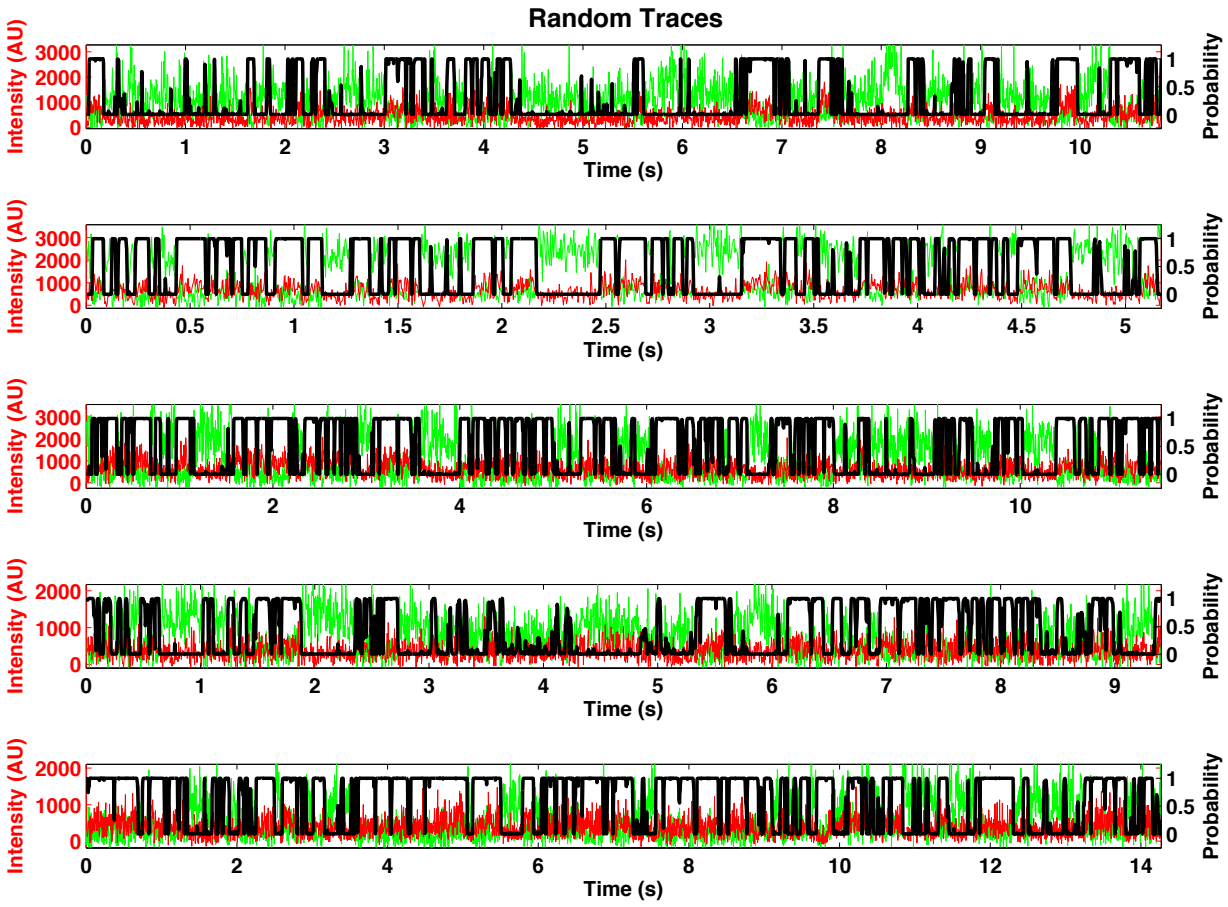


Figure S50-3. Randomly selected FRET traces of MC/MCR$_{iso}$. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S51-1.Variant and Conditions

Variant:	L5B
$MgCl_2$ (mM)	10.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.25
Number of Traces	121

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S51-2. Folding parameters of smFRET the variant L5B inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	11.6	10.1 - 13.7	2.4
	$k_u(s^{-1})$	39.8	32.7 - 46.0	2.5
	K_{eq}	0.3	0.3 - 0.3	2.4
	SNR green	0.6	0.6 - 0.6	0.2
	SNR red	1.6	1.5 - 1.6	0.4
	ΔG(kcal/mol)	0.8	0.6 - 0.8	0.5
Fits from Cumulative Data[2]	Lifetime (s)	14.1	11.9 - 17.0	14.1
	$k_{f,\ bulk}(s^{-1})$	10.0	10.2 - 9.8	8.1
	$k_{u,\ bulk}(s^{-1})$	35.6	36.3 - 34.9	26.7
	$K_{eq,bulk}$	0.4	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.5	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

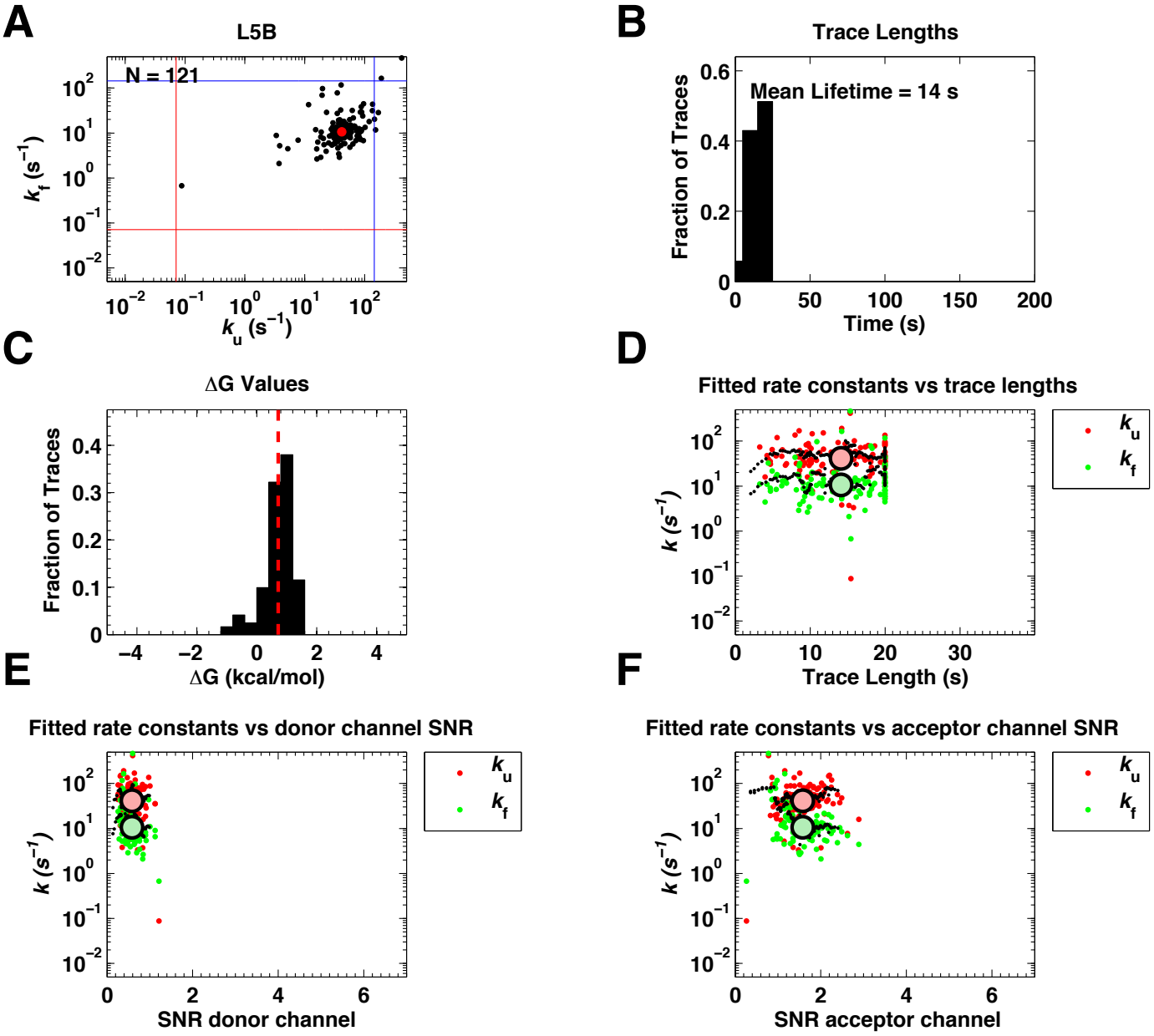


Figure S51-1. smFRET data assessment for L5B. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

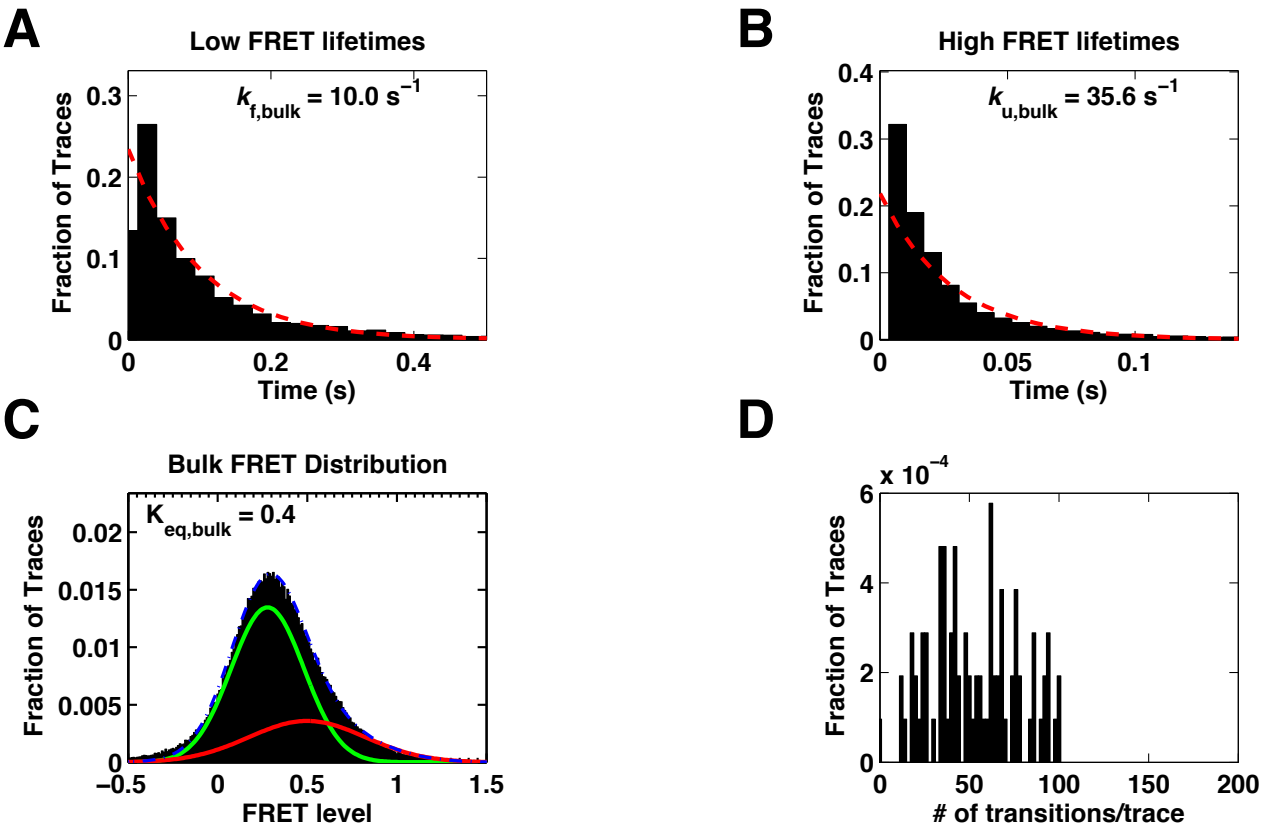


Figure S51-2. smFRET data assesment of aggregate data for L5B. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

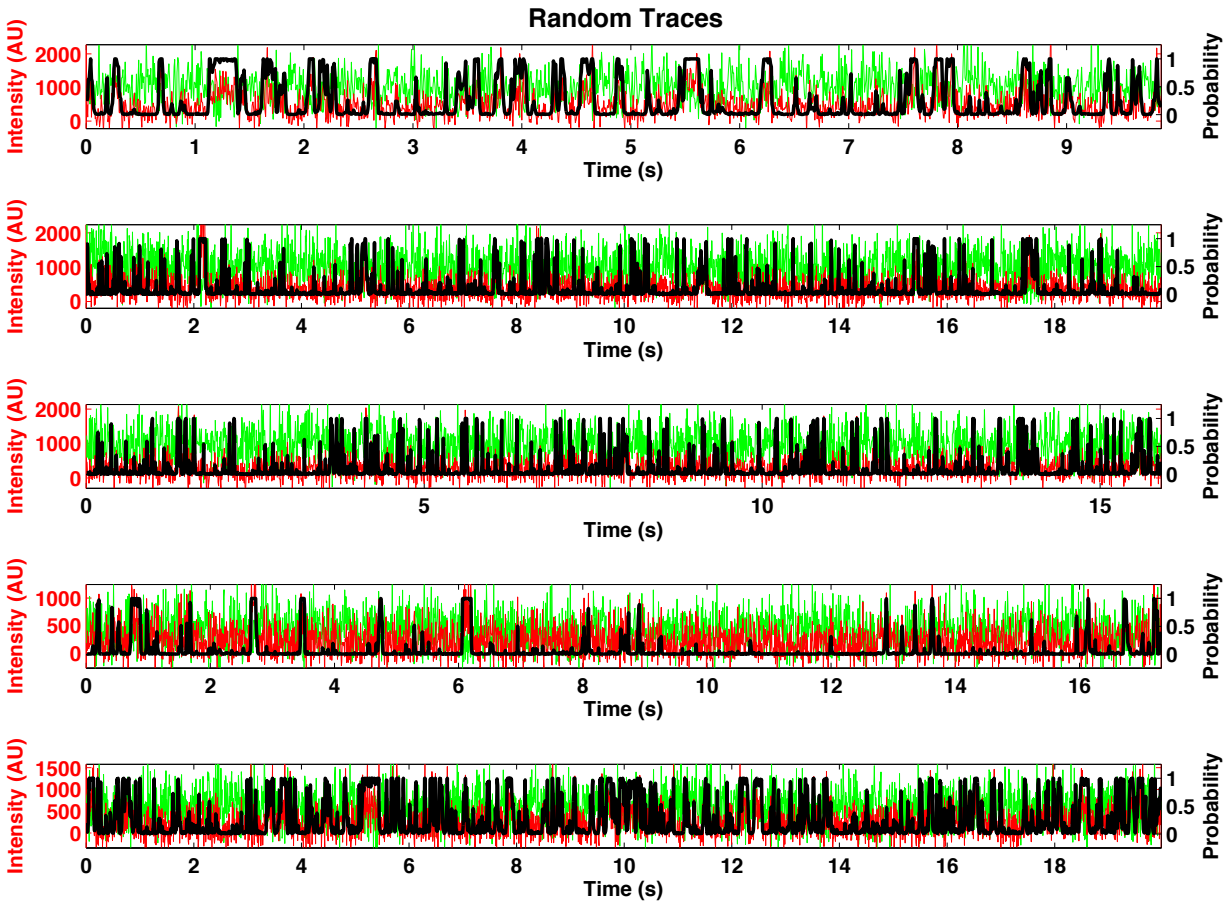


Figure S51-3. Randomly selected FRET traces of L5B. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S52-1.Variant and Conditions

