Supplemental Data

A combined PIE-FRET and FCS assay to monitor RNA dynamics and cleavage by SARS-CoV-2 Nsp15

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Table S1: The oligo sequences and label sites for the High-FRET, Mid-FRET, No-FRET, control oligos, and benchmark DNA oligos are included in the following table.

Name	Sequence (5' to 3')
RNA/DNA hybrid substrates	
High-FRET hybrid-60 (HFA/HFU)	5'- (ATTO647N) rArGrA rArAr(U or A) rArArG rGTA TCG GCA CGC TCG AGA TGA GGT ATT TCA CAC CTT AAG CCA GCC CCG ACA -3'
High-FRET complement-50	5'- (Biosg) TGT CGG GGC TGG CTT AAG GTG TGA AAT ACC TCA TCT CGA GCG TGC CGA TA (ATTO550) -3'
Mid-FRET hybrid/ Single-labeled hybrid-60 (MFA/MFU)	5'- (ATTO647N) rArGrA rArArA rArGrA rArAr(U or A) rArArG AGA ATA TCG GCA CGC TCG TGA GGT ATT TCA CAC CTT AAG CCA GCC -3'
Mid-FRET complement-45	5'- (Biosg) GGC TGG CTT AAG GTG TGA AAT ACC TCA CGA GCG TGC CGA TA/iAlex546N/ TCT -3'
No-FRET hybrid/ Single-labeled hybrid-60 (NFA/NFU)	5'- (ATTO647N) rArGrA rArArA rArGrA rArAr(U or A) rArArG AGA ATA TCG GCA CGC TCG TGA GGT ATT TCA CAC CTT AAG CCA GCC -3'
No-FRET complement-45	5'- (Biosg) GGC TGG CTT AAG GTG TGA AAT ACC TCA CGA GCG TGC CGA TAT TCT -3'
Short Oligo I (SO12)	5'- ATTO647N/rArGrA rArArA rArGrA rArArU -3'
Short Oligo II (SO15)	5'- ATTO647N/rArGrA rArArA rArGrA rArArU rArArG -3'
Benchmark DNA	
Acceptor strand	5' - biotin - CCA GAC AAA CAC TCA AAC AAA CTC GAC ACT <u>T</u>TC AGC TC - 3' [Amino dt position (linker): T31(C6)]
Donor strand (Lo FRET)	5' - GAG CTG AAA GTG TCG AGT TTG TTT GAG TGT <u>T</u>TG TCT GG - 3' [Amino dt position (linker): T31(C6); Annealed D-A Separation: 23 bp]
Donor strand (Mid FRET)	5' - GAG CTG AAA GTG TCG AGT TTG T<u>T</u>T GAG TGT TTG TCT GG - 3' [Amino dt position (linker): T23(C6); Annealed D-A Separation: 15 bp]
Donor strand (Hi FRET)	5' - GAG CTG AAA GTG TCG AGT <u>T</u>TG TTT GAG TGT TTG TCT GG - 3' [Amino dt position (linker): T19(C6); Annealed D-A Separation: 11 bp]

Table S2: The leakage factors were determined for both AF546 (Mid-FRET donor) and Atto550 (High-FRET donor), while the direct excitation factor was determined for Atto647N (all substrates). The confocal volume aspect ratio and effective volume were determined for the 531-nm 636-nm lasers via FCS calibration with Atto550 and Atto655-maleimide respectively.

PIE-FRET	**Leakage (α)**	**Direct Excitation (δ)**	**Fluorescence Lifetime – Free Dye (τ), ns**	**Fluorescence Lifetime – Oligo Attached (τ), ns**
RNA/DNA hybrid substrates				
Mid-FRET Donor (AF546)	0.061	--	4.0	3.8
High-FRET Donor (Atto550)	0.069	--	3.6	4.1
Mid-FRET Acceptor (Atto647N)	--	0.030	3.5	4.3
High-FRET Acceptor (Atto647N)	--	0.030	3.5	4.5
Benchmark DNA				
Donor Lo – Atto550	0.053	--	3.58 ± 0.05	4.05 ± 0.01
Donor Mid – Atto550	0.060	--	3.58 ± 0.05	4.12 ± 0.07
Donor Hi – Atto550	0.054	--	3.58 ± 0.05	4.14 ± 0.01
Acceptor-Atto647N	--	0.019	3.53 ± 0.01	4.35 ± 0.06
Calibration				
FCS	Confocal Volume Aspect Ratio (κ)		Effective Volume (V_{eff})	
Green Laser (531 nm)	7.1		1.54	
Red Laser (636 nm)	4.4		1.7	

