Supporting Information.

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Table S1: Summary of US simulations carried out for this study: US flavor, fosmidomycin orientation, initial pulling direction, number of umbrella windows N_{win}, simulation time per window t_{sim}, number of parallel replicas during US-HREX, number of repetitions (including initial pulling).

US flavor	orientation	Pulling dir	N_{win}	t_{sim} (ns)	N_{repl}	Repetitions
Standalone US	1	EC-to-PP	144	200	-	2
	1	PP-to-EC	144	200	-	2
US-HREX	1	EC-to-PP	144	9	24	2
	1	PP-to-EC	144	9	24	2
STeUS	1	EC-to-PP	144	200	-	2
	1	PP-to-EC	144	200	-	2
REUS	1	EC-to-PP	144	200	-	2
	1	PP-to-EC	144	200	-	2
	2	EC-to-PP	144	200	-	2
	2	PP-to-EC	144	200	-	2

A

umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)
-3.35	-2.64	-1.95	-1.23	-0.54	0.21	0.89	1.59	2.28	2.98
-3.31	-2.60	-1.91	-1.18	-0.48	0.26	0.94	1.63	2.33	3.02
-3.26	-2.55	-1.86	-1.12	-0.41	0.30	0.99	1.68	2.38	3.07
-3.21	-2.51	-1.81	-1.08	-0.34	0.35	1.04	1.72	2.42	3.11
-3.16	-2.46	-1.75	-1.06	-0.32	0.38	1.09	1.77	2.47	3.16
-3.12	-2.41	-1.71	-1.01	-0.28	0.43	1.13	1.82	2.51	3.20
-3.07	-2.37	-1.66	-0.95	-0.22	0.48	1.17	1.86	2.56	3.25
-3.02	-2.32	-1.62	-0.90	-0.17	0.52	1.21	1.91	2.61	3.29
-2.97	-2.27	-1.57	-0.86	-0.14	0.57	1.26	1.96	2.65	3.34
-2.93	-2.23	-1.52	-0.81	-0.09	0.61	1.31	2.01	2.70	
-2.88	-2.19	-1.47	-0.76	-0.04	0.66	1.36	2.05	2.75	
-2.83	-2.14	-1.41	-0.71	0.00	0.70	1.41	2.10	2.79	
-2.78	-2.09	-1.36	-0.65	0.05	0.75	1.45	2.15	2.84	
-2.74	-2.04	-1.32	-0.61	0.10	0.80	1.49	2.19	2.88	
-2.69	-1.99	-1.27	-0.57	0.15	0.84	1.54	2.23	2.93	

B

umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)
-3.40	-2.67	-1.96	-1.26	-0.56	0.12	0.85	1.58	2.27	2.97
-3.35	-2.62	-1.91	-1.22	-0.51	0.19	0.89	1.62	2.32	3.01
-3.30	-2.58	-1.87	-1.18	-0.47	0.25	0.94	1.67	2.37	3.06
-3.25	-2.53	-1.82	-1.13	-0.42	0.29	1.00	1.72	2.41	3.10
-3.20	-2.48	-1.77	-1.09	-0.37	0.33	1.05	1.76	2.46	3.15
-3.16	-2.43	-1.73	-1.04	-0.34	0.38	1.10	1.81	2.51	3.19
-3.11	-2.38	-1.69	-0.98	-0.30	0.43	1.14	1.86	2.55	3.24
-3.06	-2.34	-1.64	-0.94	-0.25	0.47	1.19	1.91	2.60	3.29
-3.01	-2.30	-1.60	-0.89	-0.20	0.52	1.24	1.95	2.64	3.34
-2.96	-2.24	-1.56	-0.85	-0.15	0.56	1.28	2.00	2.69	
-2.91	-2.20	-1.50	-0.80	-0.10	0.61	1.33	2.04	2.74	
-2.87	-2.15	-1.46	-0.74	-0.06	0.66	1.39	2.08	2.79	
-2.82	-2.10	-1.41	-0.70	-0.01	0.71	1.44	2.13	2.83	
-2.77	-2.05	-1.36	-0.66	0.03	0.77	1.48	2.17	2.87	
-2.72	-2.00	-1.31	-0.62	0.08	0.81	1.53	2.22	2.92	

C

umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)	umbrella window centers (nm)
-3.45	-2.72	-2.01	-1.31	-0.59	0.10	0.81	1.52	2.22	2.93
-3.40	-2.67	-1.97	-1.26	-0.55	0.14	0.86	1.57	2.27	2.98
-3.35	-2.62	-1.92	-1.21	-0.50	0.20	0.90	1.62	2.32	3.02
-3.30	-2.58	-1.87	-1.17	-0.45	0.27	0.95	1.66	2.37	3.07
-3.25	-2.53	-1.82	-1.12	-0.40	0.33	0.99	1.71	2.42	3.12
-3.20	-2.48	-1.77	-1.07	-0.36	0.37	1.04	1.75	2.46	3.16
-3.16	-2.43	-1.72	-1.03	-0.32	0.40	1.09	1.80	2.50	3.21
-3.11	-2.38	-1.68	-0.97	-0.27	0.44	1.15	1.84	2.55	3.26
-3.06	-2.34	-1.63	-0.92	-0.22	0.49	1.20	1.89	2.60	3.31
-3.01	-2.29	-1.59	-0.88	-0.17	0.54	1.24	1.94	2.65	
-2.96	-2.24	-1.54	-0.83	-0.13	0.57	1.29	1.99	2.70	
-2.91	-2.20	-1.49	-0.79	-0.08	0.62	1.33	2.03	2.75	
-2.87	-2.15	-1.44	-0.74	-0.03	0.67	1.38	2.08	2.79	
-2.82	-2.11	-1.39	-0.70	0.01	0.71	1.43	2.13	2.84	
-2.77	-2.06	-1.36	-0.64	0.05	0.76	1.48	2.17	2.88	

Figure S1: Umbrella window centers for EC-to-PP (A) and PP-to-EC (B) in orientation 1, and for both EC-to-PP and PP-to-EC in orientation 2 (C). The same window centers were used for all umbrella sampling flavor.