Variant:	L5B
$MgCl_2$ (mM)	30.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.25
Number of Traces	213

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S52-2. Folding parameters of smFRET the variant L5B inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	13.4	12.5 - 14.3	1.7
	$k_u(s^{-1})$	24.1	22.3 - 26.1	1.8
	K_{eq}	0.6	0.5 - 0.6	1.9
	SNR green	0.7	0.7 - 0.8	0.2
	SNR red	2.1	2.1 - 2.3	0.5
	ΔG(kcal/mol)	0.3	0.3 - 0.4	0.4
Fits from Cumulative Data[2]	Lifetime (s)	12.7	11.2 - 14.6	12.7
	$k_{f,\ bulk}(s^{-1})$	11.5	11.7 - 11.3	9.7
	$k_{u,\ bulk}(s^{-1})$	24.2	24.6 - 23.8	19.1
	$K_{eq,bulk}$	3.6	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

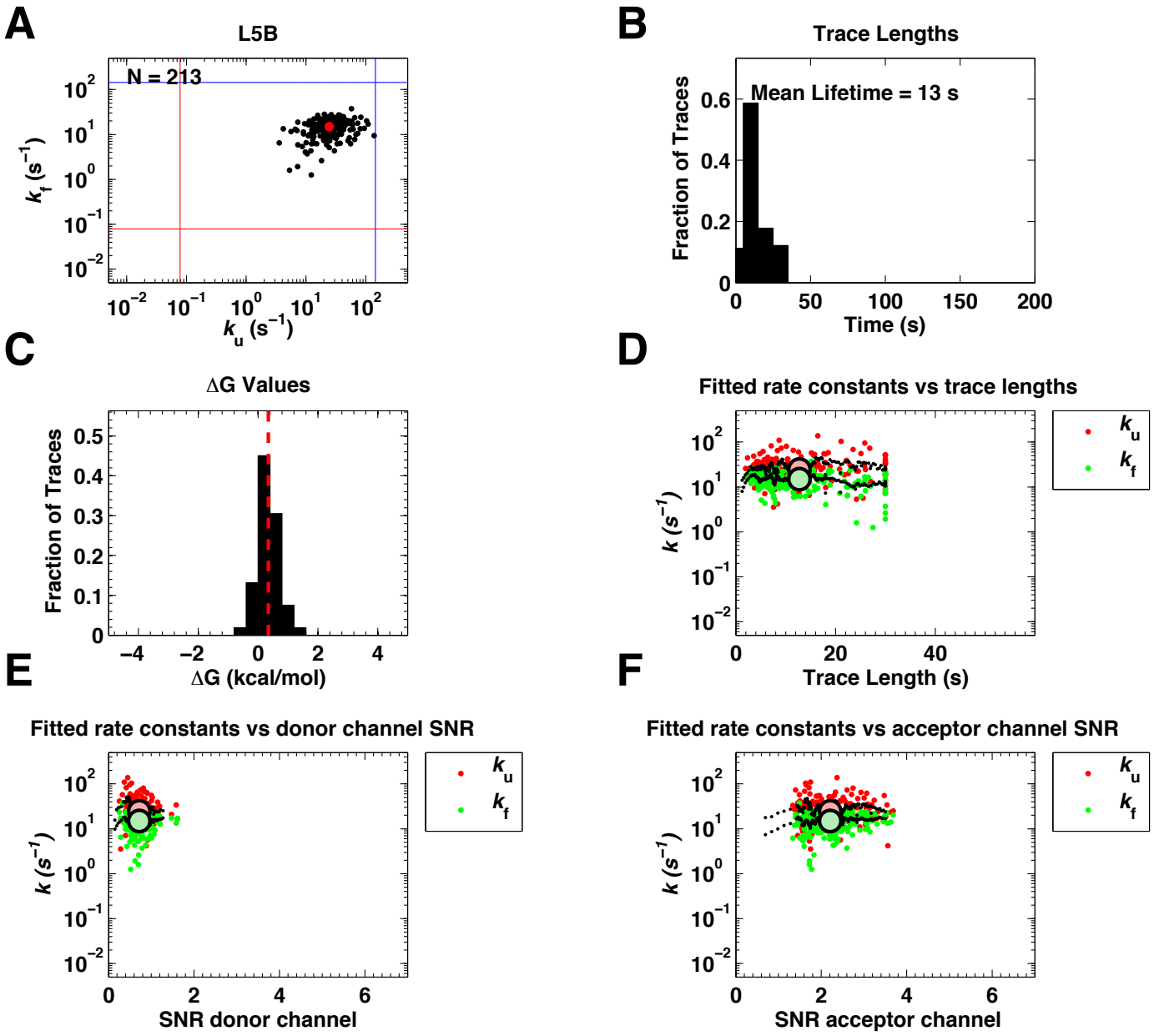


Figure S52-1. smFRET data assessment for L5B. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

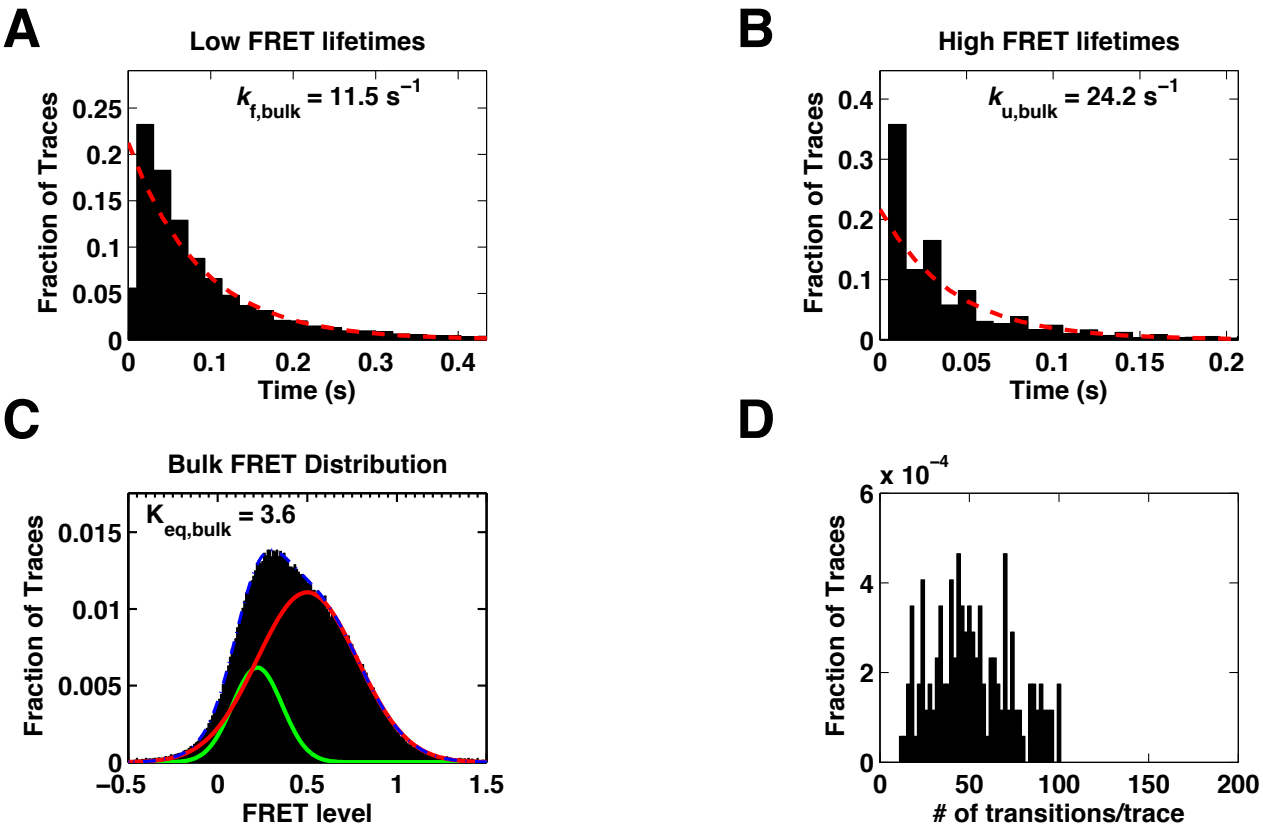


Figure S52-2. smFRET data assesment of aggregate data for L5B. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

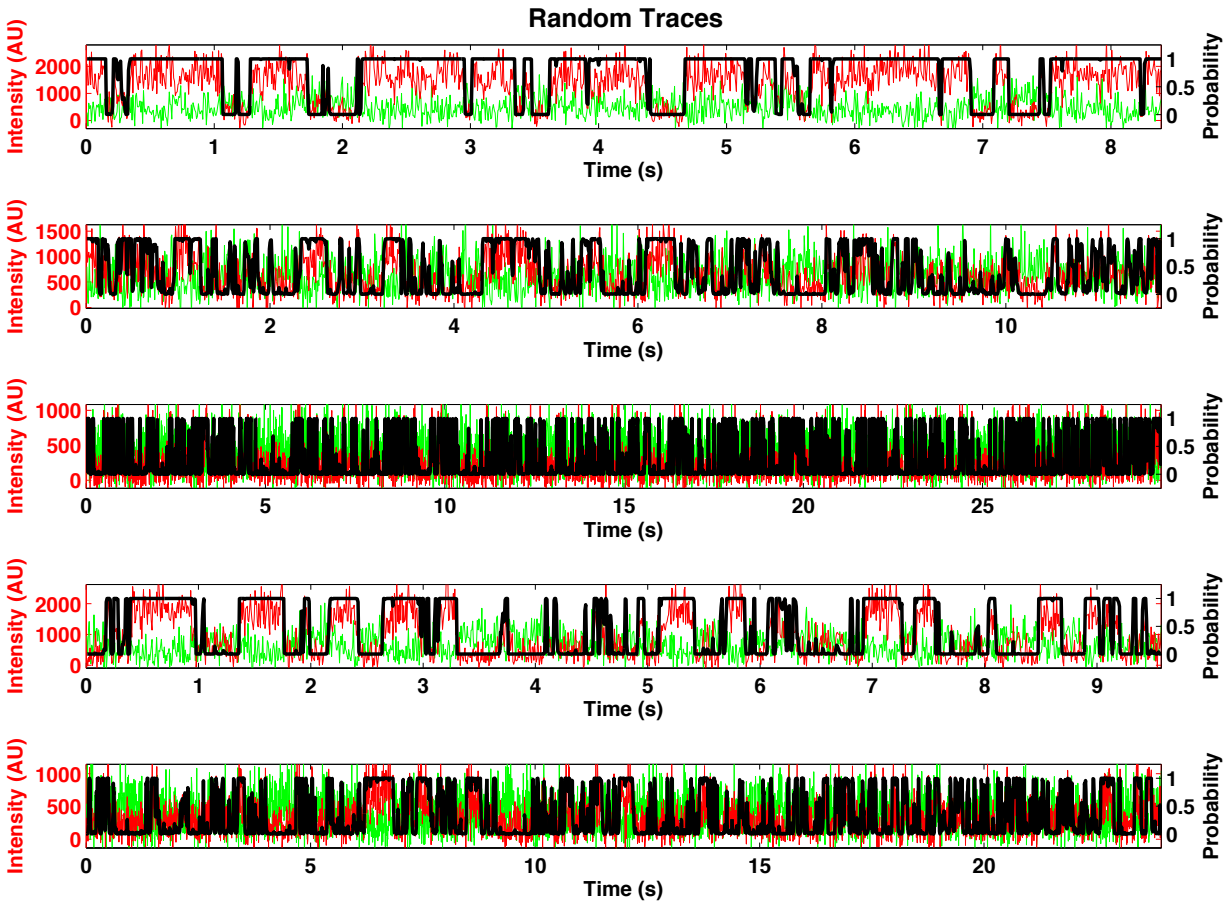


Figure S52-3. Randomly selected FRET traces of L5B. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Tabls S53-1. Variant and Conditions

Variant:	L5B
$MgCl_2$ (mM)	40.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.25
Number of Traces	185

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S53-2. Folding parameters of smFRET the variant L5B inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	13.0	11.9 - 14.0	1.8
	$k_u(s^{-1})$	18.4	16.8 - 20.2	1.9
	K_{eq}	0.7	0.6 - 0.8	1.9
	SNR green	0.6	0.7 - 0.7	0.2
	SNR red	2.1	2.1 - 2.3	0.5
	ΔG(kcal/mol)	0.2	0.2 - 0.3	0.4
Fits from Cumulative Data[2]	Lifetime (s)	11.0	9.6 - 12.8	11.0
	$k_{f,\ bulk}(s^{-1})$	12.8	13.0 - 12.5	10.3
	$k_{u,\ bulk}(s^{-1})$	19.2	19.6 - 18.9	15.9
	$K_{eq,bulk}$	1.1	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.1	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