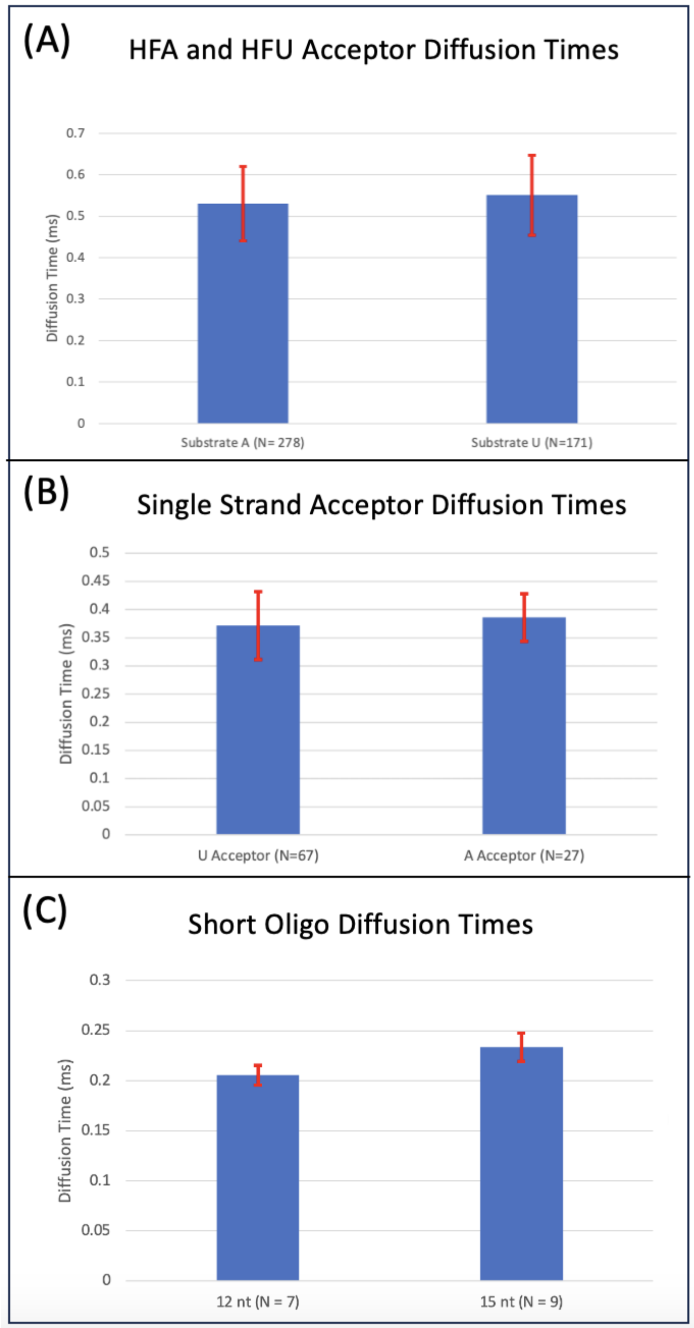

Figure S1: Diffusion times of oligos and annealed high-FRET (HFU and HFA) substrates (See Table S1 for sequences). **(A)** Annealed HFA and HFU substrates. The average diffusion time for HFA was 0.53 +/- 0.09 ms, and 0.55 +/- 0.10 ms for HFU. **(B)** Unannealed individual acceptor oligo strands of high-FRET substrates. The average diffusion time for the A acceptor was 0.39 +/- 0.05 ms and 0.37 +/- 0.06 ms for the U acceptor. **(C)** Short oligo controls. The average diffusion time for the 12 nt short oligo was 0.21 +/- 0.01 ms, and 0.23 +/- 0.01 ms for the 15 nt. A conformational FCS fitting model (Equation 3 in the main text) was used to fit the annealed substrate and single strand control data in A and B, and a pure diffusion model (Equation 2 in the main text) was used to fit the short oligo control data in C. Collection times were between 1 and 2 minutes, and sample concentrations were 1 - 10 nM.

Table S3: Comparison of diffusion coefficient and diffusion time fit parameters from conformational fits of Figure 2 data (main text) in SymPhoTime 64, Python, and PAM. The diffusion coefficient, D, was calculated using $D = {\omega_0^2}/{4\tau_D}$ and $V_{eff} = \pi^{3/2}\omega_0^3\kappa$, where, τ_D is the characteristic diffusion time, ω_0 is the diameter of the laser beam waist, and z_0 is the axial length of the laser beam. The ratio ${z_0}/{\omega_0}$ is the confocal volume aspect ratio, represented by κ. Calibration values for determining D are in Table S2.

τ_D (ms)				D (${\mu m^2}/{s}$)			
	SymPhoTime	Python	PAM		SymPhoTime	Python	PAM
WT_initial	0.470	0.488	0.444	WT_initial	63.0	60.7	66.7
W333A_initial	0.449	0.462	0.417	W333A_initial	66.0	64.2	71.1
H235A_initial	0.494	0.525	0.512	H235A_initial	60.0	56.5	57.9
WT_final	0.361	0.390	0.313	WT_final	82.0	76.0	94.5
W333A_initial	0.326	0.373	0.349	W333A_initial	91.0	79.5	84.9
H235A_final	0.529	0.555	0.559	H235A_final	56.0	53.4	53.0

Table S4: Conformational fit parameters used as constraints for mixed model fitting in Figure 3 of the main text, with β_C = 1. The average parameters were determined from a conformational fit (Eq. 3) of the triplicate measurements taken at 0-min. The data were fit in Python**.**

WT_U					
	1/N	τ_D *(ms)*	A_c	τ_C *(μs)*	*D (μm²/s)*
	1.832	0.488	0.571	5.77	60.7
	2.459	0.477	0.464	7.47	62.2
	2.383	0.473	0.521	6.59	62.7
avg	2.225	0.479	0.518	6.61	61.8
st_dev	0.342	0.008	0.054	0.852	1.05
W333A_U					
	0.893	0.462	0.363	6.91	64.2
	1.368	0.456	0.374	6.27	65.1
	2.192	0.441	0.401	6.45	67.1
avg	1.484	0.453	0.379	6.54	65.4
st_dev	0.657	0.010	0.019	0.327	1.5
H235A_U					
	0.748	0.525	0.557	6.09	56.5
	1.251	0.499	0.470	6.67	59.4
	1.339	0.527	0.408	7.37	56.2
avg	1.112	0.517	0.478	6.71	57.3
st_dev	0.319	0.016	0.075	0.640	1.8
WT_A					
	0.689	0.545	0.621	6.28	54.4

	0.980	0.554	0.672	5.49	53.5
	1.150	0.537	0.654	5.87	55.2
avg	0.940	0.545	0.649	5.88	54.4
st_dev	0.233	0.009	0.026	0.396	0.9
H235_A					
	0.877	0.580	0.661	6.39	51.1
	1.053	0.567	0.591	6.31	52.3
	1.156	0.531	0.525	6.34	55.8
avg	1.029	0.559	0.592	6.35	53.0
st_dev	0.141	0.025	0.068	0.0419	2.4

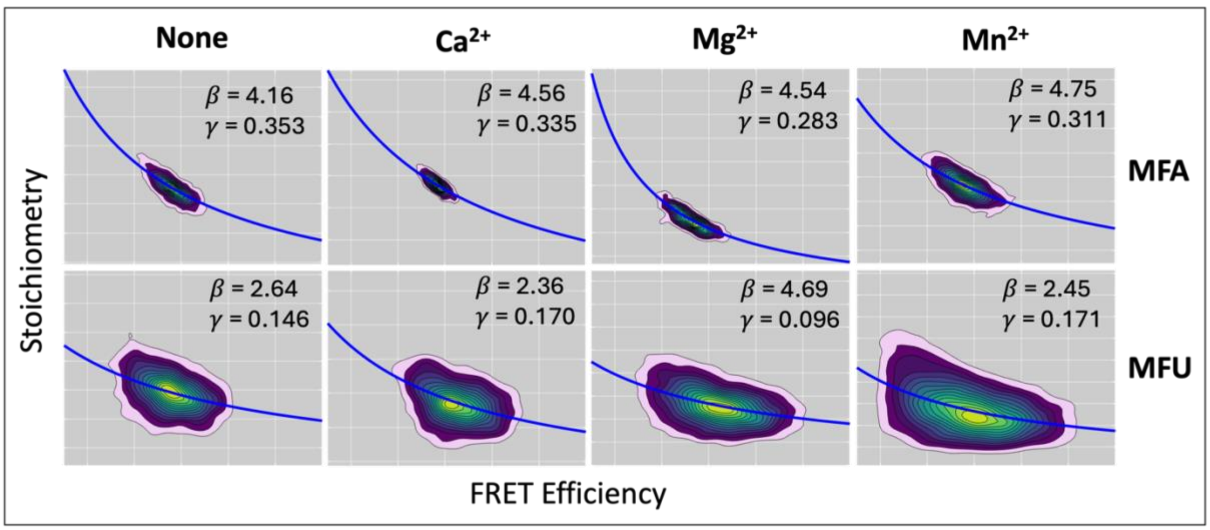

Figure S2: Beta and gamma corrections for PIE-FRET analysis were determined by fitting photon data to a reciprocal stoichiometry versus FRET efficiency curve for each population and relating fit parameters to beta and gamma correction factors (details described in Materials and Methods).