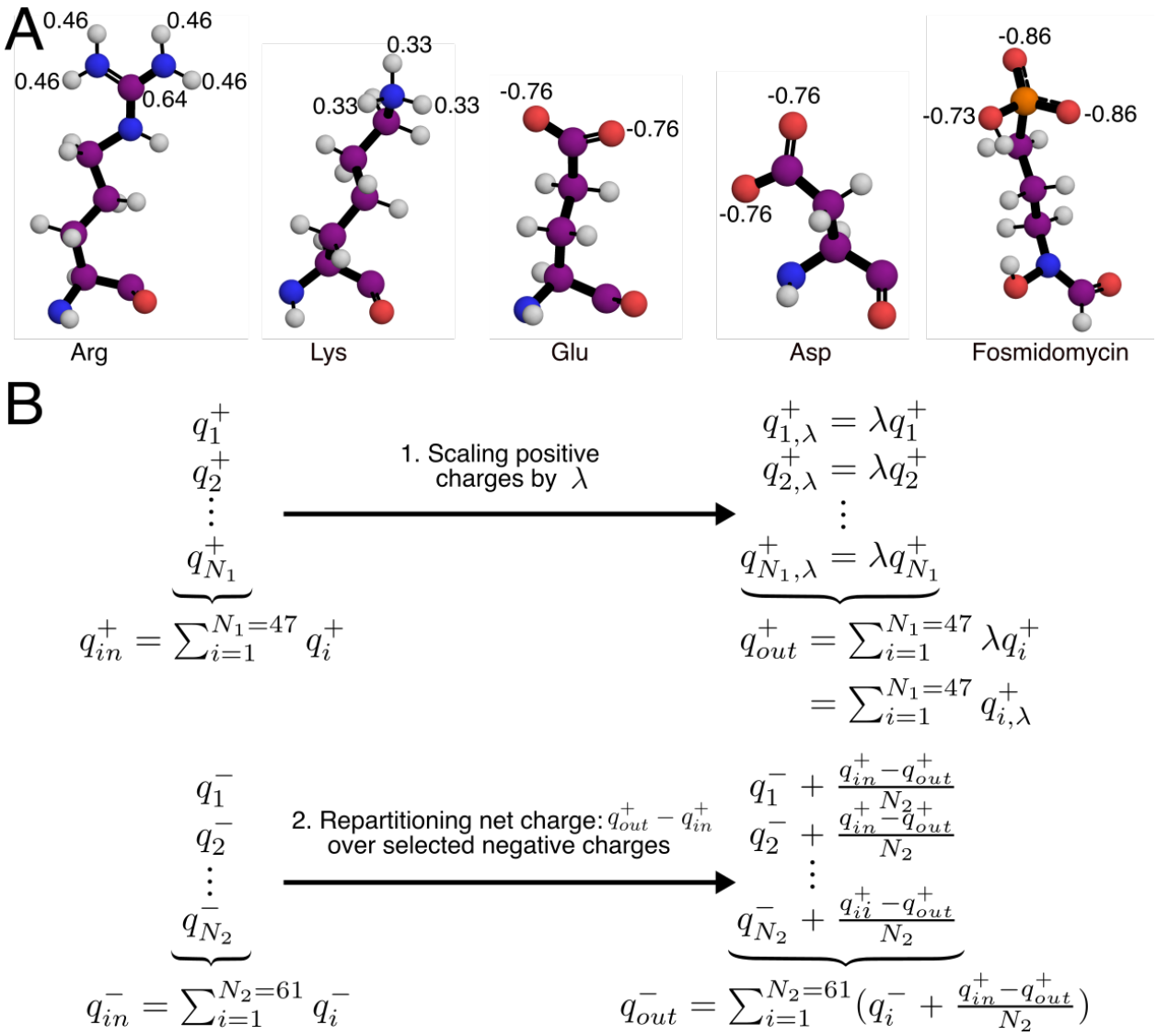

Figure S2: Hamiltonian replica-exchange protocol. (A) Residue types involved in charge scaling are depicted as balls and sticks, partial charges involved in charge scaling are written next to the respective atoms. (B) Scheme of our scaling protocol. First, positive charges q_1^+ through $q_{N_1}^+$ were scaled by a factor λ, leading to a net charge of $q_{out}^+ - q_{in}^+$. To maintain charge neutrality, a set of N_2 negative charges was corrected by $-(q_{out}^+ - q_{in}^+)/N_2$.

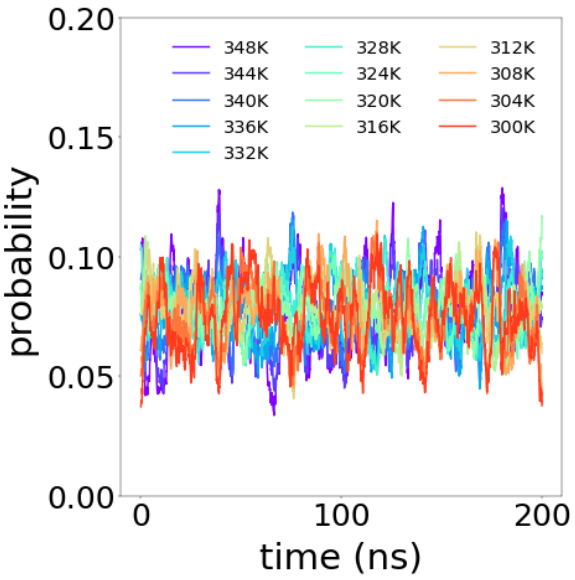

Figure S3: Temperature state probabilities versus time for window $z = 0\,\mathrm{nm}$, revealing approximately uniform probabilities for all temperature states. Curves were smoothed with the Scipy module uniform_filter1d[1] with a filter size of 500 points.

