Nanosecond chain dynamics of single-stranded nucleic acids

Mark F. Nüesch^a, Lisa Pietrek^b, Erik D. Holmstrom^{a,c,d*}, Daniel Nettels^a, Valentin von Roten^a, Rafael Kronenberg-Tenga^a, Ohad Medalia^a, Gerhard Hummer^{b,e*}, Benjamin Schuler^{a,f*}

^aDepartment of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

^bDepartment of Theoretical Biophysics, Max Planck Institute of Biophysics, Max-von-Laue Straße 3, 60438 Frankfurt am Main, Germany

^eInstitute for Biophysics, Goethe University Frankfurt, 60438 Frankfurt am Main, Germany

^fDepartment of Physics, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Abstract: The conformational dynamics of single-stranded nucleic acids are fundamental for nucleic acid folding and function. However, their elementary chain dynamics have been difficult to resolve experimentally. Here we employ a combination of singlemolecule Förster resonance energy transfer, nanosecond fluorescence correlation spectroscopy, fluorescence lifetime analysis, and nanophotonic enhancement to determine the conformational ensembles and rapid chain dynamics of short single-stranded nucleic acids in solution. To interpret the experimental results in terms of end-to-end distance dynamics, we utilize the hierarchical chain growth approach, simple polymer models, and refinement with Bayesian inference of ensembles to generate structural ensembles that closely align with the experimental data. The resulting chain reconfiguration times are exceedingly rapid, in the 10-ns range. Solvent viscosity-dependent measurements indicate that these dynamics of single-stranded nucleic acids exhibit negligible internal friction and are thus dominated by solvent friction. Our results provide a detailed view of the conformational distributions and rapid dynamics of singlestranded nucleic acids.

Keywords: Single-molecule Förster resonance energy transfer, FRET, Molecular simulations, Zero-mode waveguides, Nanophotonics

Nucleic acids and proteins have very different chemical compositions. However, as linear biopolymers, both can sample a myriad of chain configurations, and the resulting dynamics play an essential role in their folding and function. The chain dynamics of unfolded and disordered proteins have been characterized extensively with a broad range of methods²⁻⁹ because of their importance for protein folding^{10, 11} and the behavior of intrinsically disordered proteins¹². The chain dynamics of single-stranded nucleic acids (ssNAs) are less well characterized, despite their importance for many biological processes, particularly those associated with gene expression and RNA folding¹³. While doublestranded nucleic acids are very stiff, with persistence lengths in the range of tens of nanometers¹⁵, ssNAs rapidly sample conformationally more heterogeneous ensembles¹⁶⁻²². Fluorescence quenching experiments have yielded end-to-end contact rates of $\sim 10^6$ s⁻¹ for ssDNA with lengths between 2 to 20

1

nucleotides, demonstrating their pronounced flexibility and rapid dynamics^{5, 23-25}. However, the quantitative interpretation of contact rates in terms of chain dynamics requires detailed knowledge of both the distance dependence of the quenching process and the short-distance tail of the end-to-end distance distribution, which is sensitive to local structure formation and steric accessibility²⁶⁻²⁸. Modeling the behavior of ssNAs with molecular simulations has also been more challenging than for proteins, primarily because it has been difficult to capture the subtle balance of interactions such as base stacking with sufficient accuracy^{29, 30}.

To improve our quantitative understanding of chain dynamics in ssNAs, we used single-molecule Förster resonance energy transfer (FRET) combined with nanosecond fluorescence correlation spectroscopy (nsFCS) to probe the long-range intramolecular dynamics of short homopolymeric single-stranded RNA (ssRNA) and DNA (ssDNA) oligonucleotides. To interpret the results in terms of distance dynamics, we combine them with distance distributions obtained from the recently developed hierarchical chain growth (HCG) approach³¹, which produces structural ensembles that for RNA have been shown to be in good agreement with experiments, including nuclear magnetic resonance (NMR), small-angle X-ray scattering (SAXS), and single-molecule FRET³². We also compare the resulting distributions from HCG to distance distributions of polymer models commonly used to interpret single-molecule FRET data.