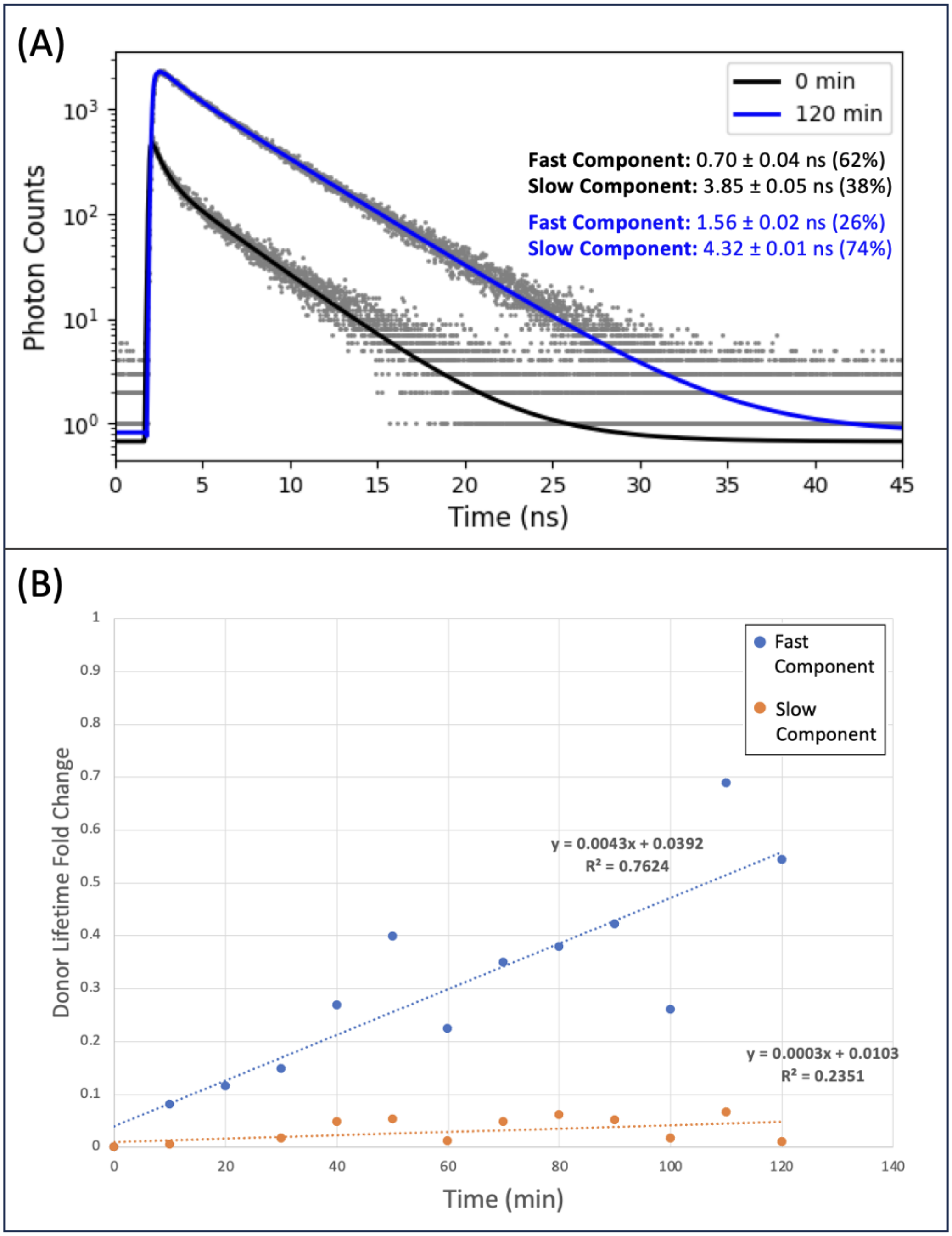

Figure S3: Fluorescence lifetime analysis of the ensemble for wildtype Nsp15 incubated with HFU substrate. **(A)** Time Correlated Single Photon Counting (TCSPC) histograms for the donor signal in a WT Nsp15-HFU experiment at 0 and 120 minutes. Data acquisition was for 90 seconds. The fast and slow components of the average donor lifetime were determined from reconvolution fitting of the histograms in SymPhoTime. We assigned the fast component to the donor lifetime due to FRET in presence of the acceptor, and the slow component to the donor lifetime in the absence of the acceptor **(B)** Fold change ($^{\Delta\tau_{fast/slow}}/_{\tau_{fast/slow,initial}}$) in Atto550 fast and slow components of average lifetimes for High-FRET substrate U (HFU) incubated with WT Nsp15 for 2 hours.