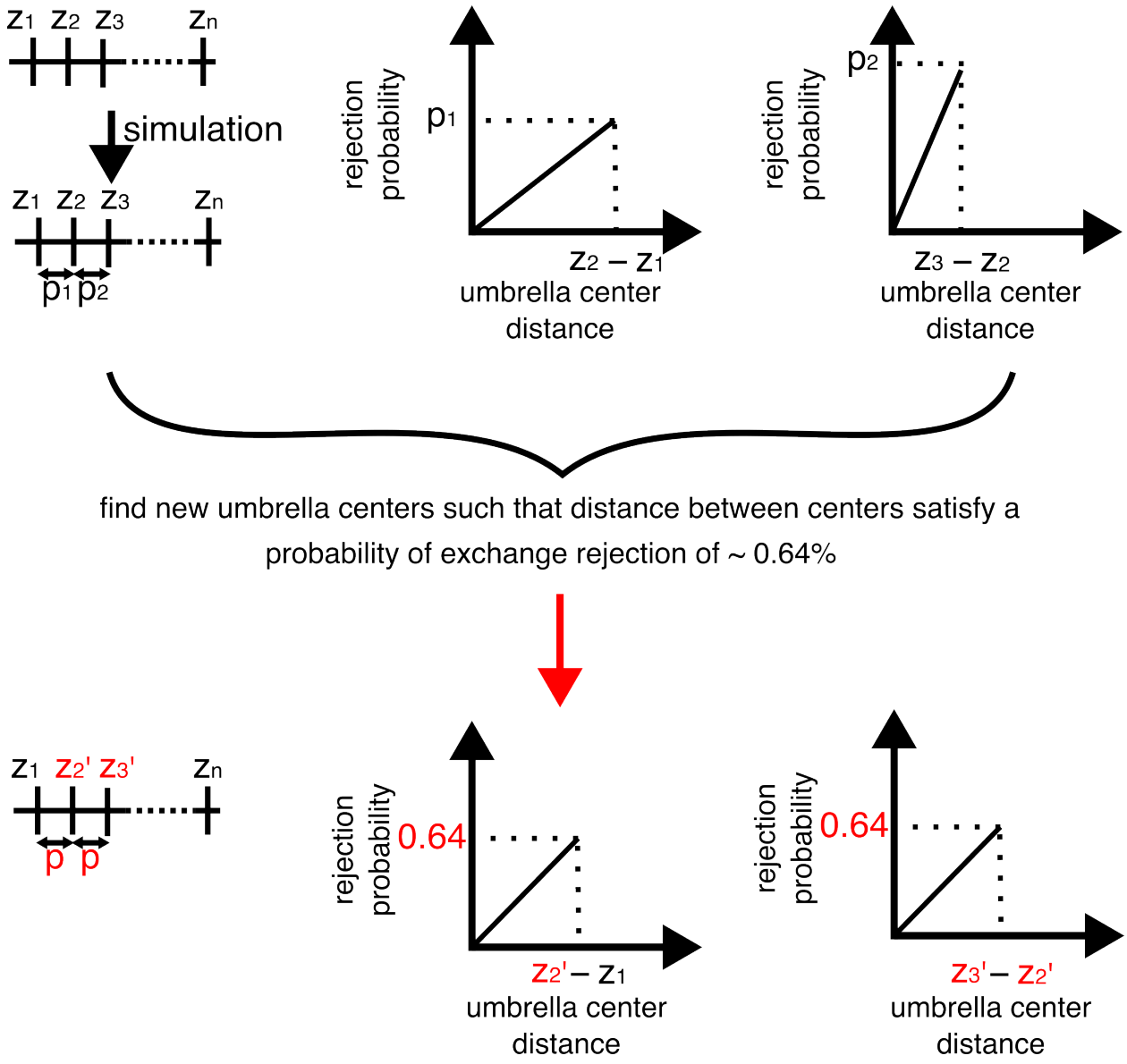

Figure S4: Protocol for optimizing the distance between neighboring umbrella windows for REUS. Series of simulations were carried out to compute average probabilities of exchange rejection between windows. From these probabilities, linear relationships between probabilities and distances between US windows were assumed. From this linear relationships, we selected centers of umbrella windows such that a rejection probability of approx. 0.64% was expected (acceptance probability of approx. 0.36%).

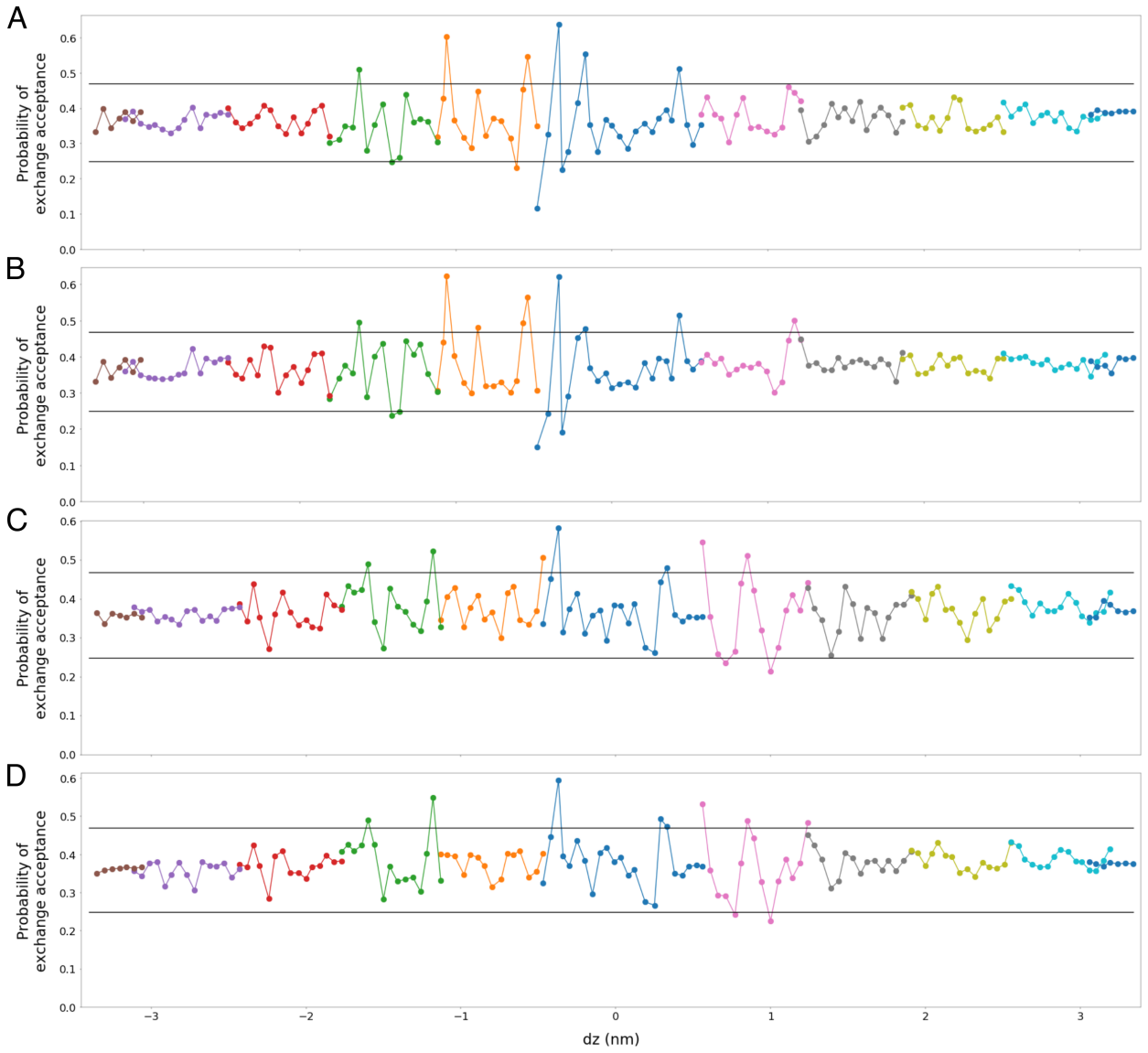

Figure S5: Average exchange probabilities between umbrella windows after 200 ns of production simulation for two forward replicates (A–B), and two reverse replicates (C–D) in orientation 1. Umbrella windows that were allowed to exchange configurations are shown with the same color. Each point shows the average exchange probability between the umbrella window centered at the respective z value with the preceding umbrella window along the z-axis. Probability region between the two horizontal black lines indicates the target region used for optimizing the umbrella window spacing.