Our approach is illustrated in Fig. 1. Confocal single-molecule FRET measurements of freely diffusing molecules are used to obtain transfer efficiencies, which can be related to the average distance between the FRET donor and acceptor attached to the ends of the oligonucleotides (Fig. 1a). We focused on ssNAs with 19 nucleotides terminally labeled with Alexa Fluor 488 and 594, which at near-physiological ionic strengths of 153 mM yield transfer efficiencies close to 0.5, where the sensitivity for distance fluctuations is optimal. To probe the influence of sequence composition on chain dynamics, we studied homopolymeric 19mer ssDNA and ssRNA oligonucleotides, with cytosine (dC_{19}, rC_{19}) , adenine (dA_{19}, rA_{19}) , and thymine or uracil (dT_{19}, rU_{19}) as nucleobases. Guanine was excluded from our study because of its propensity to form stable quadruplex structures³³. To explore the impact of chain length on dynamics, we included a double-length 38-mer of deoxythymidine (dT_{38}). Additionally, we examined a partially abasic sequence, consisting of ten thymine bases alternating with nine sites lacking the base (dT_{19}^{ab}) . This sequence

^cDepartment of Chemistry, University of Kansas, Lawrence, KS, USA

^dDepartment of Molecular Biosciences, University of Kansas, Lawrence, KS, USA



Figure 1. Quantifying chain reconfiguration dynamics of single-stranded nucleic acids (illustrated for rA₁₉). (a) Transfer efficiency, *E*, histogram of freely diffusing terminally labeled rA₁₉ at 150 mM NaCl in 10 mM HEPES pH 7, with the FRET-active population at $E \approx 0.55$ and the donor-only population at $E \approx 0$ from molecules with inactive acceptor (gray: measured; black line and cyan shading: shot noise-limited photon distribution analysis¹) with inset of schematic representation of FRET on ssNA. (b) Normalized nsFCS of the FRET subpopulation shaded in panel (a) with donor (green) and acceptor (red) fluorescence autocorrelations and donor–acceptor crosscorrelation (blue; black lines: fits with eq. 11 (SI Methods), with resulting fluorescence correlation time, τ_{cd}). (c) Normalized subpopulation-specific complete correlation functions (black lines: global fits with eq. 8). (d) Representative structures from the HCG ensemble of rA₁₉ with explicit donor and acceptor dyes, Alexa Fluors 488 (green) and 594 (red). (e) Distributions of relative donor (green contours) and acceptor fluorescence lifetimes (red contours) versus transfer efficiency⁹ for all detected bursts (white points: average lifetime and efficiency). The straight line shows the theoretical dependence for fluorophores at a fixed distance (static line); curved lines show the dependences for dynamic systems based on analytical polymer models: Gaussian chain⁹ (GC, blue), worm-like chain^{5, 9} (WLC, orange), modified self-avoiding walk polymer^{9, 14} (SAW-v, red); upper lines, donor lifetime; lower lines, acceptor lifetime. Gray (HCG) and cyan dots (HCG_{BloEn}) show the values from the HCG ensemble and the reweighted ensemble, respectively. (f) Dye-to-dye distance distributions inferred from the mean and variance of the transfer efficiency distributions of rA₁₉ for the polymer models and the HCG_{BloEn} ensemble (cyan), with root mean square end-to-end distance, *R*, indicated (dashed lines) and potential of mean force (PMF) from th

allowed us to assess the effect of the nucleobases on chain dynamics and the influence of base stacking.