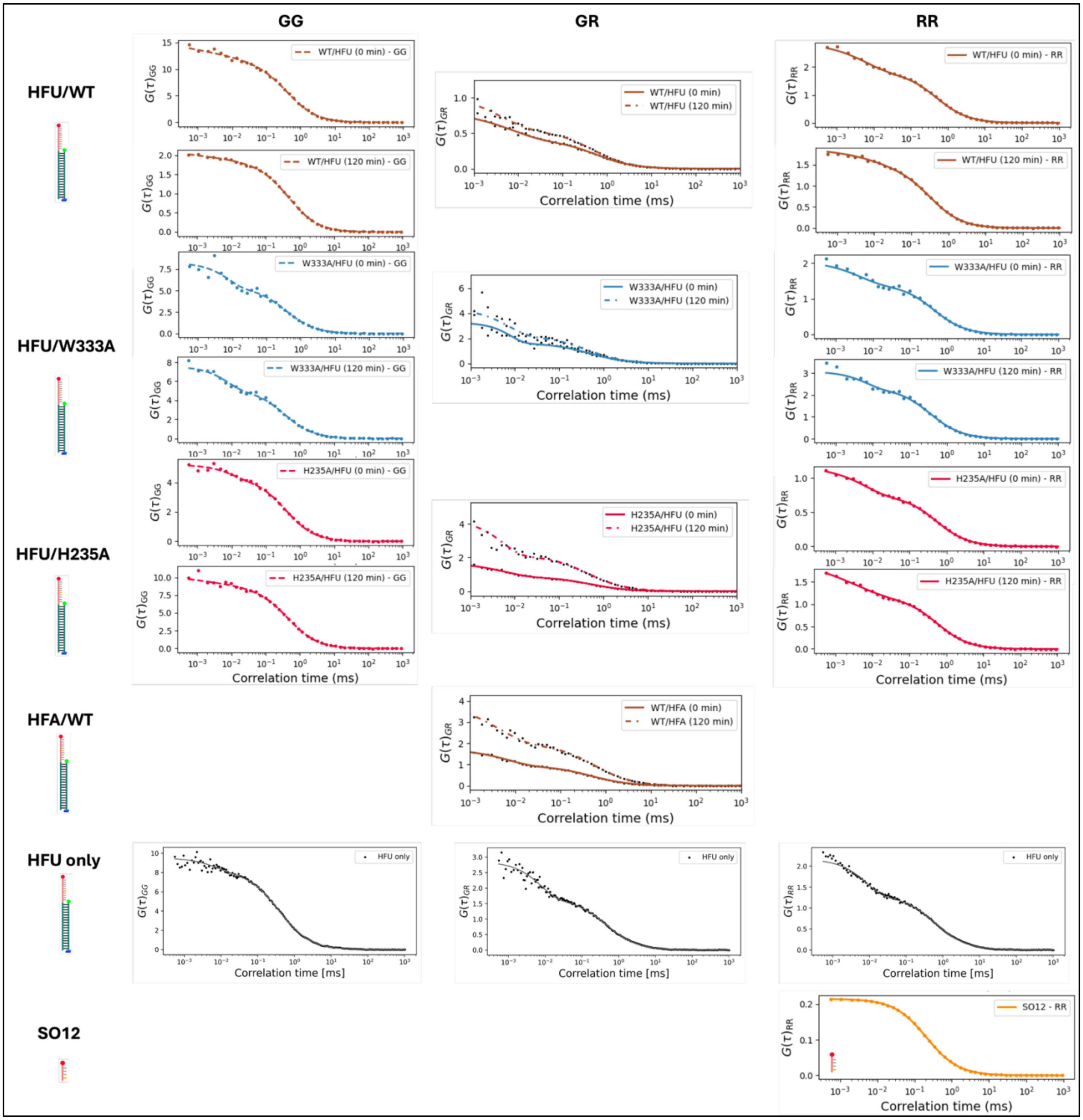

Figure S4: Autocorrelations of I_{GG}, I_{GR}, and I_{RR} signals for HFU substrate incubated with WT, W333A, and H235A Nsp15 at 0-min incubation and 120-min incubation, shown in Figure 2B of the main text. HFA in the presence of WT Nsp15 and SO12 curves are also shown. In the main text, we show only fits to the fits to autocorrelations of I_{RR}, which tracks the RNA-Atto647N molecules before and after cleavage. Each curve was fit to an appropriate model, either pure diffusion or conformational (Eq. 2 or Eq. 3), and the fit parameters are in Table S5.

Table S5: Fit parameters for autocorrelation curves in Figure S4. HFU incubated with Nsp15 variants, HFA, and SO12. Data were fit in Python or SymPhoTime using Eq. 2 (SO12 only) or Eq. 3 as a fitting model, with $\beta_C = 1$. Slight variations in the parameters for different signals may result from the presence of some unannealed oligos. I_{GG} and I_{RR} signals may contain contributions from unannealed oligos.

HFA									
	$1/N$	τ_D (ms)	A_c	τ_c (μs)		$1/N$	τ_D (ms)	A_c	τ_c (μs)
WT (0 min)					**WT (120 min)**				
GG	7.7	0.416	0.204	6.4	**GG**	8.26	0.50	0.203	12
GR	1.00	0.472	0.718	7.7	**GR**	2.09	0.561	0.79	6.3
RR	0.688	0.574	0.519	8.1	**RR**	1.8	0.682	0.445	11
HFU									
WT (0 min)					**WT (120 min)**				
GG	11.76	0.381	0.159	11.0	**GG**	1.83	0.468	0.096	11.0
GR	2.19	0.465	0.712	8.8	**GR**	1.41	0.481	0.628	9.6
RR	1.83	0.499	0.426	9.3	**RR**	1.51	0.311	0.168	14.0
W333A (0 min)					**W333A (120 min)**				
GG	5.52	0.357	0.583	5.3	**GG**	3.14	0.415	0.481	6.0
GR	2.03	0.487	1.3	5.0	**GR**	1.87	0.436	1.2	4.5
RR	1.42	0.452	0.365	5.6	**RR**	2.28	0.381	0.398	6.0
H235A (0 min)					**H235A (120 min)**				
GG	4.29	0.379	0.2	13.0	**GG**	10.38	0.431	0.151	5.8
GR	0.885	0.493	0.830	7.4	**GR**	1.46	0.497	0.6	9.8
RR	0.735	0.564	0.441	9.8	**RR**	1.59	0.559	0.42	8.0
SO12					**No Protein (HFU alone)**				
GG	--	--	--	--	**GG**	8.26	0.371	0.102	5.3
GR	--	--	--	--	**GR**	1.67	0.46	0.713	8.0
RR	0.211	0.229	--	--	**RR**	1.3	0.639	0.675	8.0

Table S7: FCS mixed model fit parameters for Figure 3B of the main text. Un-cleaved weighting (*A)* and *1/N* parameters (Eq. 2, 3 and 4) were used to fit FCS data using a custom Python script. The τ_D, A_C, and τ_C parameters were calculated from averages from fits of triplicate measurements at each timepoint (Table S4).