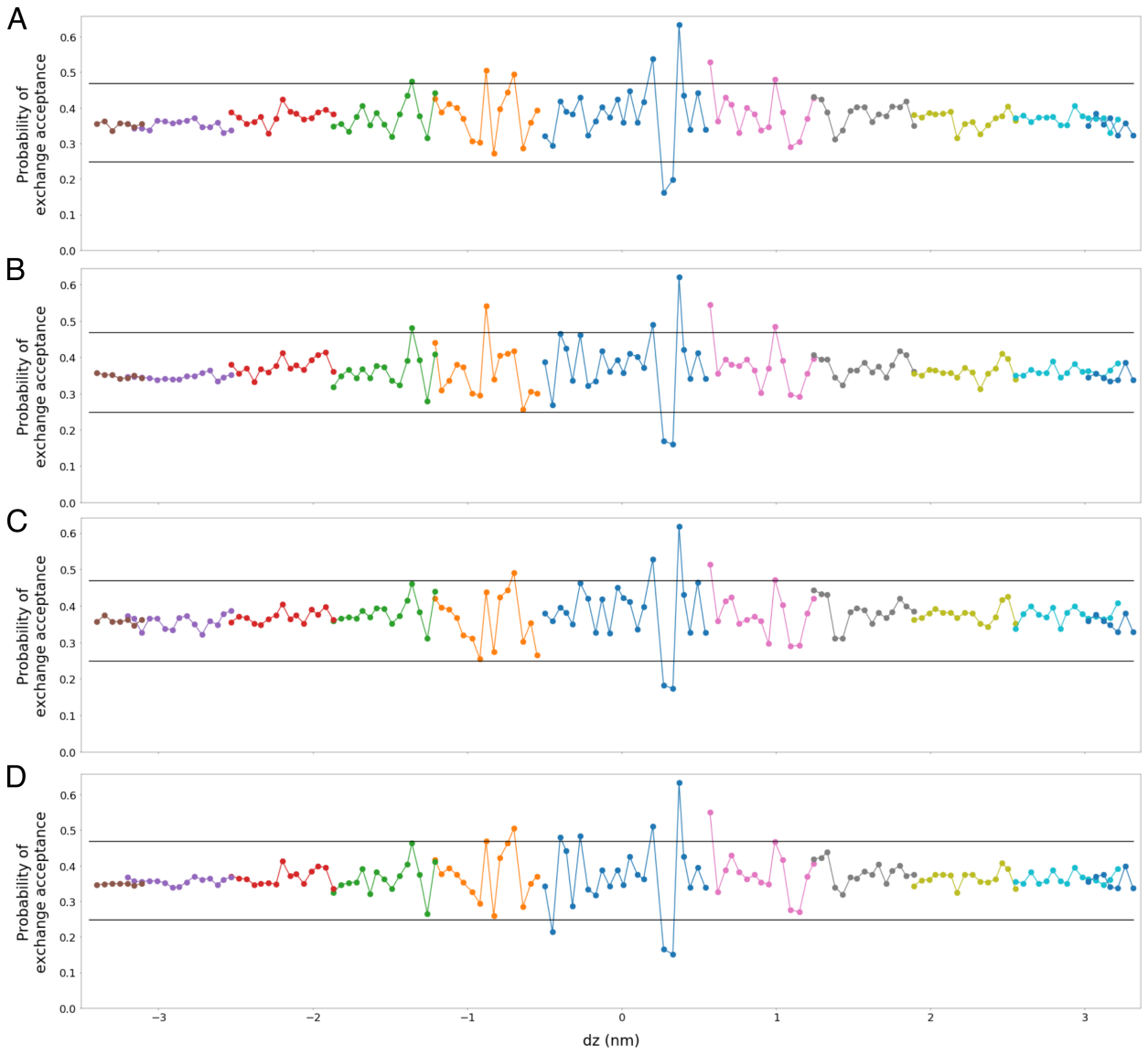

Figure S6: Average exchange probabilities between umbrella windows after 200 ns of production simulation for two forward replicates (A–B), and two reverse transition (C–D) in orientation 2. For more details, see legend of Fig. S5

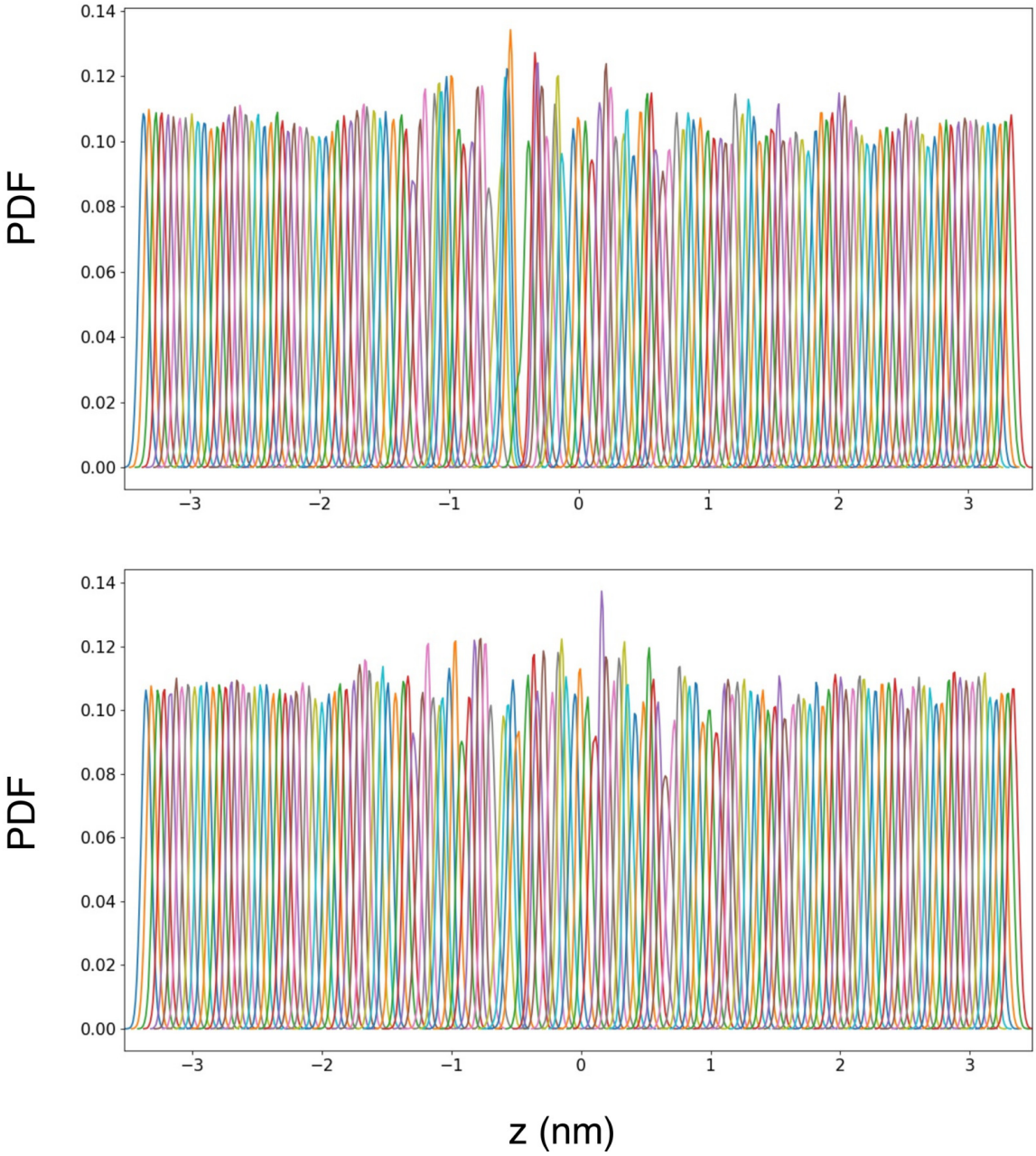

Figure S7: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 1, PP-to-EC, replica 1.