Based on the transfer efficiency histograms, we can single out the FRET-active subpopulation and exclude the contribution of donoronly-labeled molecules and any unwanted subpopulations associated with compact structures (Fig. S1) in the analysis of distance dynamics (Fig. 1a,b). The widths of the transfer efficiency peaks of the unstructured ssNAs are close to the photon shot noise limit (Fig. 1a, S1), indicating that inter-dye distance fluctuations are averaged out during the diffusion time of the molecules through the confocal volume of ~1 ms. Time-resolved fluorescence anisotropy measurements indicate high mobility of the fluorophores (Fig. S4, Table S2). To quantify the timescale of these dynamics, we used subpopulation-specific nsFCS^{6, 9}, which probes distance dynamics based on the fluorescence fluctuations of donor and acceptor emission down to the nanosecond range (Fig. 1b).

A characteristic signature of distance dynamics in FRET — in contrast to intensity fluctuations owing to contact quenching or triplet blinking — is that both the donor and acceptor

autocorrelations, as well as the donor-acceptor crosscorrelation relax with the same time constant, but with positive correlation amplitudes for the autocorrelations and a negative amplitude for the crosscorrelation⁹ (Figs. 1b-c, 2c, S2). Global fitting of the three correlation functions (see Methods for details) indicates that the relaxation corresponding to distance fluctuations is remarkably rapid, with relaxation times of $\tau_{cd}\approx 10\,\text{ns}$ (~20 ns for dT_{38}). To facilitate measurements of these rapid dynamics, we employed zero-mode waveguides³⁴ (ZMWs), which speed up data collection for nsFCS by orders of magnitude through fluorescence enhancement³⁵. Even more importantly, they lead to reduced fluorescence lifetimes, which improves the time separation between photon antibunching and distance dynamics in the correlations³⁵. On longer timescales, the crosscorrelations do not exhibit additional components with negative amplitude, indicating the absence of slower distance dynamics (Fig. 1c, 2c), in line with the near shot noise-limited width of the transfer efficiency histograms (Fig. 1a, S1). The decay components of the autocorrelations in the microsecond range are not accompanied by

a detectable crosscorrelation component, and are thus most likely to be caused by triplet blinking, which commonly occurs on this timescale³⁵ (Fig. 1c).

To obtain from the measured nsFCS relaxation times, τ_{cd} , the chain reconfiguration times, τ_r , i.e., the decorrelation time of the end-toend distance, we approximate the chain dynamics in terms of diffusion in a potential of mean force derived from the end-to-end distance distribution sampled by the chain^{6, 9, 36} (Fig. 1f, S5). For unfolded and disordered proteins as well as double-stranded DNA, simple analytical models based on polymer theory have been shown to provide suitable approximations of their distance distributions^{9, 37, 38}, but for ssNAs, the applicability of such simple models has not been established. Indeed, obvious candidates for analytical distance distributions, such as the worm-like chain, a self-avoiding walk polymer, or the Gaussian chain, are not in accord with a combined analysis of transfer efficiencies and fluorescence lifetime data (Fig. 1e, 2a, S3). In particular, their deviations from the diagonal static FRET line³⁹ are greater than observed experimentally, which indicates that these simple models overestimate the widths⁴⁰ of the distance distributions in ssNAs.

To address this deficiency, we used the recently developed hierarchical chain growth (HCG) approach³¹, which has been shown to yield conformational ensembles of single-stranded oligoribonucleotides that are in accord with small-angle X-ray scattering and FRET results³². Briefly, HCG creates a pool of short oligonucleotide structures that are then combined at random into polymers by fragment assembly. By structurally aligning the individual fragments and rejecting fragment pairs that are poorly aligned or involve steric clashes, ensembles with a high quality of both local and global structural properties are obtained (Fig. 1d-f). To account for the FRET dyes, a library of dye and linker configurations from molecular dynamics simulations²⁹ was used to