	WT/HFU			**W333A/HFU**			**H235A/HFU**		
Time (min.)	***A***	***1/N₁***	***1/N₂***	***A***	***1/N₁***	***1/N₂***	***A***	***1/N₁***	***1/N₂***
0	0.999753	1.850844	1.327115	0.994687	0.889481	0.768465	1	0.760035	1.19E-06
2	0.95018	2.500359	2.06966	1	1.356338	0.001095	0.961539	1.266768	0.949659
4	1	2.39601	0.368718	1	2.185654	0.255925	0.948938	1.344982	1.230501
10	0.896743	1.318692	1.155731	0.999999	0.947921	0.170545	1	0.862909	0.001134
12	0.87511	2.000953	1.730673	0.90905	1.459303	1.136893	1	1.193288	0.000746
14	0.841076	2.848327	2.056754	0.949703	1.759556	1.393946	0.894469	1.611865	1.2722
20	0.767245	1.307152	1.08812	0.996167	0.859225	0.557701	1	0.76516	0.000395
22	0.720112	2.324876	2.24996	0.934608	1.366951	1.294879	0.956478	1.015454	0.829156
24	0.545756	3.14329	3.20789	0.910428	2.049857	1.664751	0.850677	1.177097	0.90735
30	0.69253	1.33487	1.265683	0.999666	0.915842	0.643511	1	0.573283	0.000239
32	0.603215	1.8681	1.768379	1	1.526972	0.240712	1	0.841944	0.000489
34	0.593829	2.02809	2.040837	1	2.307168	2.746299	0.96323	0.872926	0.757214
40	0.684489	1.425792	1.370221	0.892329	1.007159	0.810236	1	0.723074	0.000126
42	0.569411	2.088302	2.11573	0.772614	1.50769	1.314532	1	1.102111	0.000265
44	0.531972	2.383477	2.567179	0.676947	1.717573	1.487357	1	1.354017	0.001981
50	0.607677	1.536832	1.32309	0.847396	1.028907	0.859621	1	0.743077	0.303826
52	0.678498	2.466219	2.475545	0.746784	1.476706	1.247916	0.897748	0.98133	0.780448
54	0.611274	3.092176	3.140738	0.689145	1.731664	1.519098	0.879122	0.779368	0.648598
60	0.541126	1.14347	1.092303	0.868146	1.153124	0.989705	0.894805	0.741915	0.128456
62	0.454422	1.370353	1.372305	0.765417	1.750607	1.458395	0.826046	0.654961	0.46565
64	0.352791	1.557271	1.565513	0.784195	2.468305	2.300523	0.860877	0.436235	0.162984
70	0.546357	1.417352	1.398745	0.732824	1.582065	1.335667	1	0.871597	0.012479
72	0.542894	2.175378	2.206199	0.624304	1.864014	1.765455	1	1.526439	1.84E-05
74	0.541127	2.658633	2.836827	0.420406	1.86309	2.2199	0.999999	1.70838	0.009323
80	0.515517	1.491421	1.481524	0.765263	1.238067	1.155418	1	0.731937	0.000222
82	0.49795	2.1224	2.193292	1	2.148548	0.003412	0.909371	0.975561	0.765919
84	0.459379	2.403327	2.452283	0.633831	2.126618	2.115856	0.880294	0.784308	0.665797
90	0.530417	1.608938	1.597545	0.683329	0.875931	0.799359	0.999999	0.722383	0.001191
92	0.492415	2.223645	2.281249	0.581021	1.08693	1.040191	0.988104	1.021027	0.855443
94	0.365044	2.578368	2.651608	0.466747	1.034492	0.997777	0.896884	1.17014	1.050771
100	0.467789	1.6754	1.725237	0.75928	1.181883	1.09211	1	0.925493	0.000723
102	0.396347	2.282537	2.437616	0.710674	1.892929	1.732937	1	1.40751	5.02E-06
104	0.413453	2.644633	2.741297	0.678761	2.667014	2.691135	0.939723	1.739104	0.995401
110	0.431965	1.39367	1.427748	0.740631	1.082647	0.951547	0.999999	1.045647	0.000902

112	0.37441	1.753772	1.838582	0.675297	1.717021	1.487494	0.979341	1.282063	1.10538
114	0.274218	1.828753	2.056505	1	3.071353	0.005116	0.815717	1.598065	1.135454
120	0.423863	1.455477	1.578389	0.768018	1.259688	1.192348	1	1.174186	0.000961
122	0.376701	2.228798	2.423733	0.650334	1.970154	1.749161	1	1.589436	0.001601
124	0.354433	2.065043	2.372114	0.704384	2.361548	2.300651	0.880914	2.149317	1.819679

	WT/HFA			**H235A/HFA**		
Time (min.)	***A***	***1/N₁***	***1/N₂***	***A***	***1/N₁***	***1/N₂***
0	0.945161	0.725583	0.181858	1	0.898667	0.000204
2	0.965401	1.005541	0.656357	0.968235	1.07902	0.530222
4	1	1.159075	0.008949	0.886892	1.190766	0.927102
10	1	0.868182	5.8E-05	0.894606	1.036291	5.85E-18
12	0.96481	1.071437	0.658561	0.83128	1.300555	3.32E-12
14	0.951534	1.145809	0.653076	1	1.210876	4.53E-05
20	1	0.725621	0.001237	1	0.881977	0.000471
22	1	1.134738	0.002652	0.964686	1.111413	3.78E-15
24	0.952616	1.222298	0.946975	1	1.099457	0.001561
30	0.983876	0.685002	0.514491	1	0.970006	0.000173
32	0.98766	1.042333	0.208162	0.973793	1.172602	3.14E-21
34	0.939498	1.496066	1.082957	0.971171	1.276436	0.946559
40	0.949875	0.759977	0.523123	1	0.779122	0.001612
42	0.970443	1.156323	0.805022	1	1.116043	0.000723
44	1	1.547012	8.54E-06	1	1.182743	0.00111
50	0.994111	0.659361	0.406274	1	0.993648	0.000192
52	0.87519	1.003246	0.484865	0.90948	1.000118	4.67E-16
54	0.94388	1.152533	0.805657	1	0.986361	4.31E-05
60	0.849895	1.050592	0.357474	1	0.959452	0.074657
62	0.967178	1.464316	1.161712	0.959343	1.302937	1.01795
64	0.907696	2.048811	1.706935	1	1.362294	0.000158
70	0.898698	1.062925	0.633882	0.87113	0.98107	0.006536
72	0.925836	1.565416	0.957143	1	1.051933	0.001491
74	0.92865	1.427071	0.803214	1	1.16655	0.059355
80	1	0.876077	0.008907	1	1.010966	0.62255
82	0.896636	1.286528	1.02555	1	1.305059	0.082775
84	0.896048	1.525714	1.377571	0.987809	1.469781	1.218406
90	0.866104	1.13953	0.811308	0.979379	1.025293	0.637573
92	0.931825	1.674297	1.364441	0.965298	1.248943	0.91338
94	0.940226	2.350921	1.712251	0.993159	1.325251	2.85E-10
100	0.888773	1.121199	0.678615	1	0.940541	0.000327
102	0.928082	1.761962	1.291017	0.998634	1.313473	1.035459
104	0.904959	2.031344	1.17192	0.958209	1.64646	1.284561
110	0.869361	1.298378	1.052581	0.899614	0.941668	0.262884

112	0.842574	1.770341	1.58468	0.998636	1.154911	0.916094
114	0.801608	2.202382	1.662532	0.916906	1.381025	1.078795
120	0.866622	1.047865	0.235573	1	0.967559	0.000948
122	0.954883	1.496476	0.64578	1	1.12059	0.000735
124	1	1.854046	0.006866	1	1.162132	0.000132

Table S8: Exponential fitting parameters A and k for un-cleaved substrate fraction plotted vs time for HFU and HFA substrates with Nsp15 variants in Figure 3B of the main text. The exponential fitting function used was $f(t) = Ae^{-kt}$.

	A	k
WT/HFU	1.00	0.00782
W333A/HFU	1.00	0.00528
H235A/HFU	1.00	0.000580
WT/HFA	1.00	0.000538
H235A/HFA	1.00	0.0000605

Table S9: FCS mixed model fit parameters for Figure 3C of the main text. Un-cleaved weighting (*A)* and *1/N* parameters (Eq. 2, 3 and 4) were used to fit FCS data using a custom Python script. The τ_D, A_c, and τ_C parameters were calculated from averages from fits of triplicate measurements at each timepoint.