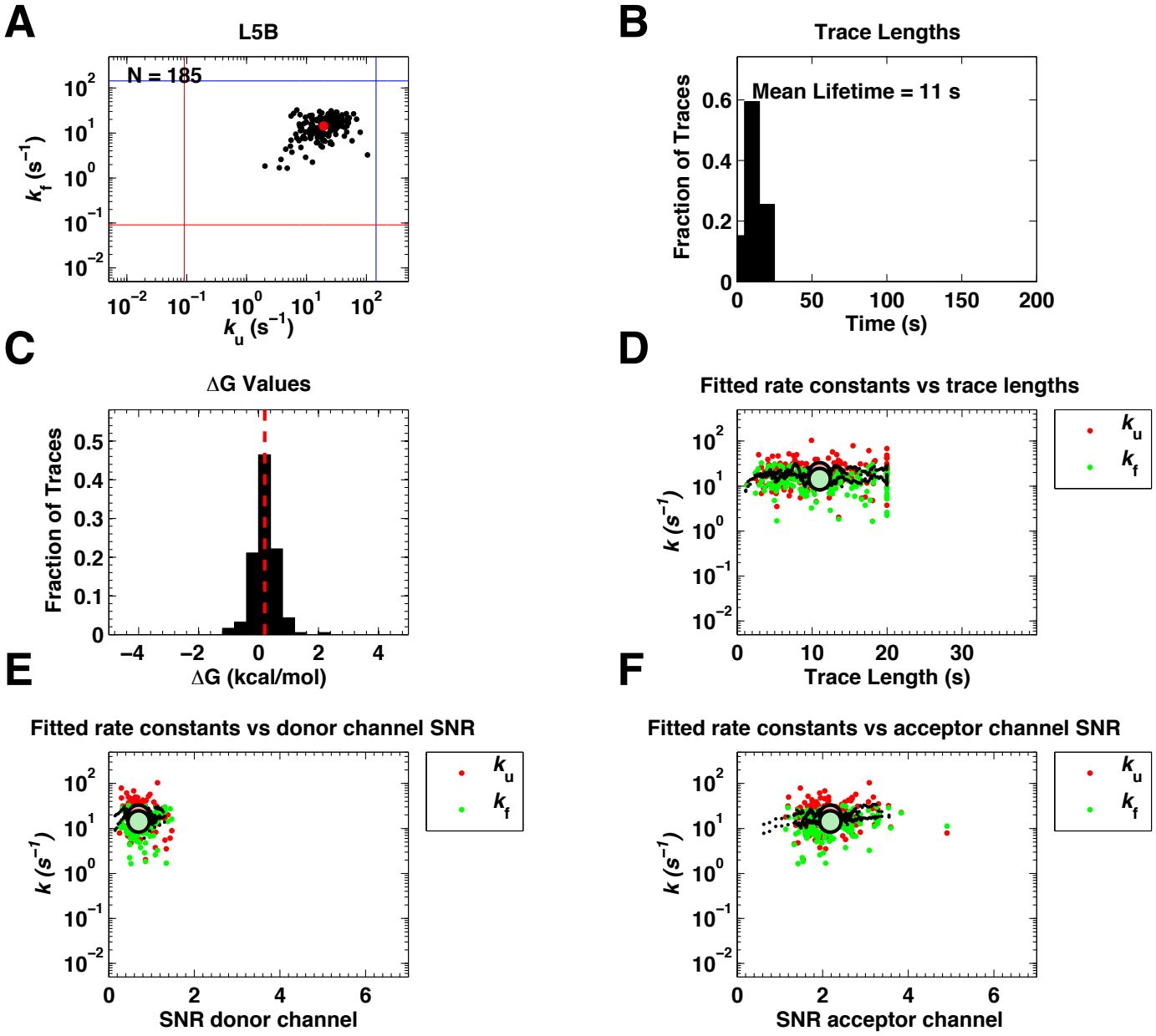


Figure S53-1. smFRET data assessment for L5B. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

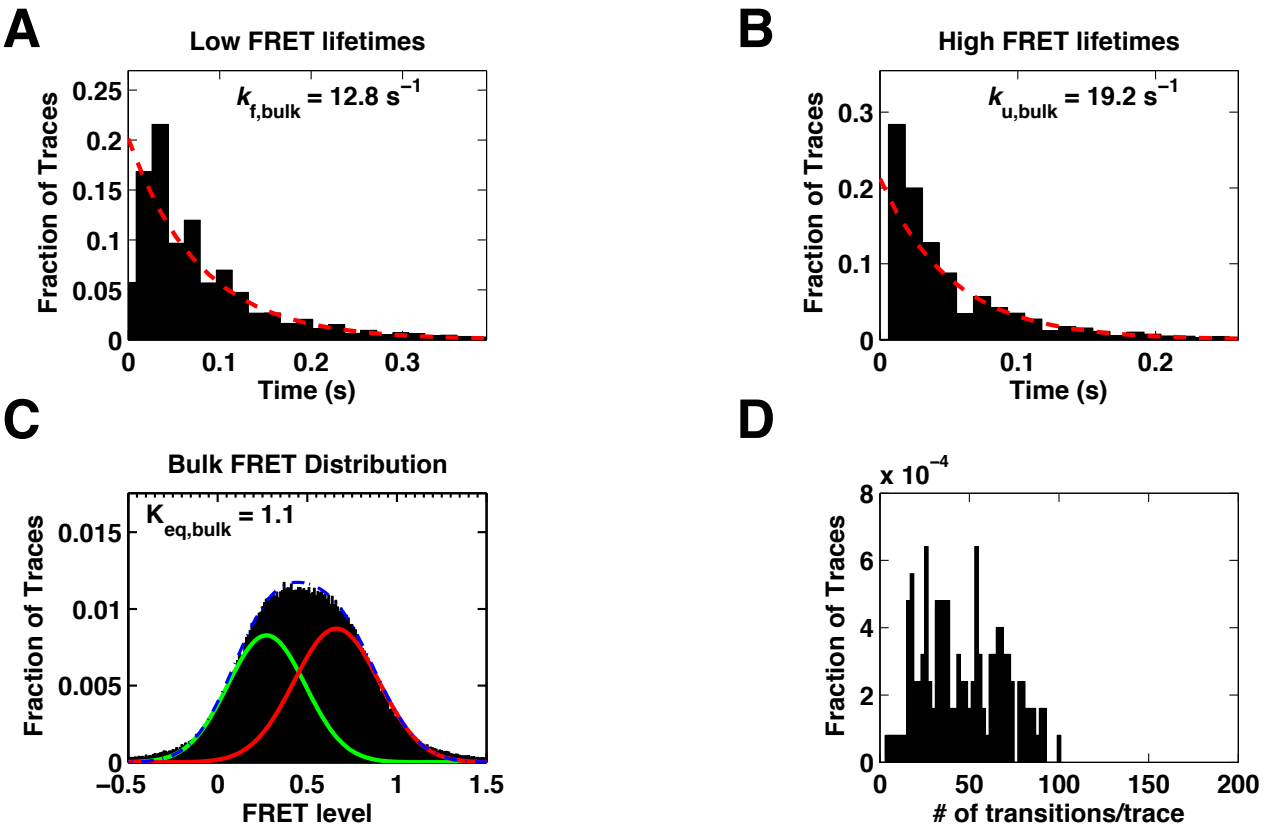


Figure S53-2. smFRET data assesment of aggregate data for L5B. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

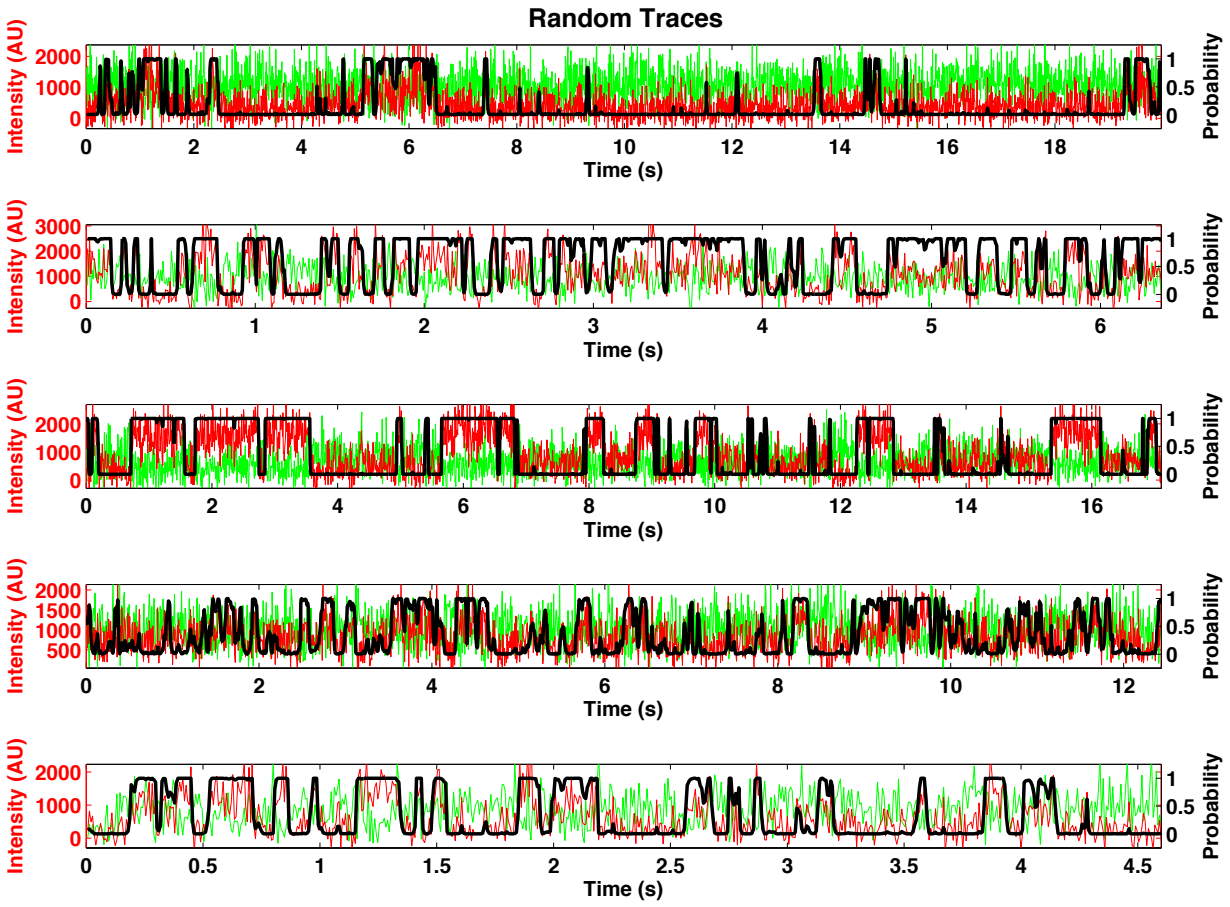


Figure S53-3. Randomly selected FRET traces of L5B. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S54-1. Variant and Conditions

Variant:	WT Transcribed
$MgCl_2$ (mM)	1.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	25
SNR Threshold[2]	0.75
Number of Traces	205

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S54-2. Folding parameters of smFRET the variant WT Transcribed inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.2	0.2 - 0.3	2.1
	$k_u(s^{-1})$	1.7	1.5 - 1.8	2.0
	K_{eq}	0.1	0.1 - 0.2	2.6
	SNR green	3.2	3.1 - 3.4	1.1
	SNR red	2.7	2.7 - 2.9	1.0
	ΔG(kcal/mol)	1.2	1.0 - 1.2	0.5
Fits from Cumulative Data[2]	Lifetime (s)	74.8	65.6 - 86.2	74.8
	$k_{f,\ bulk}(s^{-1})$	0.3	0.3 - 0.3	0.3
	$k_{u,\ bulk}(s^{-1})$	1.6	1.7 - 1.6	1.3
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.7	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

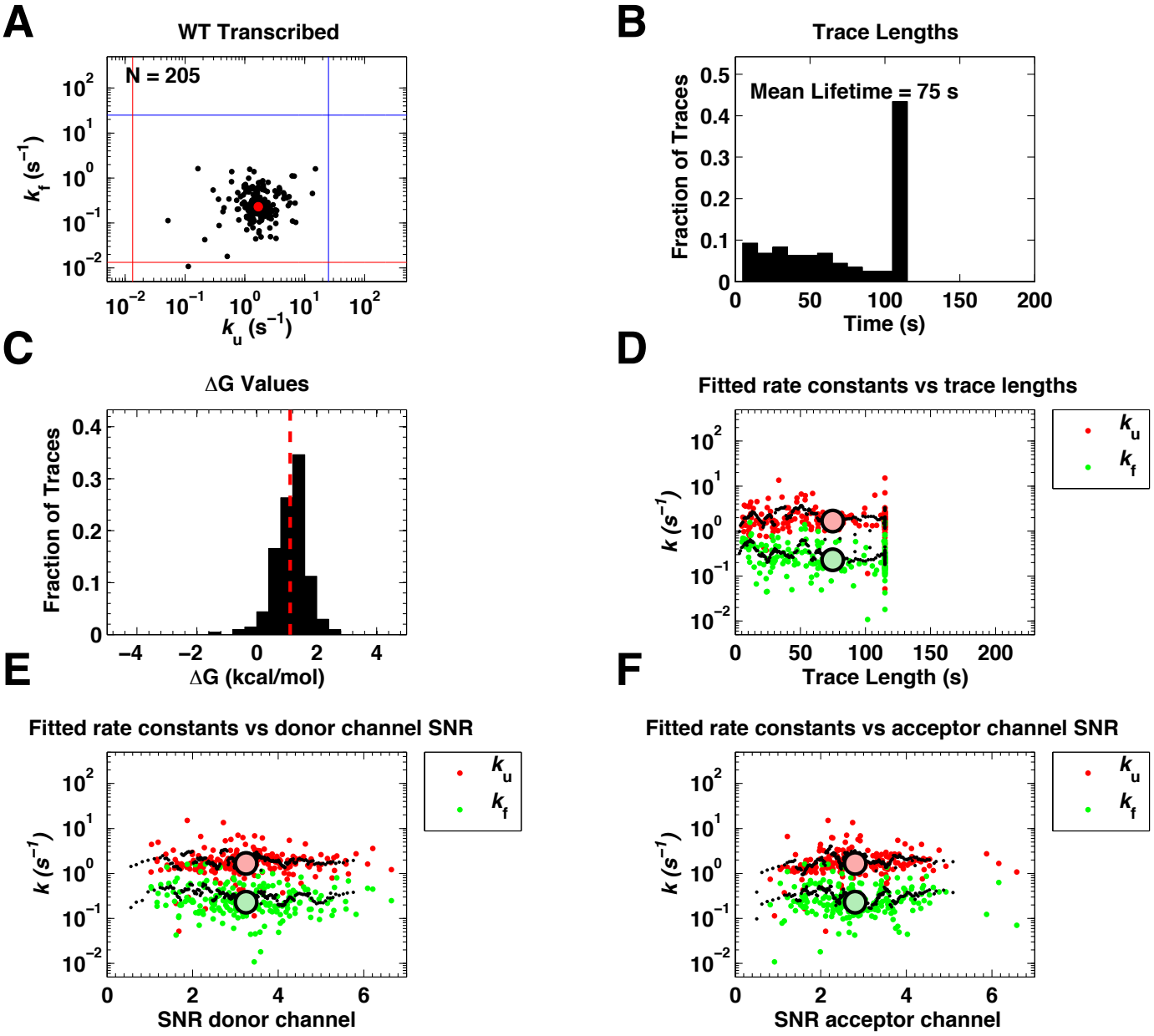


Figure S54-1. smFRET data assessment for WT Transcribed. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