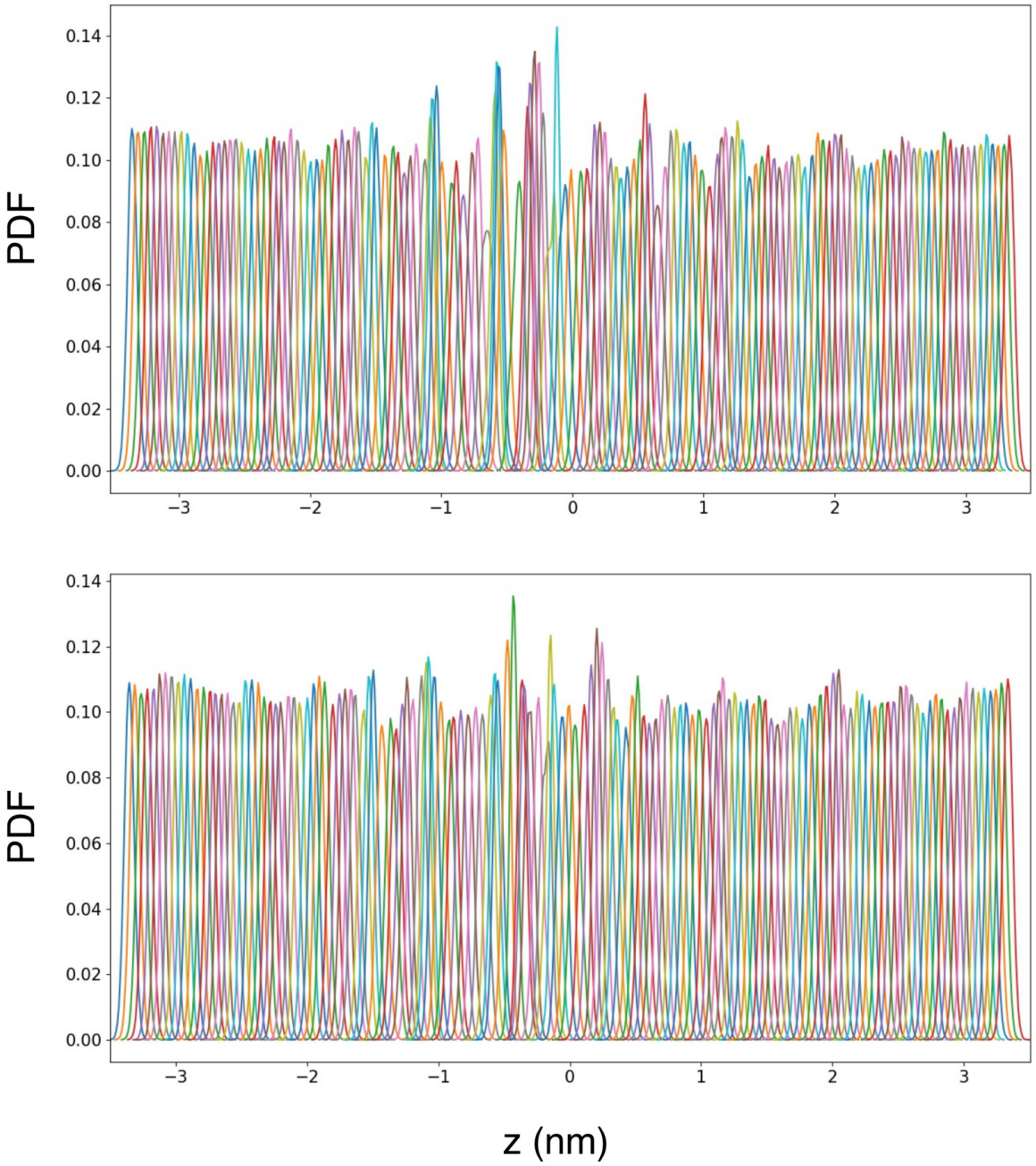

Figure S8: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 1, PP-to-EC, replica 2.