	None/HFU			**Ca/HFU**		
Time (min.)	***A***	***1/N₁***	***1/N₂***	***A***	***1/N₁***	***1/N₂***
0	0.978143	0.563882	0.133927	0.720344	0.845379	0.014606
2	0.927396	0.473087	0.271847	0.937092	1.051839	0.390122
4	0.863136	0.40704	0.270609	0.804698	1.52394	1.180887
10	0.916366	0.998116	0.548782	0.824129	0.870962	0.229083
12	0.863062	1.130501	0.787175	0.77346	1.151128	0.683025
14	0.913608	1.113111	0.623331	0.640709	1.584499	1.393556
20	1	0.797807	0.000262	0.71556	0.822184	0.159531
22	0.665911	1.120813	0.930373	0.795254	1.097848	0.640262
24	0.5782	1.306543	1.22231	0.632149	1.591919	1.408289
30	--	--	--	0.701076	0.910043	0.431166

32	--	--	--	0.646809	1.126881	0.959102
34	--	--	--	0.512132	1.29601	1.186362
40	--	--	--	0.6362	0.907141	0.623807
42	--	--	--	0.625432	1.397933	1.222711
44	--	--	--	0.483231	1.812441	1.773751
50	1	0.829074	0.000529	0.587408	0.801691	0.482727
52	0.778396	0.790928	0.63913	0.624089	1.18432	0.974613
54	0.652932	0.836534	0.464594	0.577723	1.377664	1.154133
60	1	1.214166	0.005487	0.501933	0.963684	0.817588
62	0.672911	1.349022	1.235938	0.447516	1.186316	1.066005
64	0.68451	1.376504	1.231763	0.333875	1.391085	1.425079
70	0.830292	0.918635	0.627065	0.55019	0.967915	0.790069
72	0.560136	0.893345	0.605451	0.46725	1.340302	1.247061
74	0.578307	0.835218	0.631704	0.301918	1.613114	1.929096
80	0.853738	0.975817	0.647376	0.4492	0.972904	0.846276
82	0.69774	1.073053	0.890241	0.441485	1.316782	1.224726
84	0.588181	1.03966	0.894054	0.368957	1.562668	1.616257
90	1	0.829074	0.000529	0.38507	1.031653	0.939977
92	0.778398	0.790927	0.639135	0.413622	1.319817	1.239795
94	0.653092	0.83633	0.464807	0.2782	1.376883	1.577313
100	1	1.214166	0.005487	0.397651	0.999916	0.890791
102	0.67291	1.349024	1.235934	0.352905	1.19064	1.138025
104	0.684506	1.376512	1.231747	0.257958	1.230098	1.376479
110	0.830269	0.91866	0.626981	0.448016	1.093791	0.97139
112	0.560258	0.89315	0.605619	0.380282	1.410492	1.373486
114	0.57817	0.835417	0.631498	0.369528	1.49821	1.508974
120	0.853786	0.975762	0.647589	0.370881	0.964039	0.889207
122	0.697667	1.073166	0.890026	0.281138	1.017217	0.981731
124	0.588176	1.03967	0.894042	0.233097	0.939451	0.946328

	Mg/HFU			**Mn/HFU**		
Time (min.)	***A***	***$1/N_1$***	***$1/N_2$***	***A***	***$1/N_1$***	***$1/N_2$***
0	0.966871	0.833505	0.355509	0.918775	1.049153	0.341581
2	1	1.217822	0.003895	1	1.410299	0.000522
4	0.797154	1.604257	1.328537	0.989187	2.074396	0.332769
10	0.900881	1.17279	0.933257	0.874466	1.162832	0.553799
12	0.964916	1.677225	1.403225	0.705285	2.085438	1.169169
14	0.733687	2.646422	2.070428	0.604062	3.03841	3.127846
20	0.804267	1.459571	1.172673	0.745035	1.264345	0.913098
22	0.686695	2.09785	1.38735	0.63097	1.720997	1.55597
24	0.695075	2.628139	2.56432	0.567379	2.340053	2.35734
30	0.715159	1.604622	1.472382	0.831454	1.237747	0.95042

32	0.532395	2.2232	2.24908	0.691747	1.902548	1.672259
34	0.667584	2.561189	2.514692	0.590819	2.648622	2.696693
40	0.594409	1.566522	1.429931	0.802284	1.306694	1.041077
42	0.585198	2.195452	2.142783	0.750122	1.801755	1.662562
44	0.421111	2.880354	2.973739	0.985464	0.494898	0.12596
50	0.569022	1.66826	1.568093	0.67983	1.449877	1.251156
52	0.584004	2.21536	2.168736	0.605615	2.2016	1.808055
54	0.421521	2.543506	2.721416	0.532079	2.736116	2.808223
60	0.535552	1.854456	1.812959	0.667599	1.348711	1.154516
62	0.576591	2.749407	2.806908	0.709167	1.865875	1.669234
64	0.596617	4	4	0.562251	2.51384	2.56161
70	0.38805	2.26067	2.314116	0.697407	1.297561	0.895206
72	0.477119	2.828046	2.899675	0.61227	2.475737	2.484762
74	0.412281	3.686555	3.739222	0.491426	2.431568	2.54826
80	0.398575	1.860296	2.039124	0.608885	1.42923	1.27016
82	0.463233	3.066925	3.143268	0.603144	2.026846	1.874317
84	0.636938	4	4	0.589447	2.466928	2.492181
90	0.366469	1.821662	2.072557	0.580577	1.369218	1.14866
92	0.413071	2.978695	3.063476	0.56113	1.853877	1.783193
94	0.254019	2.901504	3.151362	0.548534	2.317726	2.351417
100	0.366191	2.258506	2.332951	0.567157	1.327107	1.17853
102	0.380656	3.527267	3.603134	0.624567	2.580269	2.602831
104	0.746721	4	4	0.532198	2.268392	2.311312
110	0.311525	2.366991	2.443878	0.539834	1.215148	1.086279
112	0.24794	3.509634	3.574931	0.437077	1.51403	1.464919
114	0.244462	3.972455	3.979269	0.39608	1.56559	1.558159
120	0.348208	2.419589	2.569113	0.486097	1.338588	1.244522
122	0.281762	3.612214	3.673637	0.395449	1.428204	1.383096
124	0.916625	4	4	0.393478	1.386817	1.334223

Table S10: Exponential fitting parameters A and k for un-cleaved substrate fraction plotted vs time (Figure 3C, main text). The exponential fitting function used was $f(t) = Ae^{-kt}$.

	A	***k***
None	1.00	0.00131
Ca^{2+}/HFU	1.00	0.00587
Mg^{2+}/HFU	1.00	0.00823
Mn^{2+}/HFU	1.00	0.00473

Table S11: Gaussian fit parameters of FRET distributions in Figure 4 of the main text.