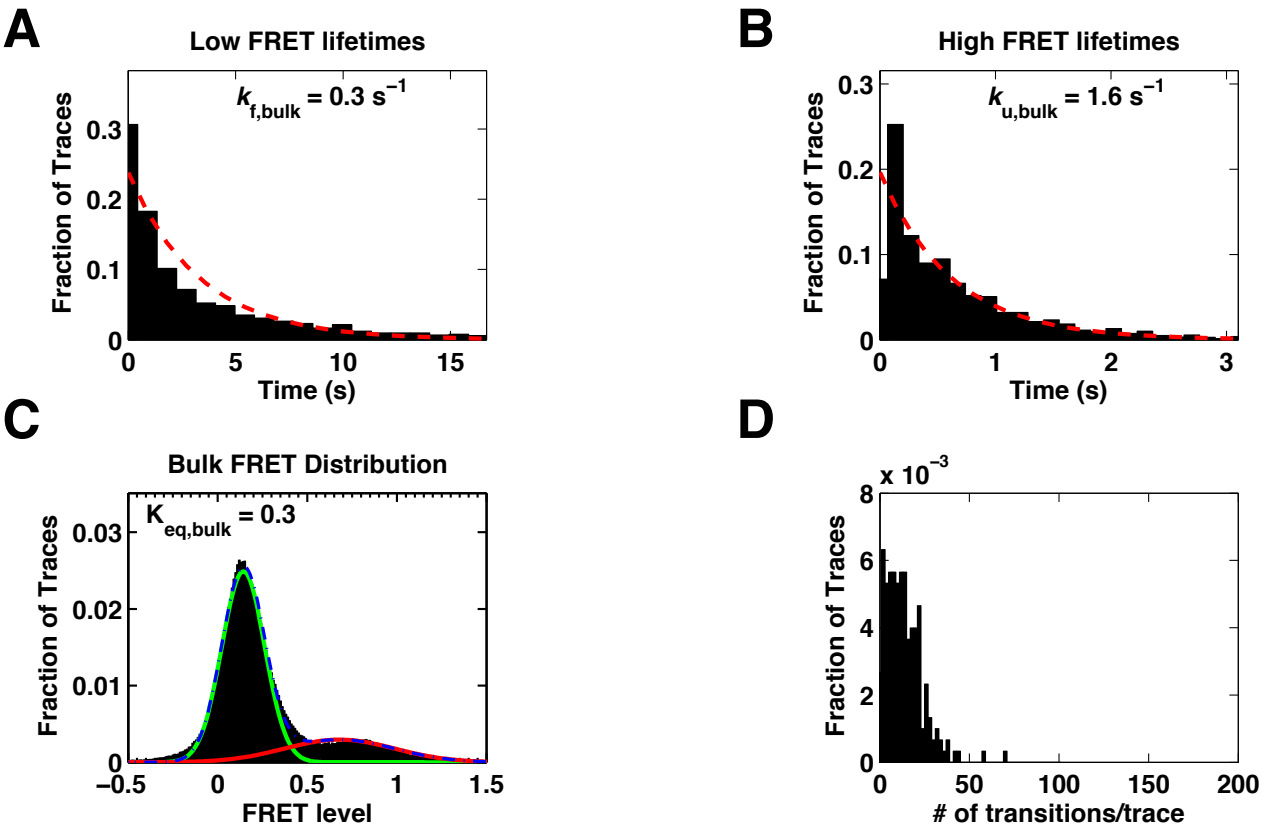


Figure S54-2. smFRET data assesment of aggregate data for WT Transcribed. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

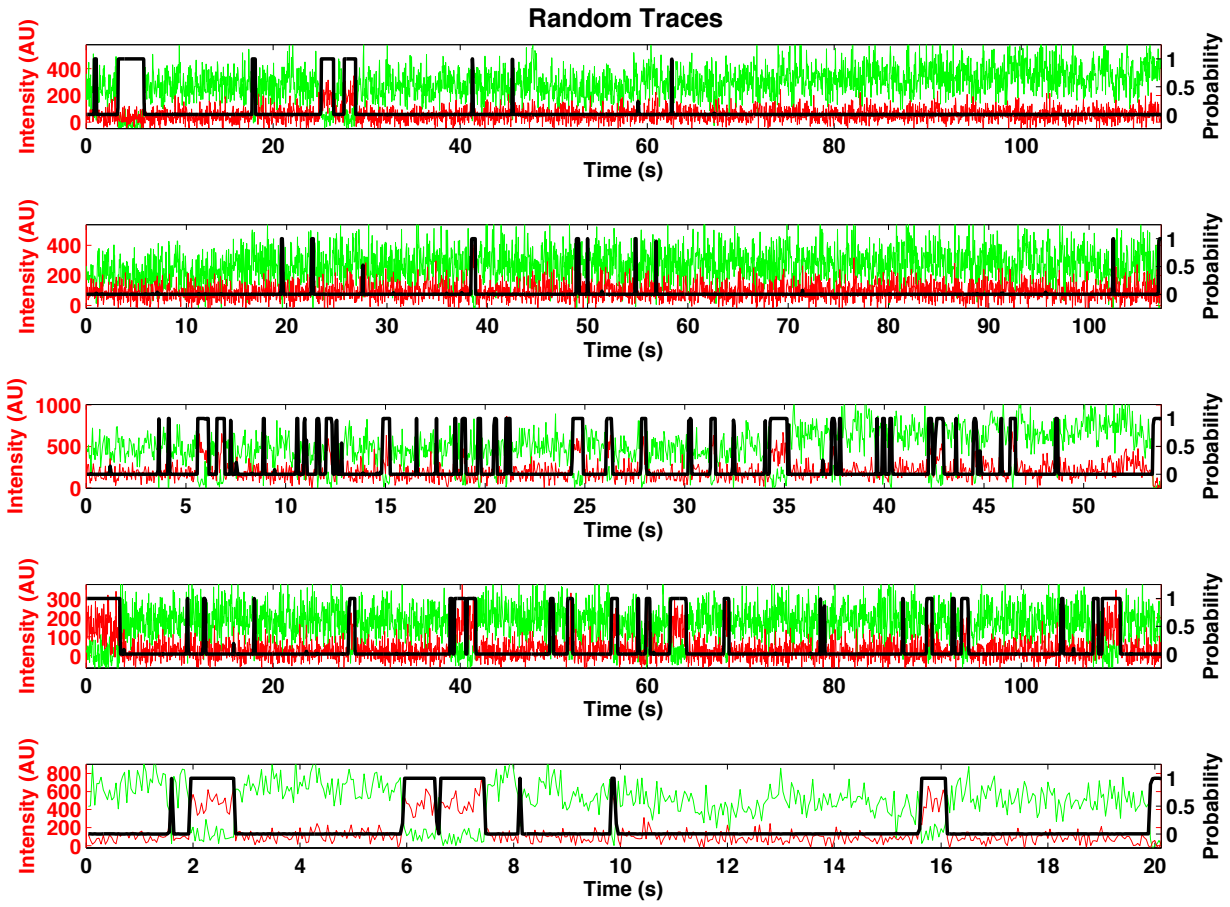


Figure S54-3. Randomly selected FRET traces of WT Transcribed. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S55-1. Variant and Conditions

Variant:	WT Synthetic
$MgCl_2$ (mM)	1.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	50
SNR Threshold[2]	0.75
Number of Traces	189

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S55-2. Folding parameters of smFRET the variant WT Synthetic inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.3	0.2 - 0.3	1.8
	$k_u(s^{-1})$	2.2	2.0 - 2.5	1.9
	K_{eq}	0.1	0.1 - 0.1	2.7
	SNR green	3.1	3.1 - 3.3	0.9
	SNR red	3.1	3.0 - 3.2	0.9
	ΔG(kcal/mol)	1.2	1.2 - 1.3	0.6
Fits from Cumulative Data[2]	Lifetime (s)	51.7	45.1 - 60.0	51.7
	$k_{f,\ bulk}(s^{-1})$	0.3	0.4 - 0.3	0.3
	$k_{u,\ bulk}(s^{-1})$	1.9	2.0 - 1.9	1.6
	$K_{eq,bulk}$	0.3	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.8	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

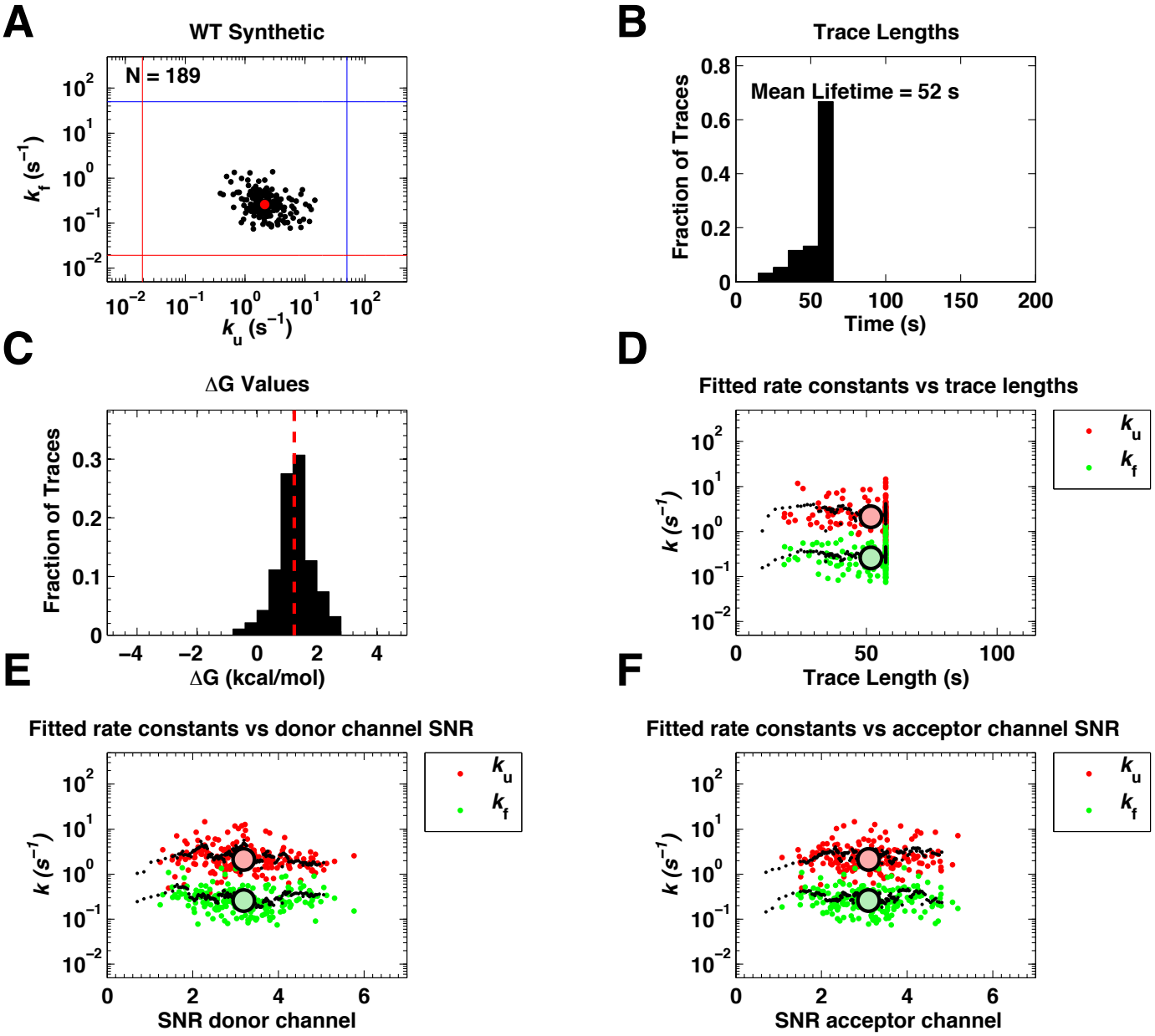


Figure S55-1. smFRET data assessment for WT Synthetic. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