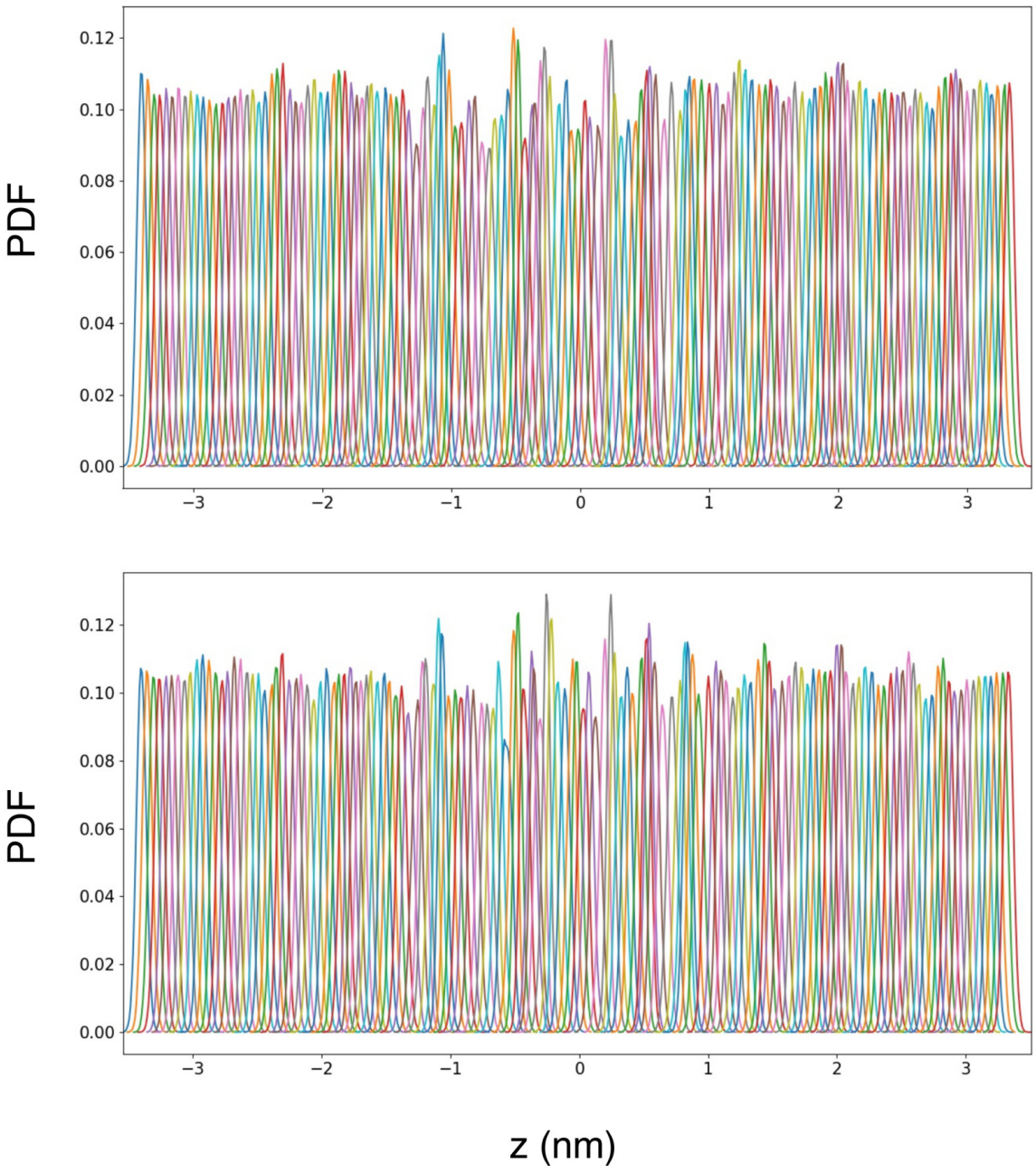

Figure S9: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 1, EC-to-PP, replica 1.

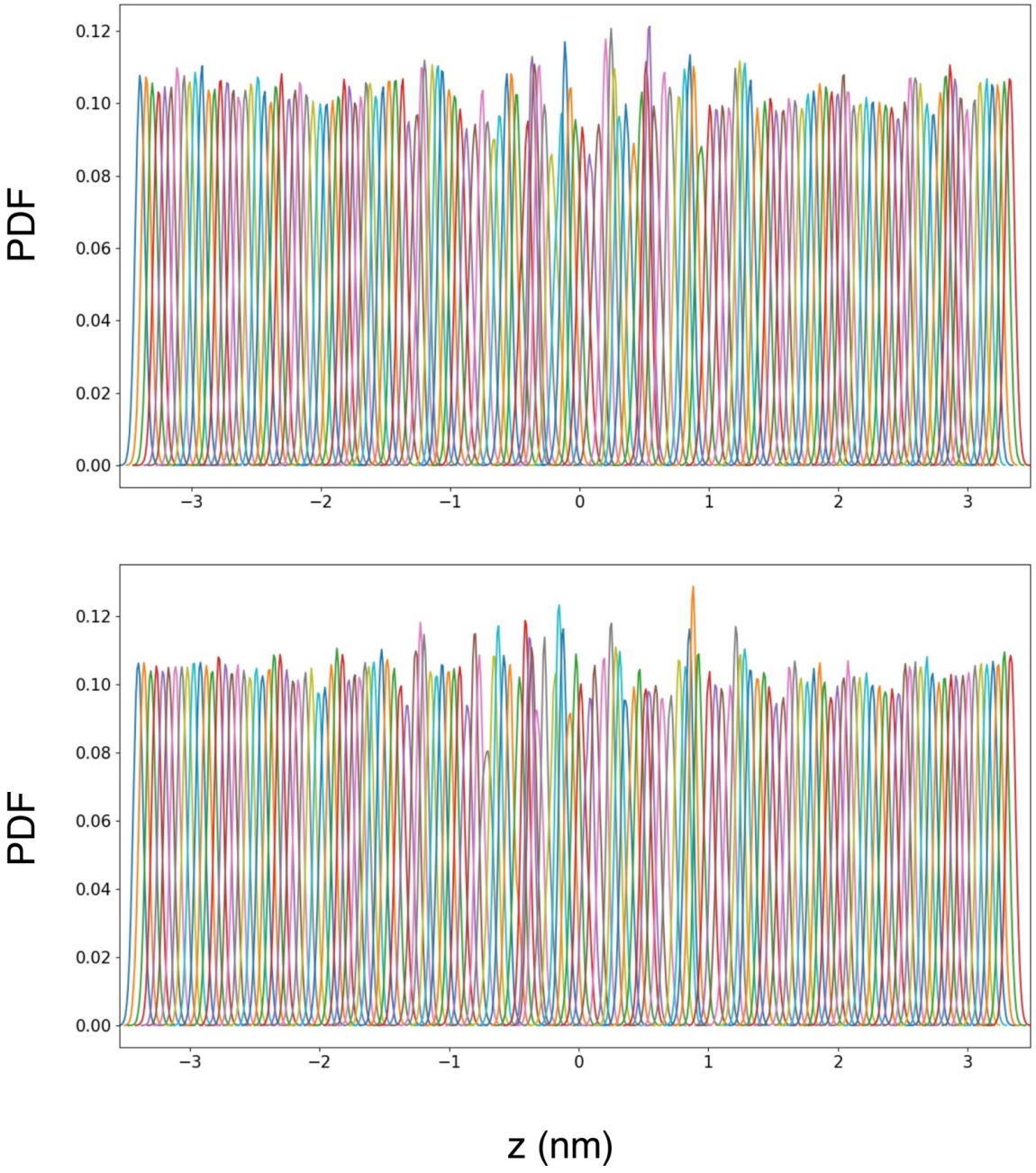

Figure S10: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 1, EC-to-PP, replica 2.