	Avg. FRET	% Population	Width
		MFA	
None/MFA			
Fraction 1	0.81	--	0.06
Ca/MFA			
Fraction 1	0.79	--	0.04
Mg/MFA			
Fraction 1	0.85	--	0.05
Mn/MFA			
Fraction 1	0.78	--	0.07
None/MFA			
Fraction 1	0.81	--	0.06
		MFU	
None/MFU			
Fraction 1	0.58	--	0.10
Ca/MFU			
Fraction 1	0.74	0.56	0.11
Fraction 2	0.60	0.44	0.20
Mg/MFU			
Fraction 1	0.77	0.64	0.09
Fraction 2	0.65	0.36	0.14
Mn/MFU			
Fraction 1	0.76	0.51	0.12
Fraction 2	0.54	0.49	0.20

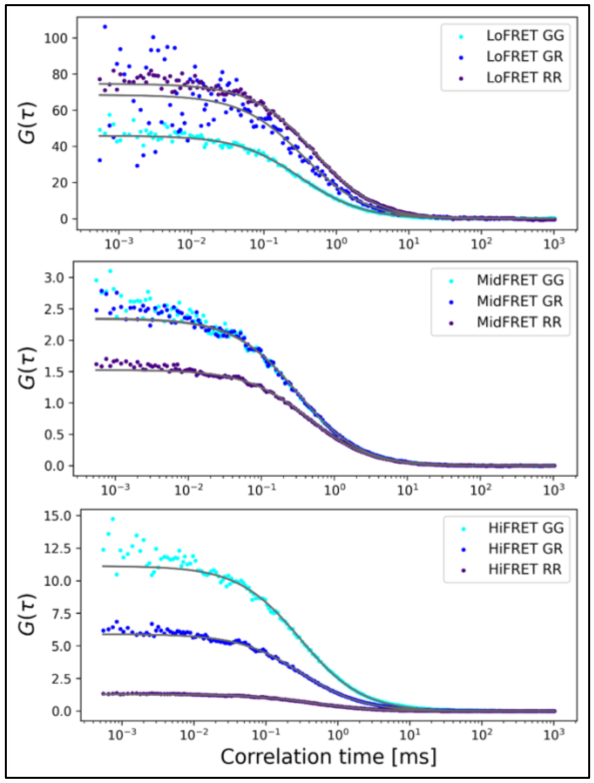

Figure S6: Representative FCS data for Lo FRET, Mid FRET, and High FRET benchmark control oligos (see Table S1 for sequences). Fitting was performed in Python using Equation 2 as a fitting model. The annealed oligos were diluted to 1 – 10 nM in 1X PBS and 20-minute acquisitions were collected. The fit parameters are in Table S12.

Table S12: Fit parameters for Lo FRET, Mid FRET, and High FRET benchmark oligo control data from Figure S6. Data were fit in Python using Equation 2 as a fitting model.

Sample	***1/N***	**τ_D (ms)**
Lo FRET		
Lo FRET_GG	45.778	0.322
Lo FRET_GR	68.342	0.365
Lo FRET_RR	74.355	0.511
Mid FRET		
Mid FRET_GG	2.348	0.311
Mid FRET_GR	2.339	0.315
Mid FRET_RR	1.525	0.454
Hi FRET		
Hi FRET_GG	11.123	0.304
Hi FRET_GR	5.900	0.310
Hi FRET_RR	1.279	0.436

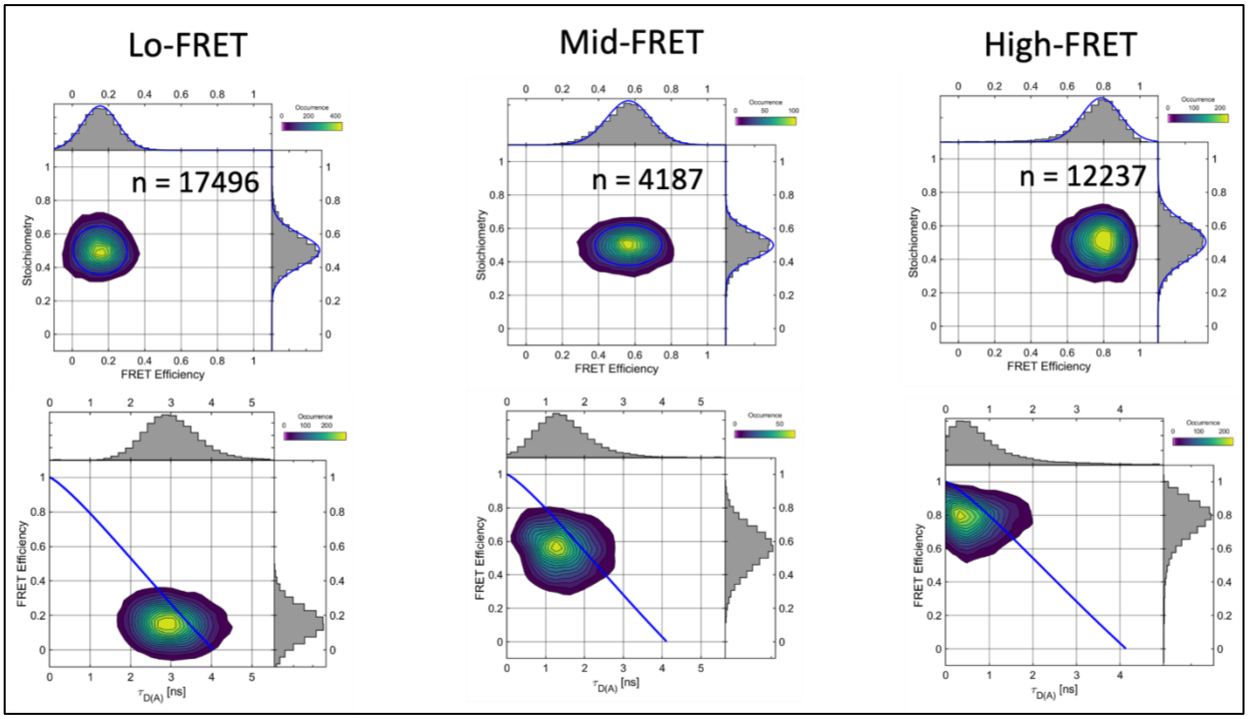

Figure S7: Stoichiometry vs FRET efficiency (*E*-*S*) and FRET efficiency vs donor lifetime (E-$\tau_{D(A)}$) histograms for: Lo-, Mid-, and High-FRET benchmark oligos[1]. See Table S1 for sequences. The populations of photon bursts for benchmark oligo samples are centered on or near the theoretical static FRET line (blue) due to static behavior, in stark contrast to the RNA substrates (Figure 5A in the main text) that exhibit microsecond timescale dynamics. These FRET efficiency vs donor lifetime plots support the interpretation that the Nsp15 substrates MFU and MFA are highly dynamic. The FRET efficiency for 2D *E*-*S* plots were calculated with Eq. 10 in the main text.

Table S13: FCS fitting parameters for Figure 5B (I_{GR}, which represents FRET signal) fit in SymPhoTime. All curves were fit to a conformational model, Eq. 3, with $\beta_C = 1$.