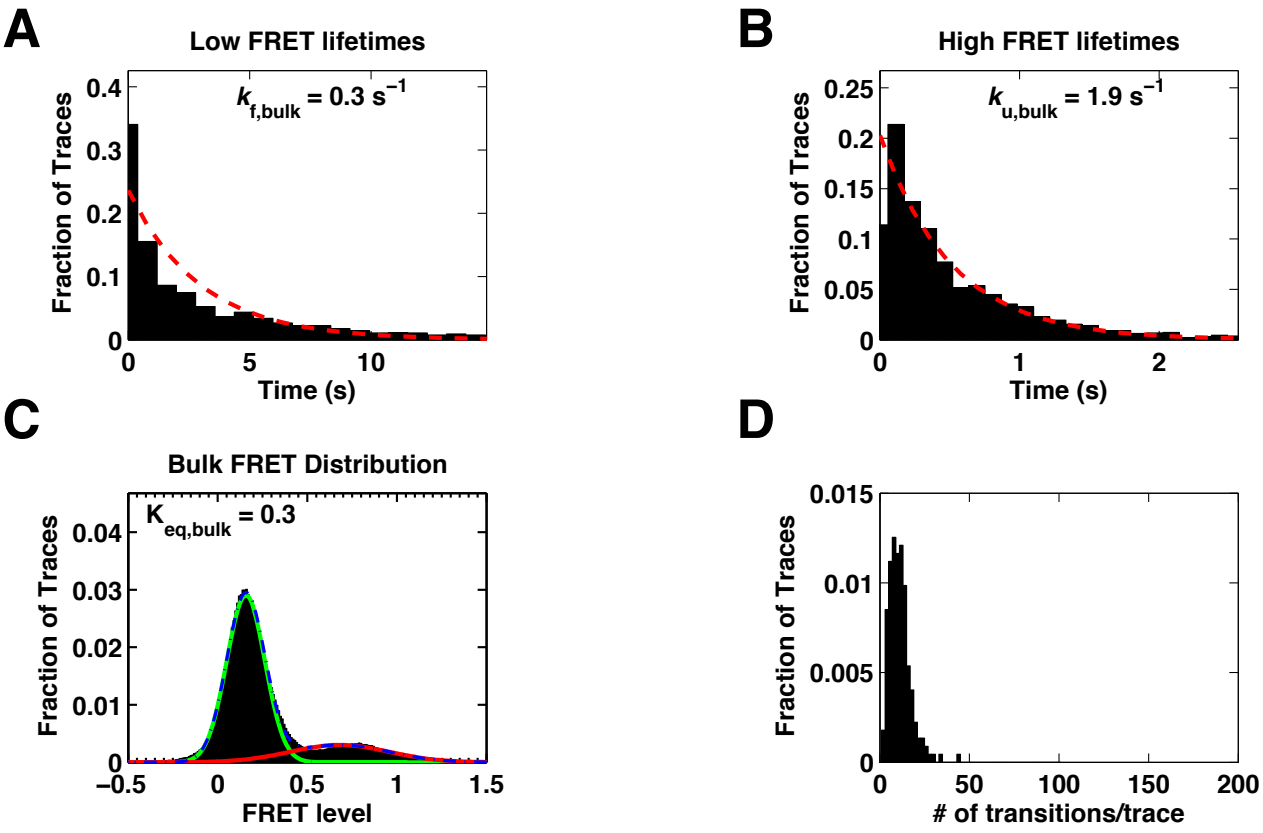


Figure S55-2. smFRET data assesment of aggregate data for WT Synthetic. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

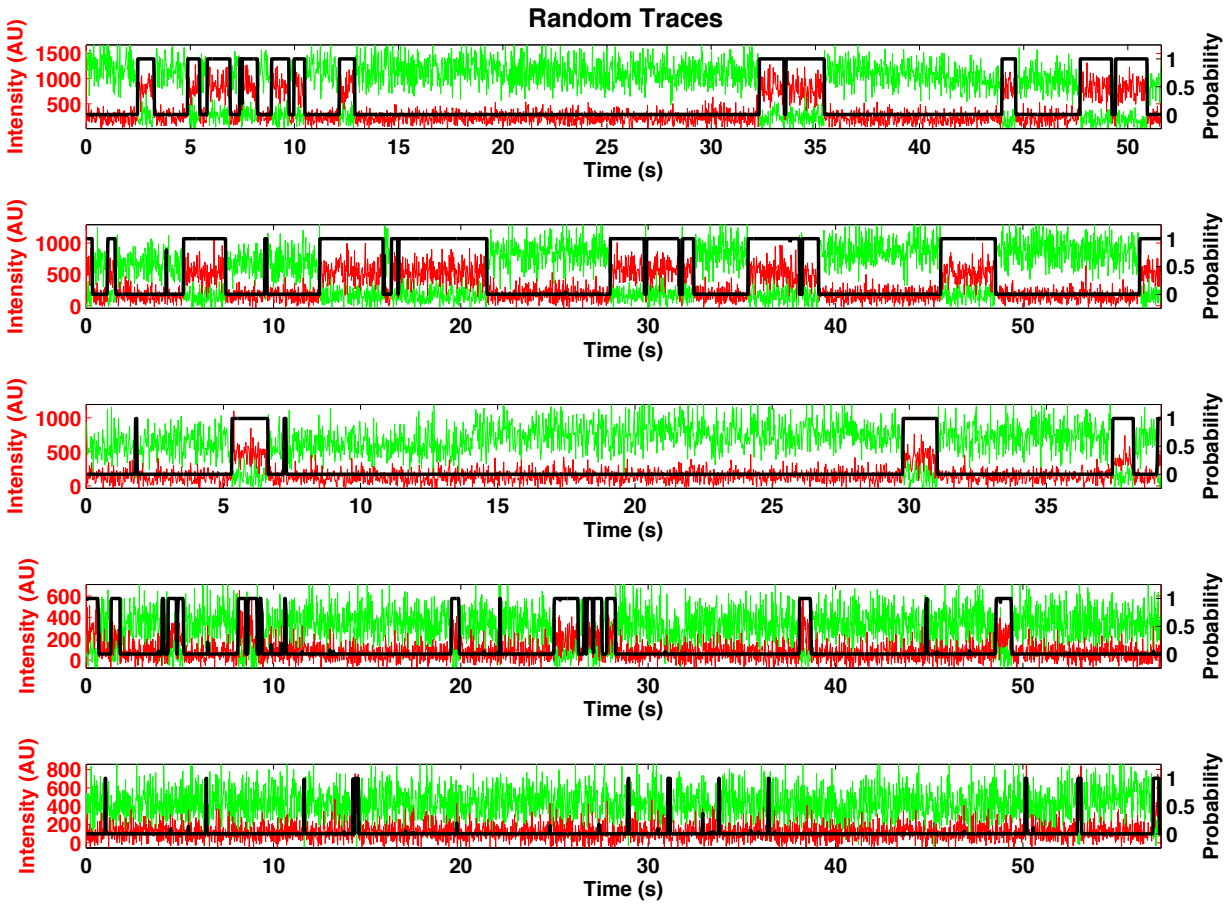


Figure S55-3. Randomly selected FRET traces of WT Synthetic. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S56-1. Variant and Conditions

Variant:	WT P4P6 Cy3b/Atto647N
$MgCl_2$ (mM)	1.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.75
Number of Traces	64

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S56-2. Folding parameters of smFRET the variant WT P4P6 Cy3b/Atto647N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	0.4	0.3 - 0.6	2.7
	$k_u(s^{-1})$	2.2	1.8 - 2.7	2.2
	K_{eq}	0.2	0.1 - 0.3	3.5
	SNR green	3.6	3.4 - 3.7	0.6
	SNR red	2.1	2.0 - 2.2	0.4
	ΔG(kcal/mol)	1.0	0.8 - 1.1	0.7
Fits from Cumulative Data[2]	Lifetime (s)	21.8	17.3 - 28.3	21.8
	$k_{f,\ bulk}(s^{-1})$	0.7	0.8 - 0.7	0.5
	$k_{u,\ bulk}(s^{-1})$	2.3	2.5 - 2.1	2.0
	$K_{eq,bulk}$	0.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	0.8	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

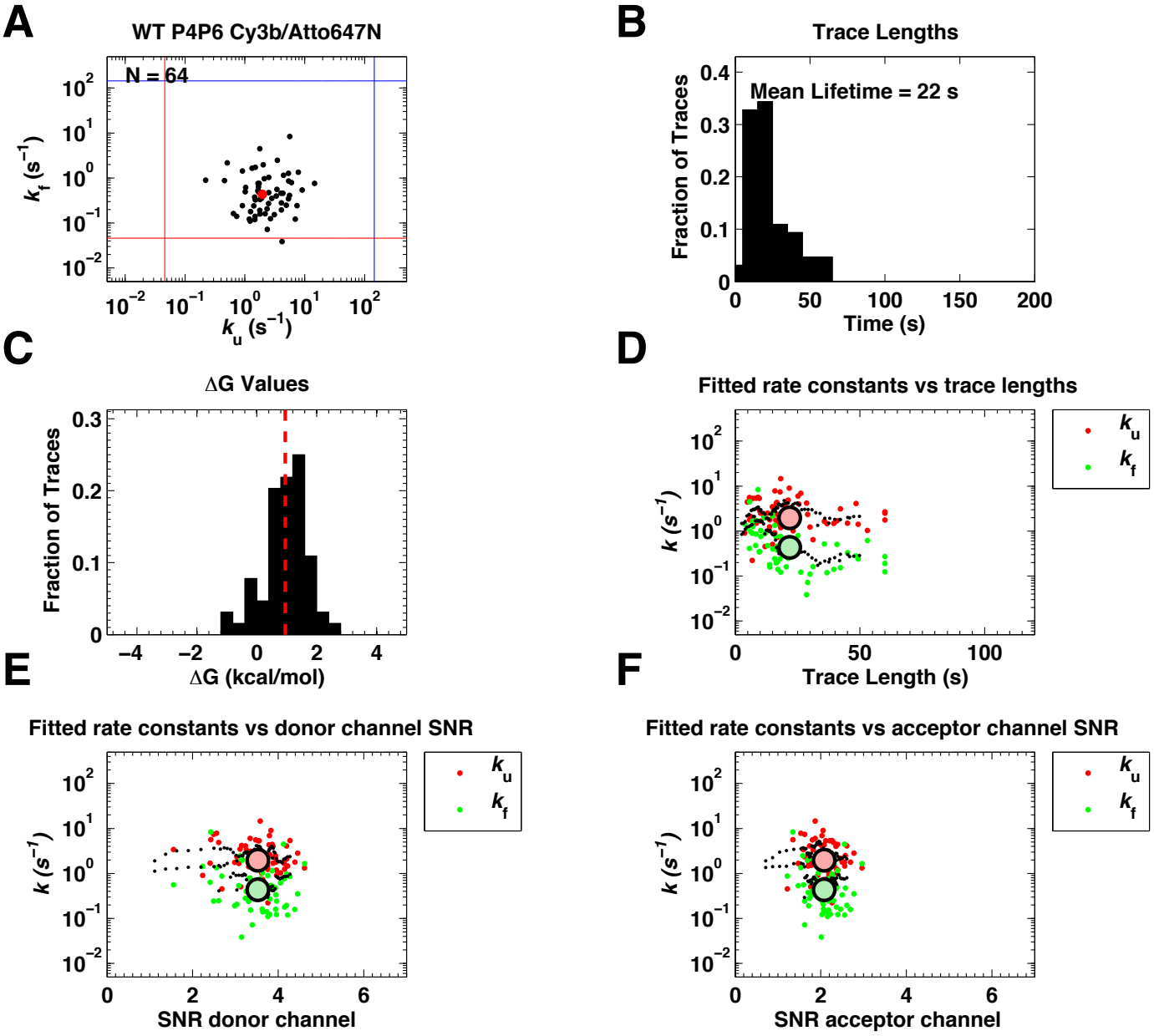


Figure S56-1. smFRET data assessment for WT P4P6 Cy3b/Atto647N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

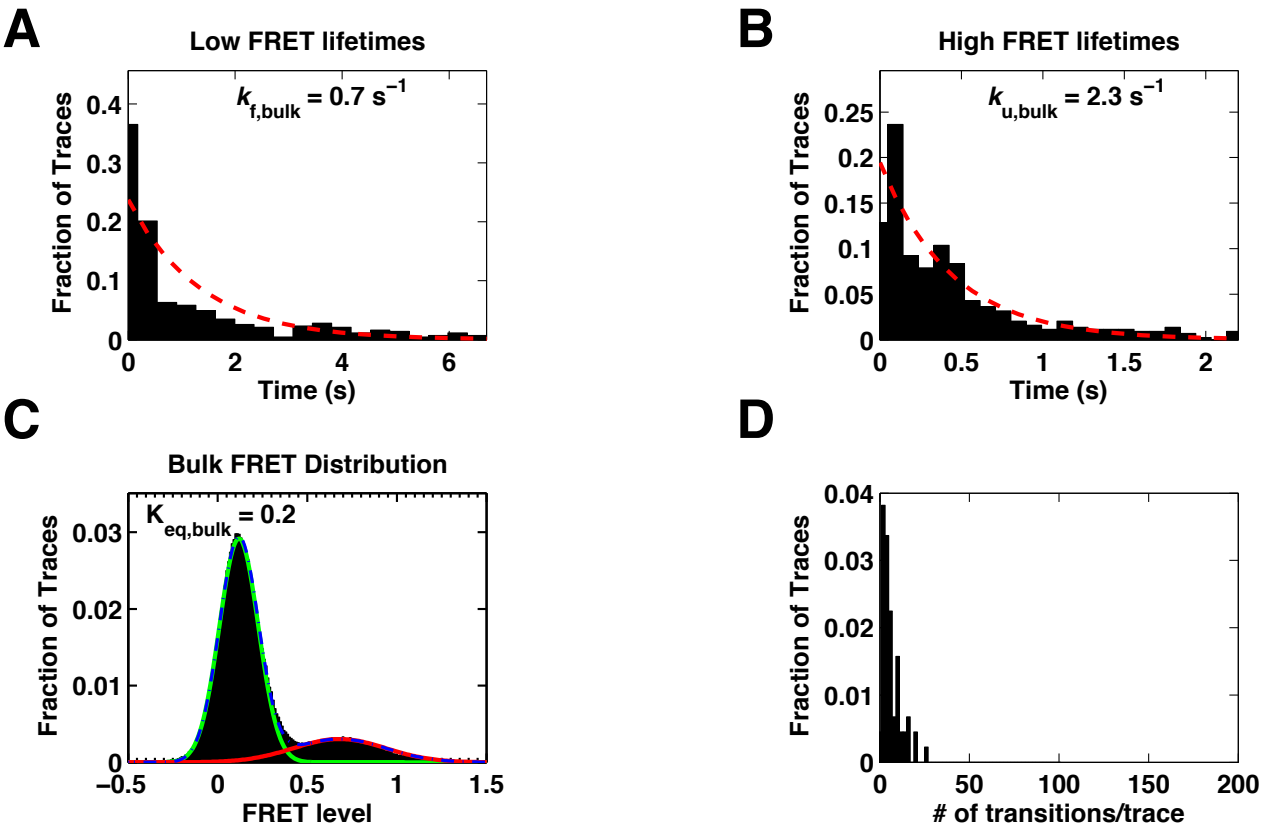


Figure S56-2. smFRET data assesment of aggregate data for WT P4P6 Cy3b/Atto647N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