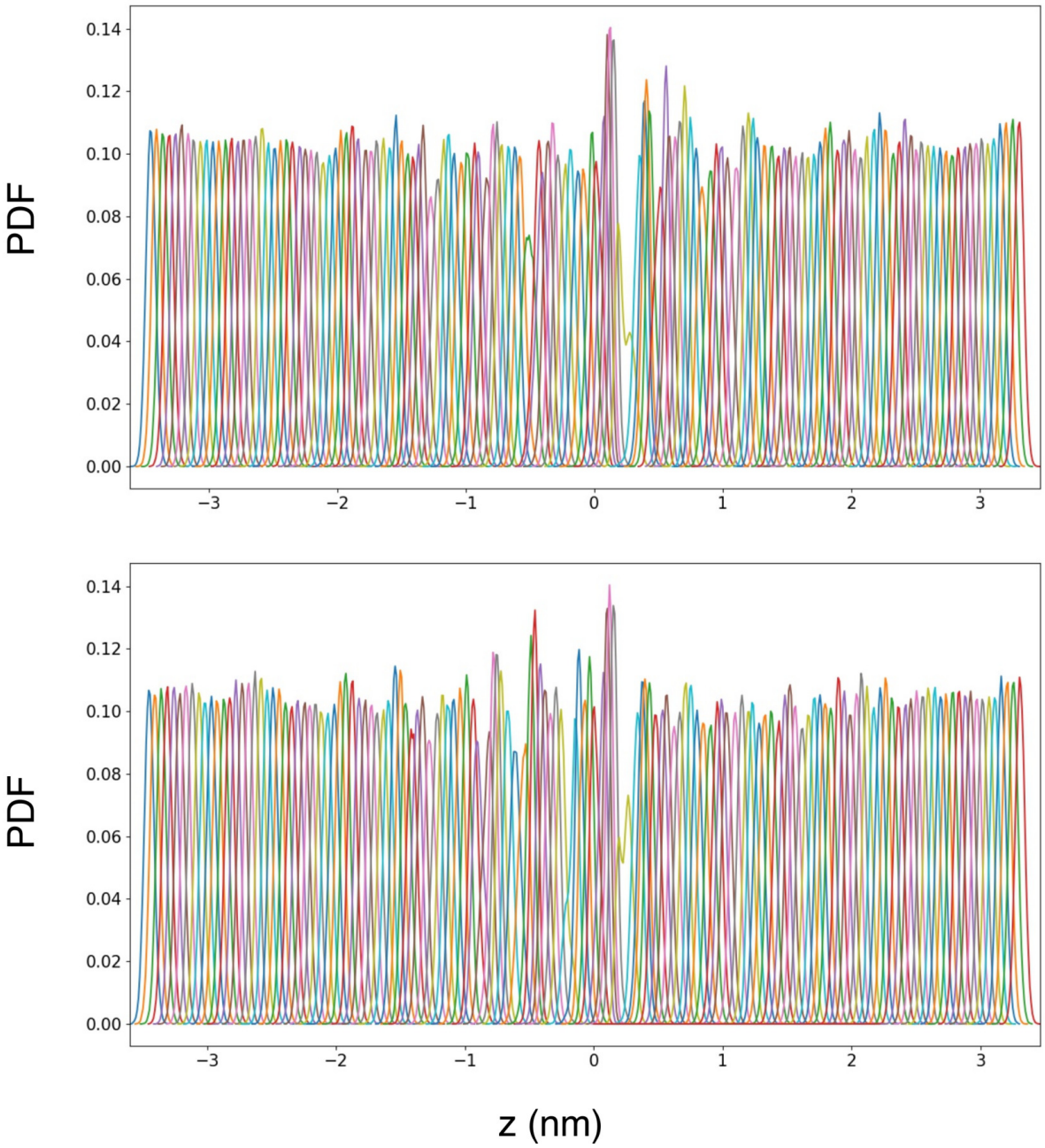

Figure S11: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 2, PP-to-EC, replica 1.

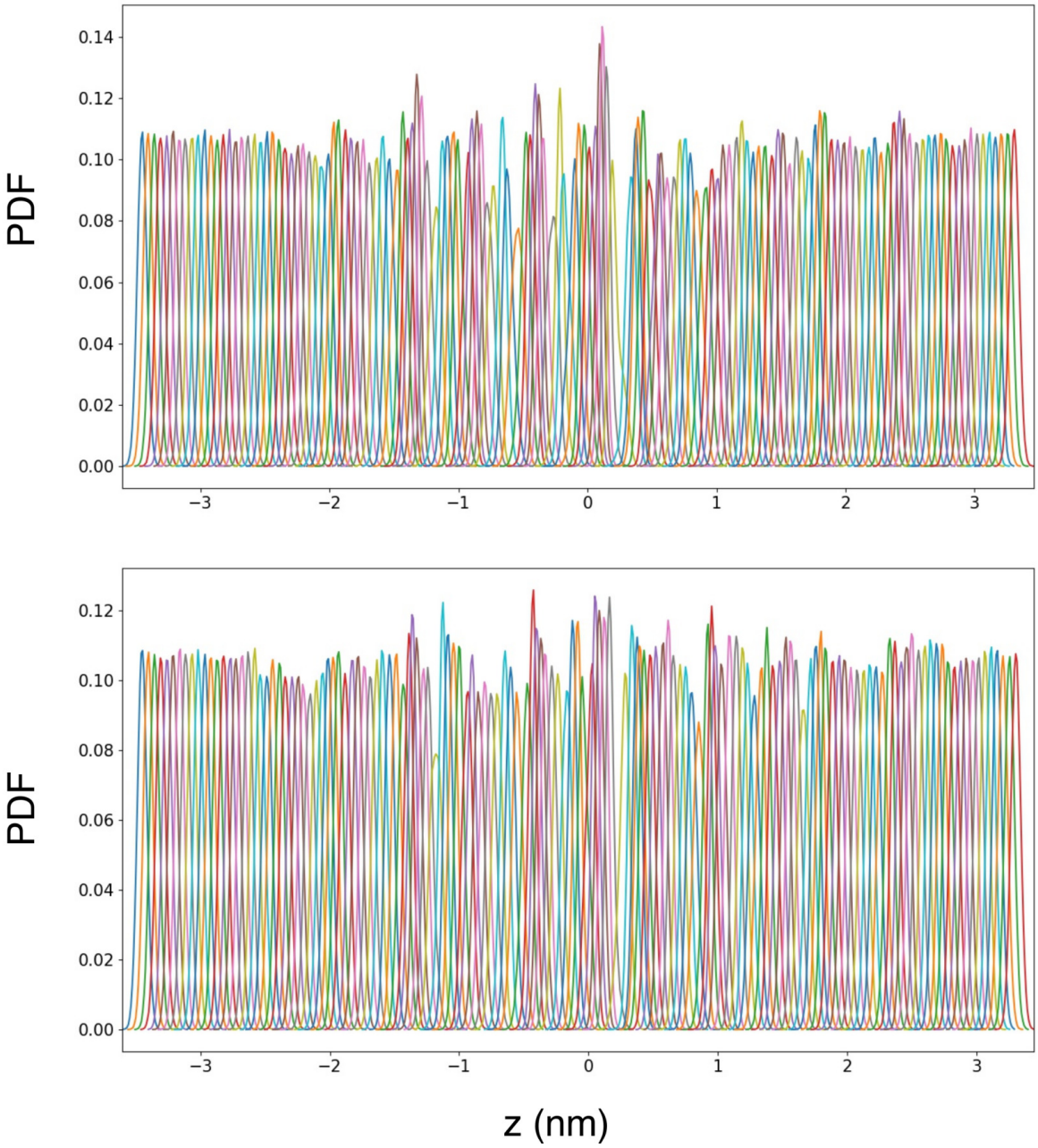

Figure S12: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 2, PP-to-EC, replica 2.