	$1/N$	τ_D (ms)	A_c	τ_c (μs)
None/MFA	0.413	0.487	0.501	4.1
Ca^{2+}/MFA	0.172	0.477	0.707	4.7
Mg^{2+}/MFA	0.461	0.525	0.783	4.9
Mn^{2+}/MFA	0.340	0.499	1.1	5.3
None/MFU	0.488	0.457	0.42	4.3
Ca^{2+}/MFU	0.671	0.469	0.71	4.9
Mg^{2+}/MFU	0.719	0.478	0.88	4.6
Mn^{2+}/MFU	0.719	0.450	1.3	4.6

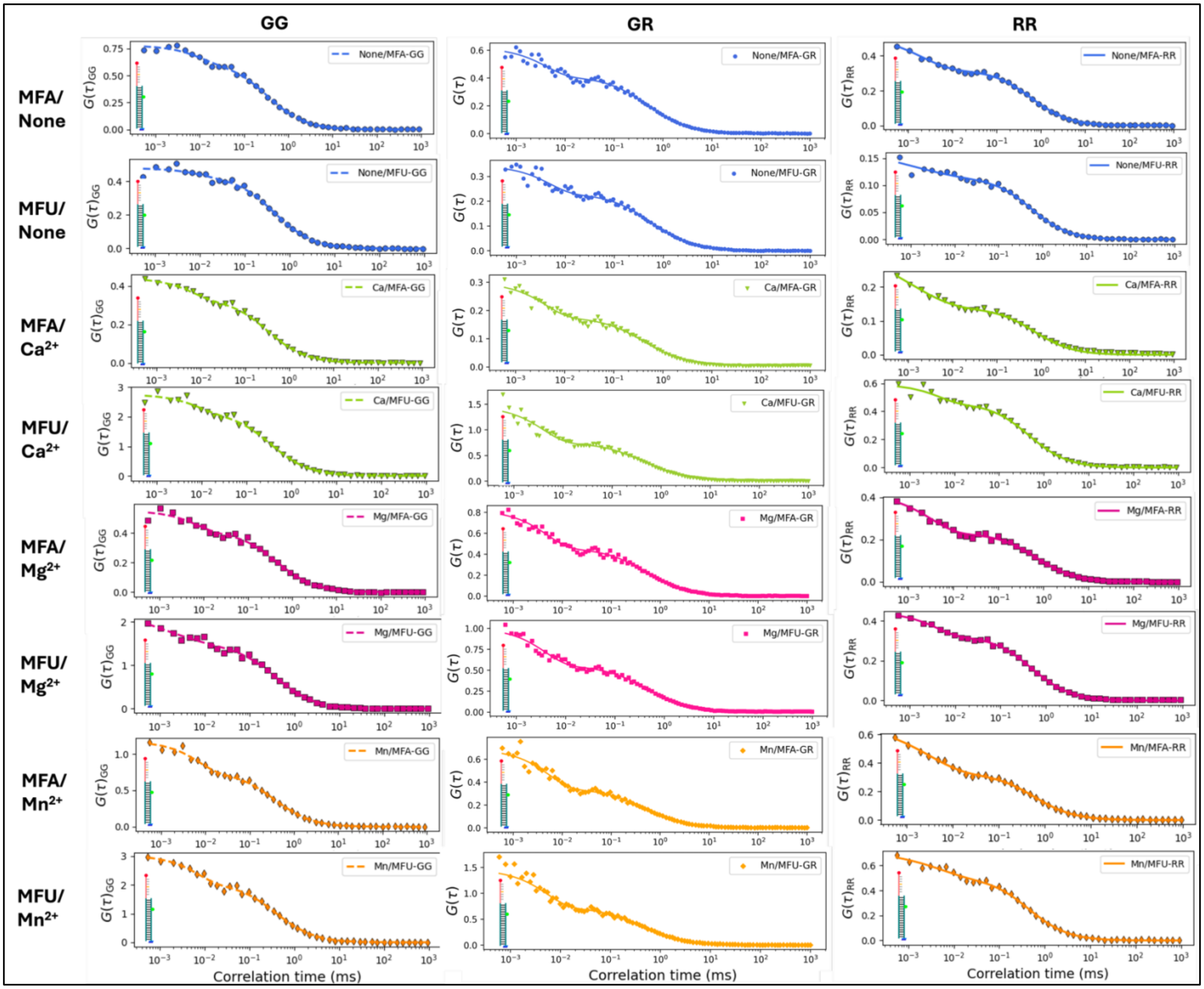

Figure S8: Autocorrelations of I_{GG}, I_{GR}, and I_{RR} signals for FCS data shown in Figure 5B of the main text. In the main text, we show only fits to the autocorrelations of I_{GR}, which represents FRET signal. Each curve was fit to a conformational model, Eq. 3, and the fit parameters are in Table S14.

Table S14: Fit parameters for autocorrelation curves in Figure S8. All curves were fit to a conformational model, Eq. 3, with $\beta_C = 1$. Slight variations in the parameters for different signals may result from the presence of some unannealed oligos. I_{GG} and I_{RR} signals may contain contributions from unannealed oligos.

None									
	$1/N$	τ_D (ms)	A_c	τ_c (μs)		$1/N$	τ_D (ms)	A_c	τ_c (μs)
MFA					**MFU**				
GG	0.629	0.338	0.266	6.9	**GG**	0.427	0.461	0.24	7.0
GR	0.413	0.487	0.501	4.1	**GR**	0.488	0.457	0.42	4.3
RR	0.316	0.629	0.422	4.0	**RR**	0.115	0.572	0.184	7.7
Ca^{2+}									
MFA					**MFU**				
GG	0.348	0.309	0.277	5.7	**GG**	2.16	0.34	0.279	7.2
GR	0.172	0.477	0.707	4.7	**GR**	0.671	0.469	0.71	4.9
RR	0.137	0.663	0.591	4.8	**RR**	0.455	0.510	0.320	5.8
Mg^{2+}									
MFA					**MFU**				
GG	0.411	0.44	0.344	6.1	**GG**	1.859	0.392	0.428	4.8
GR	0.461	0.525	0.783	4.9	**GR**	0.719	0.478	0.88	4.6
RR	0.35	0.718	0.636	4.9	**RR**	0.318	0.530	0.311	5.6
Mn^{2+}									
MFA					**MFU**				
GG	0.763	0.347	0.576	5.8	**GG**	2.1	0.359	0.515	5.1
GR	0.340	0.499	1.1	5.3	**GR**	0.719	0.450	1.3	4.6
RR	0.31	0.637	0.74	5.9	**RR**	0.498	0.481	0.341	6.8

REFERENCES

1. Hellenkamp, B. *et al.* Precision and accuracy of single-molecule FRET measurements—a multi-laboratory benchmark study. *Nat Methods* **15,** 669–676 (2018).