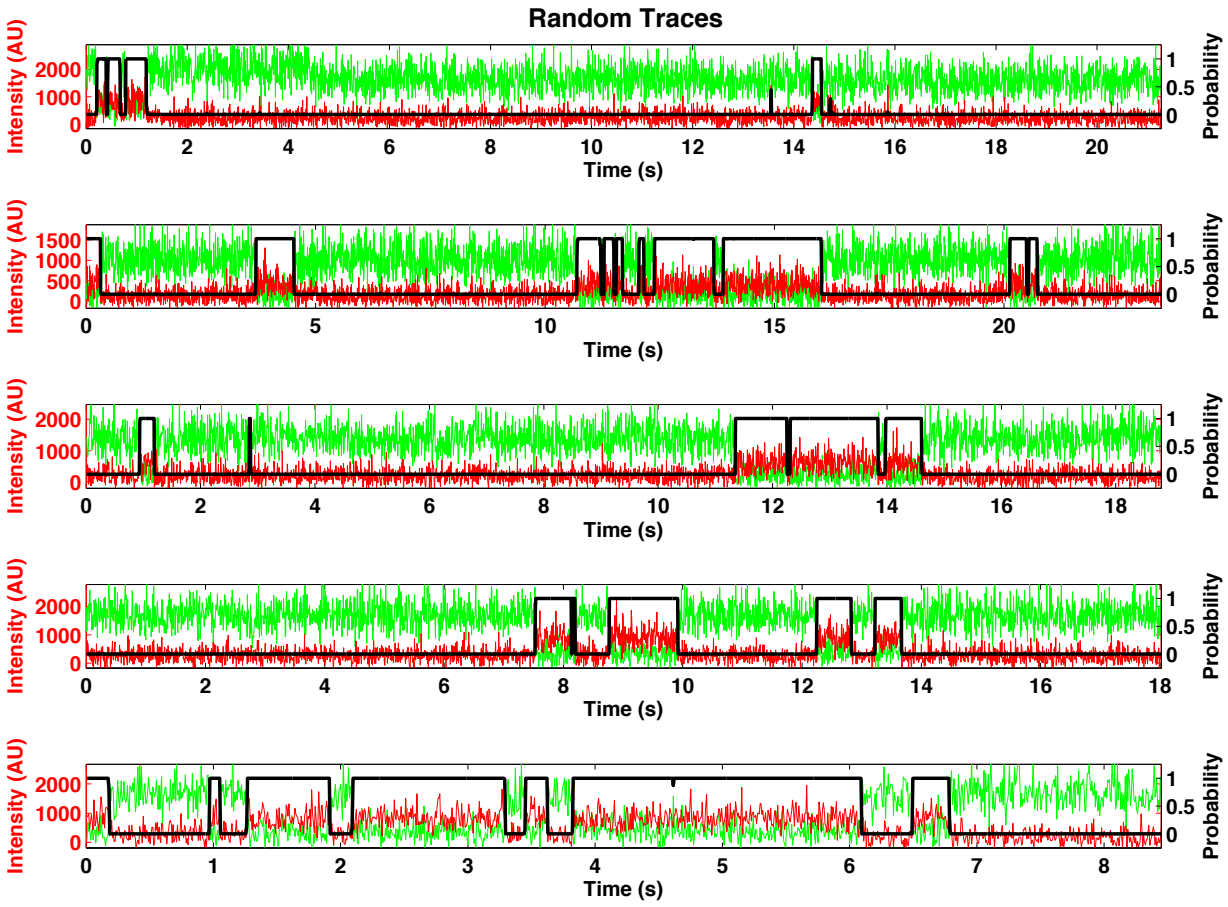


Figure S56-3. Randomly selected FRET traces of WT P4P6 Cy3b/Atto647N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S57-1. Variant and Conditions

Variant:	WT Transcribed
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	48
SNR Threshold[2]	0.75
Number of Traces	195

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S57-2. Folding parameters of smFRET the variant WT Transcribed inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	5.4	4.7 - 6.2	2.7
	$k_u(s^{-1})$	0.3	0.2 - 0.3	5.9
	K_{eq}	21.1	14.7 - 29.4	11.6
	SNR green	4.1	3.9 - 4.3	1.3
	SNR red	2.7	2.8 - 3.1	1.1
	ΔG(kcal/mol)	-2.3	-2.0 - -1.6	1.4
Fits from Cumulative Data[2]	Lifetime (s)	73.4	64.1 - 84.9	73.4
	$k_{f,\ bulk}(s^{-1})$	2.6	2.7 - 2.6	2.1
	$k_{u,\ bulk}(s^{-1})$	1.2	1.2 - 1.1	0.4
	$K_{eq,bulk}$	4.9	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-0.9	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

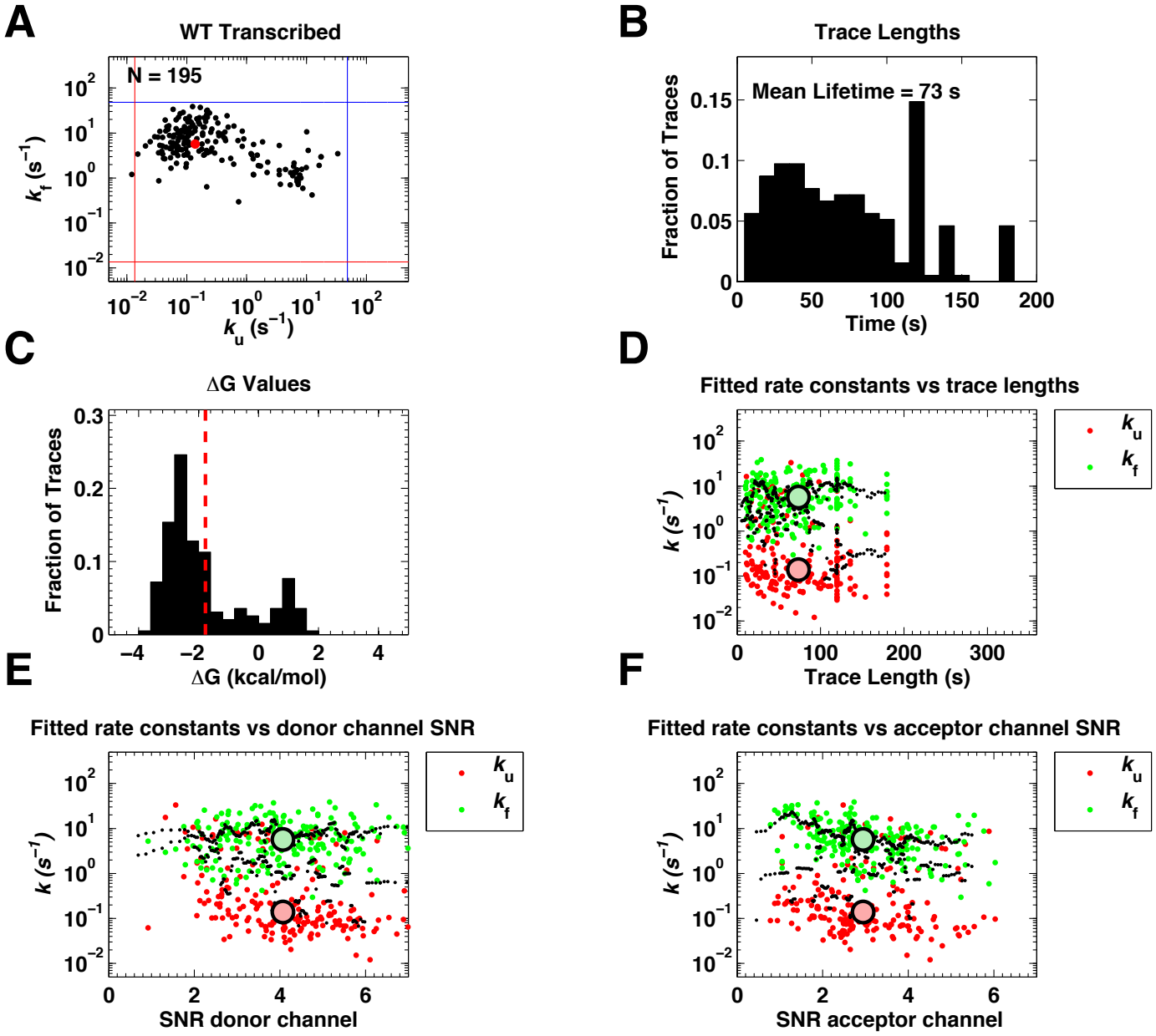


Figure S57-1. smFRET data assessment for WT Transcribed. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

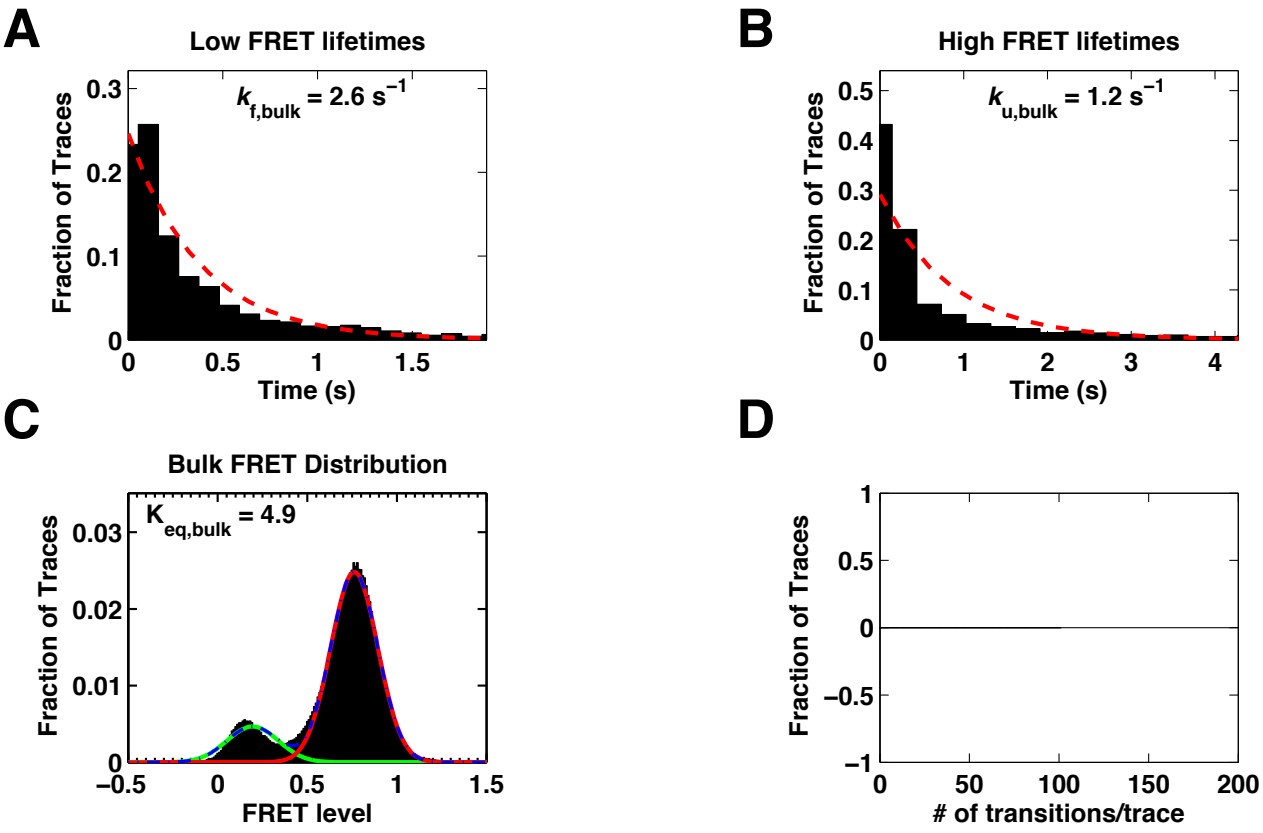


Figure S57-2. smFRET data assesment of aggregate data for WT Transcribed. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