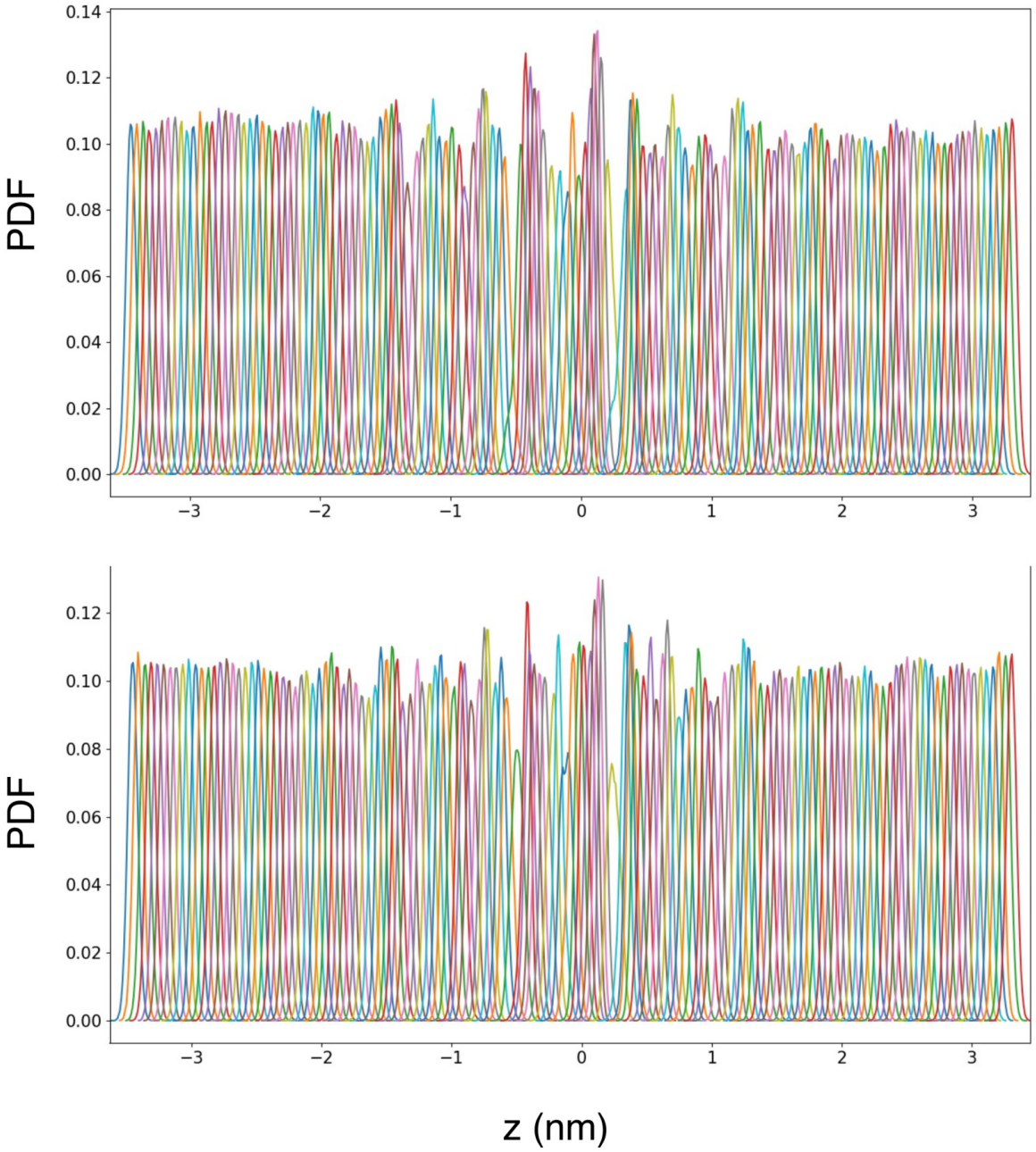

Figure S13: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 2, EC-to-PP, replica 1.

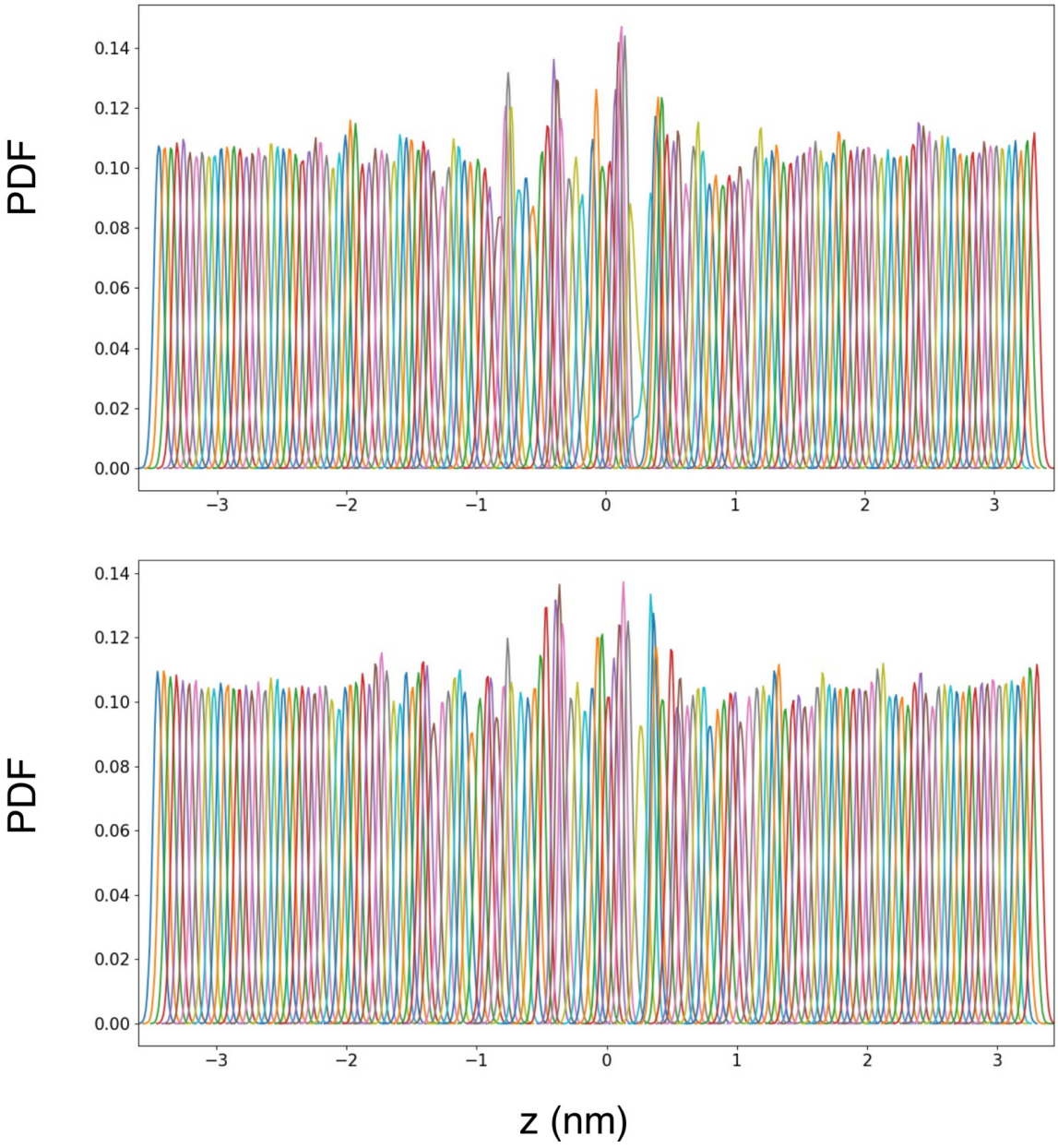

Figure S14: Umbrella windows during the first 100 ns (upper graph) and last 100 ns (lower graph) for REUS in orientation 2, EC-to-PP, replica 2.

References

(1) Virtanen, P. et al. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat. Methods* **2020**, *17*, 261–272.