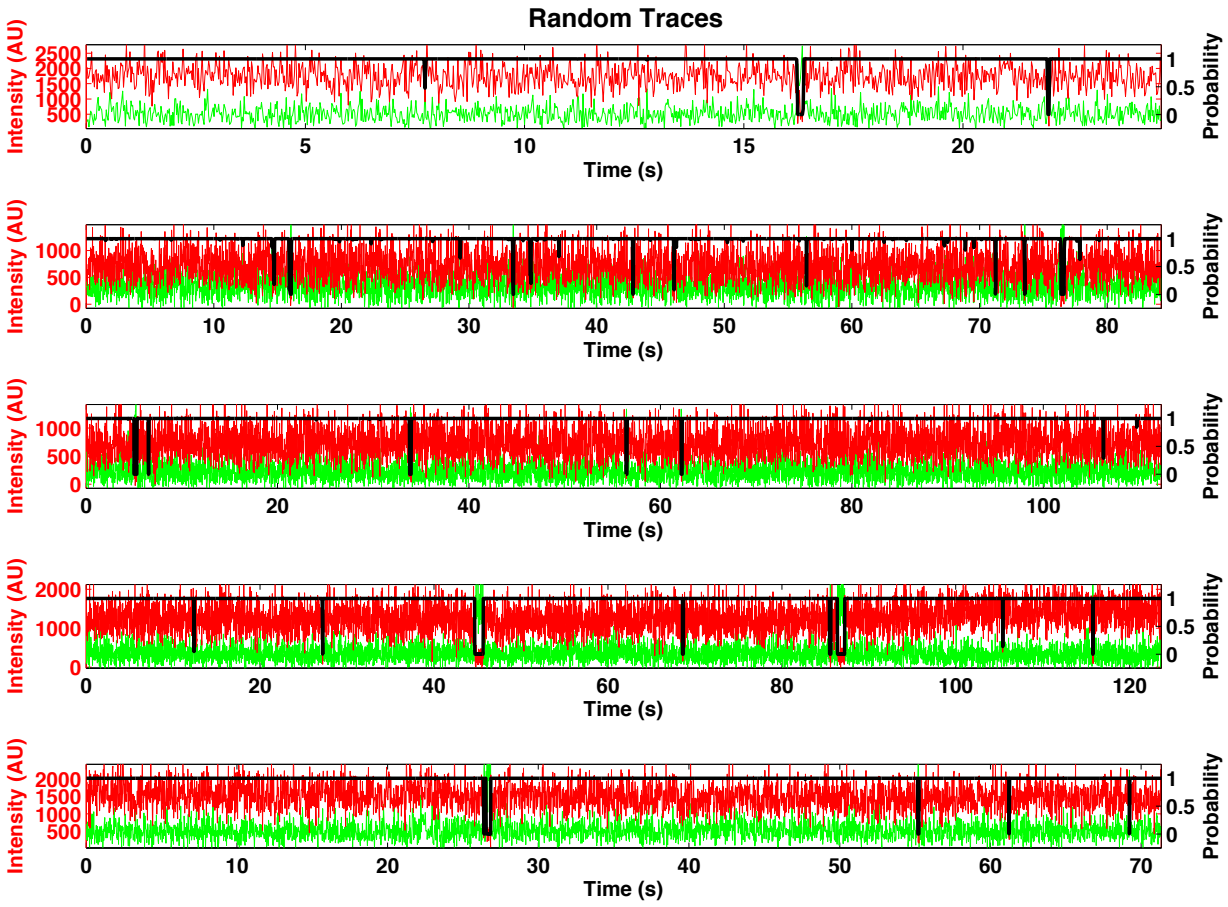


Figure S57-3. Randomly selected FRET traces of WT Transcribed. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S58-1. Variant and Conditions

Variant:	WT Synthetic
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	48
SNR Threshold[2]	0.75
Number of Traces	167

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S58-2. Folding parameters of smFRET the variant WT Synthetic inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	5.8	5.0 - 6.8	2.8
	$k_u(s^{-1})$	0.2	0.2 - 0.2	4.6
	K_{eq}	31.3	22.7 - 41.3	7.1
	SNR green	3.9	3.8 - 4.2	1.5
	SNR red	3.3	3.3 - 6.2	6.2
	ΔG(kcal/mol)	-2.3	-2.2 - -1.8	1.1
Fits from Cumulative Data[2]	Lifetime (s)	67.7	58.5 - 79.2	67.7
	$k_{f,\ bulk}(s^{-1})$	4.7	4.9 - 4.6	3.2
	$k_{u,\ bulk}(s^{-1})$	1.5	1.6 - 1.5	0.4
	$K_{eq,bulk}$	5.4	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

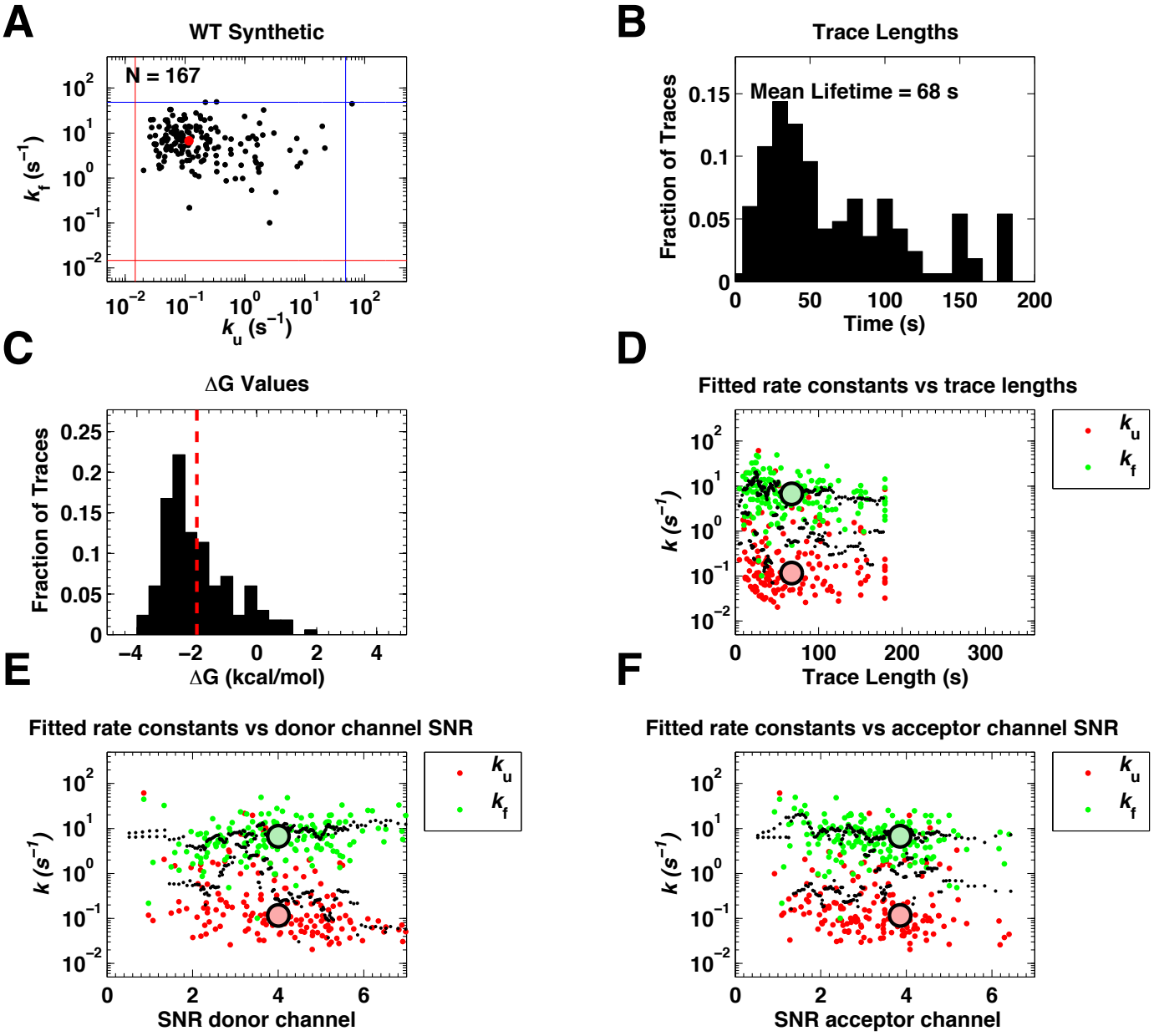


Figure S58-1. smFRET data assessment for WT Synthetic. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

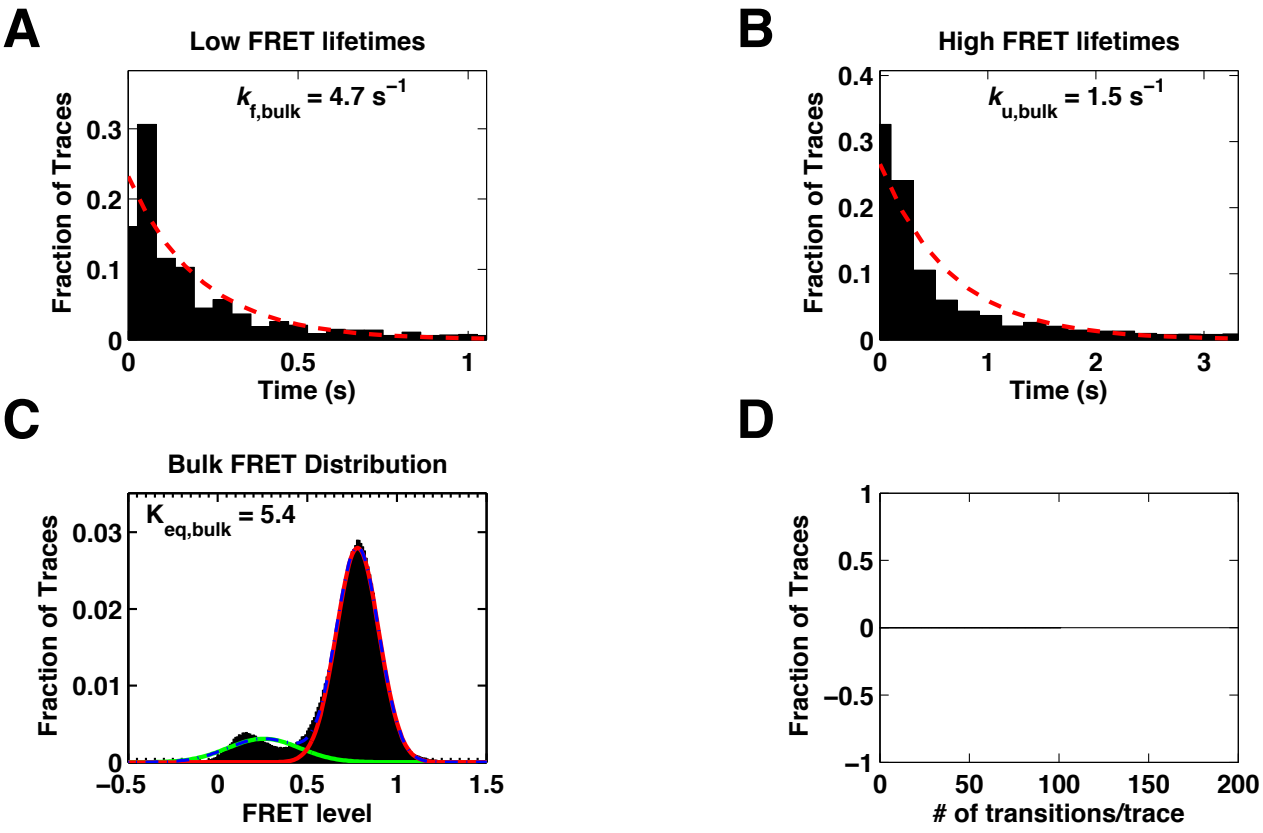


Figure S58-2. smFRET data assesment of aggregate data for WT Synthetic. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

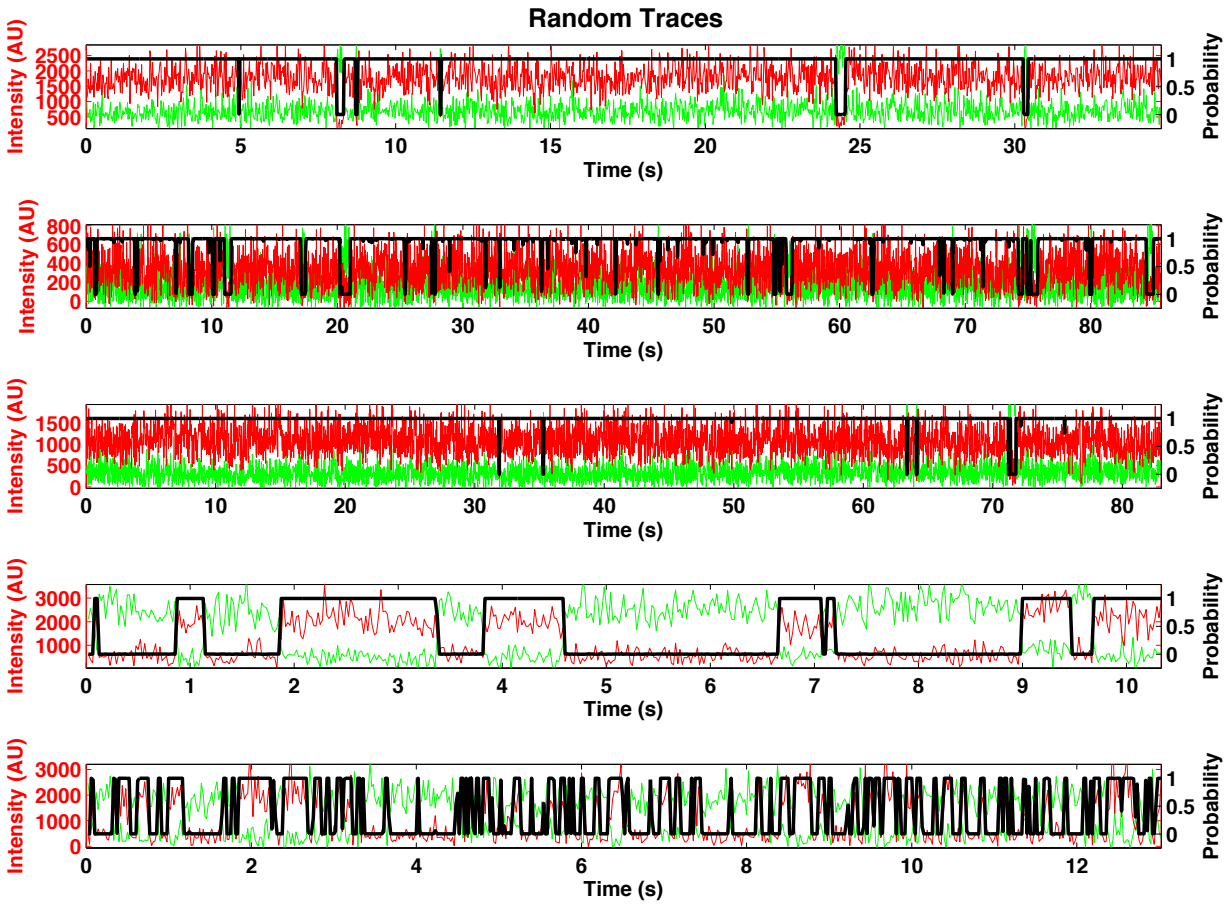


Figure S58-3. Randomly selected FRET traces of WT Synthetic. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).

Table S59-1. Variant and Conditions

Variant:	WT P4P6 Cy3b/Atto674N
$MgCl_2$ (mM)	5.0
$BaCl_2$ (mM)	0.0
KCl (mM)	100.0
pH	8.0
FPS[1]	145
SNR Threshold[2]	0.75
Number of Traces	84

[1] Frame per second, rate at which images were acquired.
[2] Signal-to-noise value used to threshold data (see Methods).

Table S59-2. Folding parameters of smFRET the variant WT P4P6 Cy3b/Atto674N inferred from fits to individual molecules and the population of molecules

	Parameter	**Value**	**Error**[3]	**SD**[4]
Fits from Individual Molecules[1]	$k_f(s^{-1})$	6.6	5.2 - 8.4	3.2
	$k_u(s^{-1})$	0.5	0.4 - 0.7	4.2
	K_{eq}	12.7	8.9 - 18.0	5.5
	SNR green	3.1	3.0 - 3.3	0.6
	SNR red	2.0	1.9 - 2.1	0.4
	ΔG(kcal/mol)	-1.7	-1.7 - -1.3	1.0
Fits from Cumulative Data[2]	Lifetime (s)	53.7	43.8 - 67.3	53.7
	$k_{f,\ bulk}(s^{-1})$	8.6	8.9 - 8.3	4.7
	$k_{u,\ bulk}(s^{-1})$	2.3	2.4 - 2.3	1.0
	$K_{eq,bulk}$	5.2	N.D.	N.D.
	ΔG_{bulk}(kcal/mol)	-1.0	N.D.	N.D.

[1] Mean values from fits of individual molecules (see SI methods).
[2] Values determined from fits to bulk data (see SI methods).
[3] Bootstrap-estimated 95% confidence intervals of the mean (see SI methods).
[4] Standard deviation.

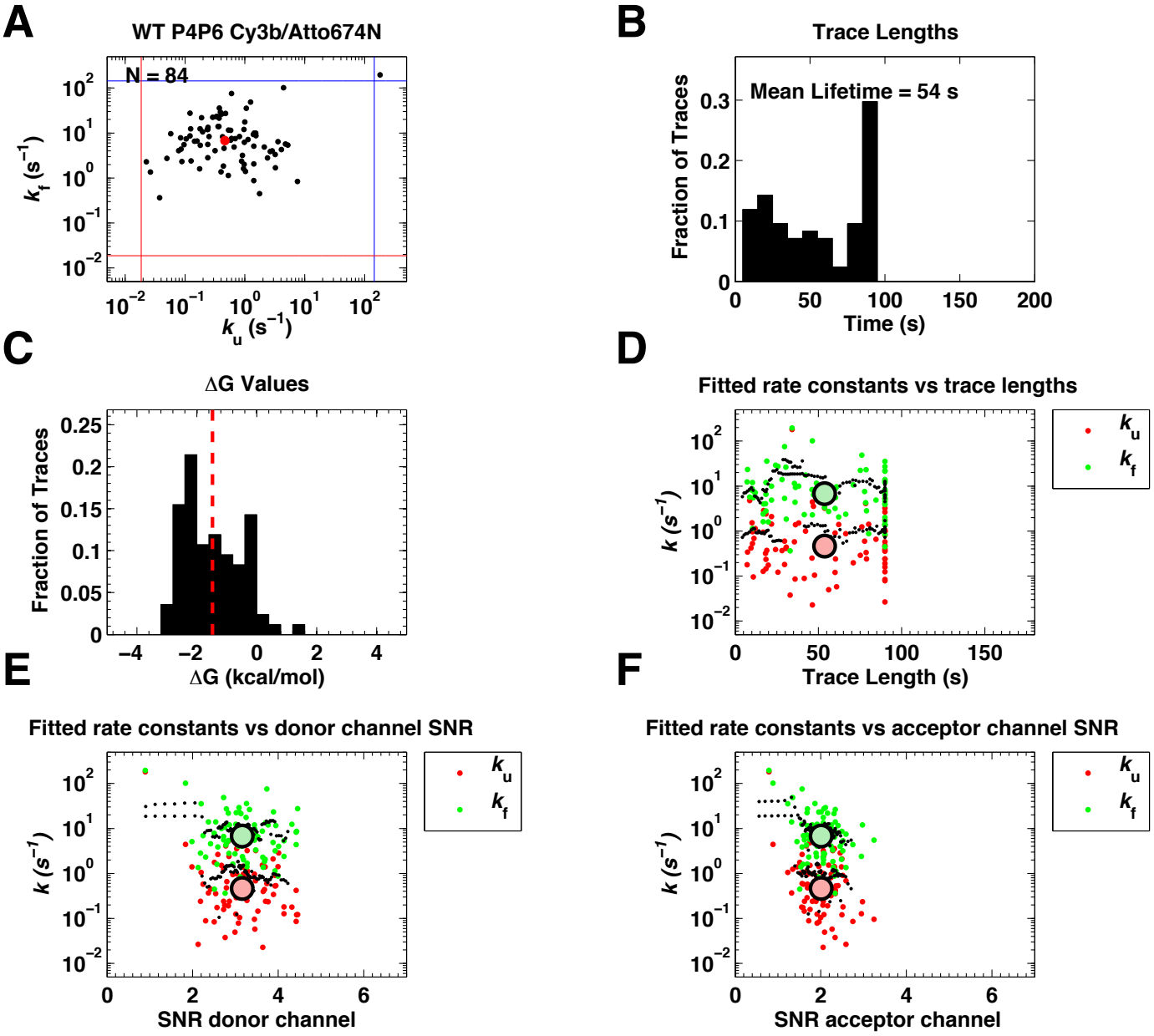


Figure S59-1. smFRET data assessment for WT P4P6 Cy3b/Atto674N. (A) Plot of fitted values of k_{fold} versus k_{unfold} rate constants for each molecule. Blue lines indicate the value of the camera frame rate and red lines indicate the value of the average lifetime of the molecules. Red dot indicates median folding and unfolding rate constants. (B) Histogram of trace lengths. Fit to exponential distribution shown in dotted red line. (C) Histogram of ΔG values. Mean value is denoted with dotted red line. (D) Scatter plot of rate constants versus trace length, k_{fold} (green) and k_{unfold} (red). The mean value for each rate constant is shown by larger light green (k_{fold}) and red (k_{unfold}) circles. Running average of ten sorted values is shown in black to guide the eye to trends in the data. (E) and (F) Rate constants versus signal-to-noise ratio (SNR) in the donor channel and acceptor channels respectively; colors as in (D).

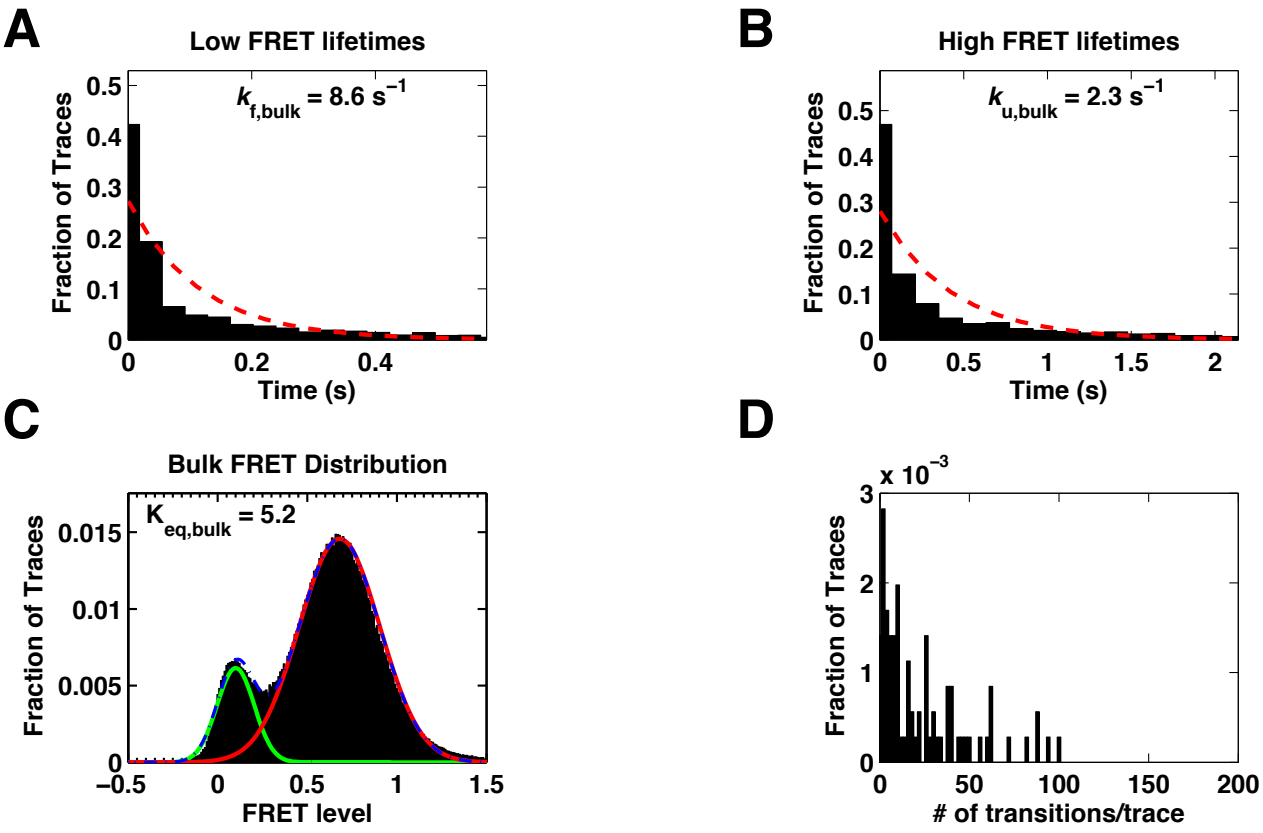


Figure S59-2. smFRET data assesment of aggregate data for WT P4P6 Cy3b/Atto674N. (A) and (B) Determination of the k_{fold} and k_{unfold} rate constants respectively from an exponential fit (dotted red lines) to the distribution of lifetimes of all molecules in the unfolded and folded states (see SI Methods). (C) Histogram of FRET distribution for all molecules. Distribution is fit to a two-Gaussian model (blue). The equilibrium constant ($K_{eq,bulk}$) was determined from the ratio of the fraction of molecules that are in the high FRET peak (red) versus low FRET peak (green) (see SI Methods). (D) Distribution of the number of transitions per trace.

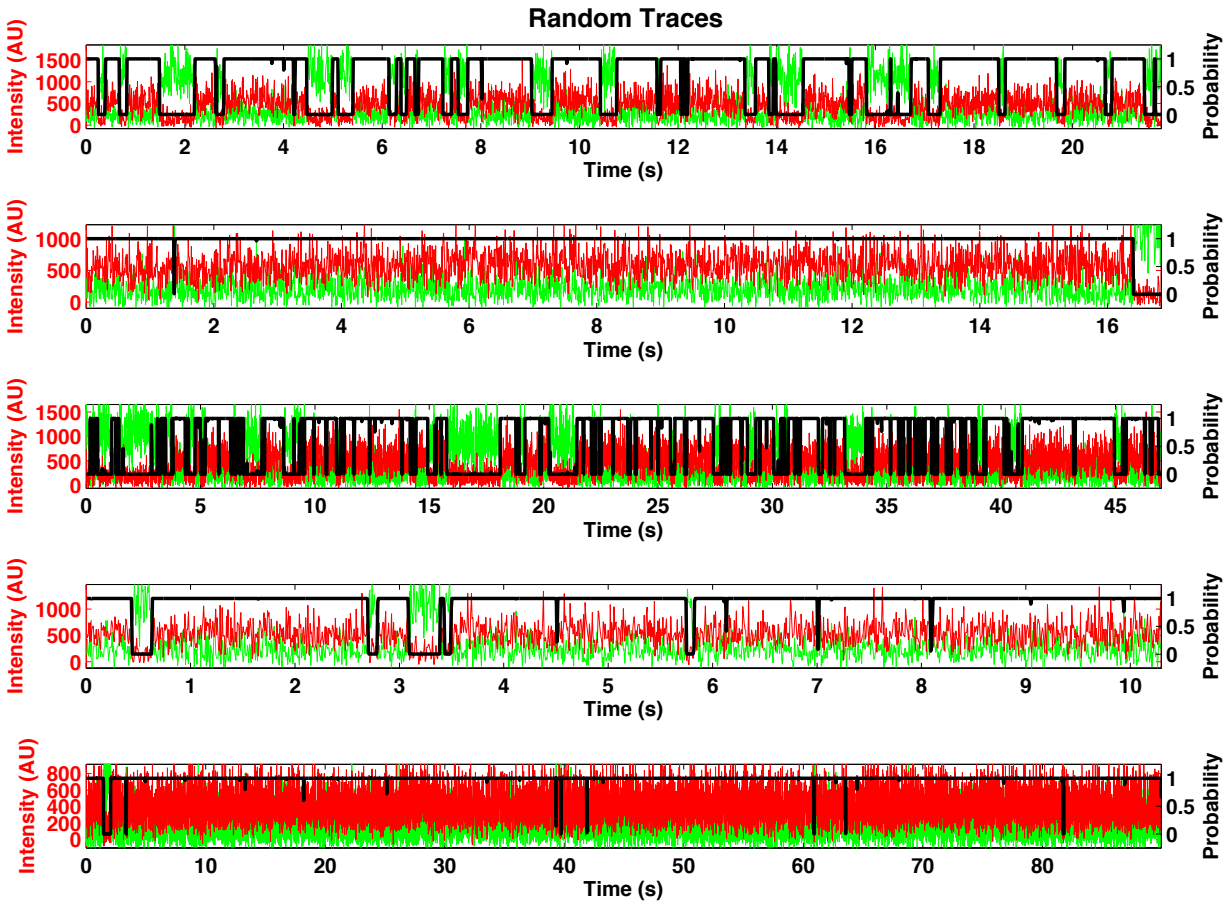


Figure S59-3. Randomly selected FRET traces of WT P4P6 Cy3b/Atto674N. The intensities of the donor dye (green) and the acceptor dye (red) are shown. The black line denotes the probability of the high FRET state determined by a hidden Markov model fit to data (Methods).