Figure 2. Sequence dependence of chain dynamics and internal friction in single-stranded nucleic acids. (a) Distributions of relative donor (green contours) and acceptor fluorescence lifetimes (red contours) versus transfer efficiency of ssDNA and ssRNA for all detected fluorescence bursts (white points: average lifetime and efficiency) compared with predictions from analytical polymer models (analogous to Fig. 1e), the HCG (gray) and HCG_{BIOEn} ensembles (cyan) for ssRNA and the reweighted polymer models (PM_{BIOEn}: blue star) for the ssDNA. (b) Dye-to-dye distance distributions from HCG_{BIOEn} ensembles (cyan) and reweighted polymer models (blue star). (c) Representative normalized subpopulation-specific nsFCS (color code as in Fig. 1) for dT₁₉ at different viscosities with fluorescence correlation times, τ_{cd} (black lines, global fit; see Methods). (d) Solvent viscosity (η) dependences of chain reconfiguration times, τ_r , of ssNAs with linear fits (shaded bands: 95% confidence intervals; for error bars, see SI Methods).

generate ensembles that not only include explicit representations of the fluorophores but also take into account that the excluded volume of the dyes affects the conformational distributions of the fluorescently labeled nucleic acid chains.

The resulting ensembles, containing 10,000 members each, were reweighted using Bayesian inference of ensembles⁴¹ (BioEn) to reach agreement with both the means and variances of the transfer efficiency distributions observed experimentally (Figs. 1e, 2a; see SI, Methods). For the ssRNA sequences, the HCG ensembles yield means and variances close to the experimental values even without reweighting, in line with recent results³²; slight reweighting thus suffices in these cases. For the ssDNA sequences, however, much stronger reweighting is required to obtain agreement with experiment, which may point to deficiencies in the current force fields available for DNA²⁹. For ssDNA, we thus employed reweighting of the distance distributions from simple analytical polymer models (see SI, Methods). The resulting distance distributions indicate that the chain dimensions are similar for the different ssNAs (SI, Methods), but some differences are noteworthy. For instance, the most expanded sequence is dA₁₉, in accord with the pronounced base stacking expected for adenine bases^{18, 42}.

The reweighted distance distributions were then converted to potentials of mean force by Boltzmann inversion (Fig. 1f, S5). The fluorescence relaxation times, τ_{cd} , from nsFCS, combined with the reweighted distance distributions, and the known distance dependence of the FRET efficiency according to Förster's theory fully define the dynamics of the chain in the framework of diffusion in a potential of mean force³⁶ (SI, Methods); the dynamics can be characterized either by the effective end-to-end diffusion coefficient or the chain reconfiguration time, τ_r (Table S4). The numerical values of τ_{cd} and τ_r are very similar, which is expected³⁶, because the average transfer efficiencies probed here correspond to distances near R₀ (Fig. 1g, Table S4). To assess the influence of the detailed shape of the potential on the values of τ_r , we compared the results based on the reweighted HCG ensembles and different analytical polymer models (Fig. 1f). The resulting maximum differences in τ_r range from 6 to 17 % for the different 19-mers (Table S4), indicating that the reconfiguration times we infer are robust, presumably because FRET with the Förster radius we use probes primarily the central parts of the distance distributions, which are similar for all models (Fig. 1f).

The values of τ_r for the different 19mers range from ~9 to ~17 ns, indicating rapid chain dynamics with a similar timescale for all sequences we investigated (Fig. 2d, Table S4), even though very different degrees of base stacking are expected for the different nucleotides. Adenine, e.g., is known to exhibit pronounced stacking, whereas thymine shows low stacking propensity^{19, 42, 43}. dT_{19} and dT_{19}^{ab} (where every other nucleotide is lacking the base) have very similar reconfiguration times and average end-to-end distances, in line with the absence of base stacking in dT₁₉ under our experimental conditions. The reconfiguration times are comparable for corresponding ssRNA and ssDNA samples, suggesting that the dynamics are dominated by the four freely rotatable bonds in the phosphodiester linkage between nucleotides rather than the identity of the nucleobase or the sugar. Nevertheless, more pronounced stacking appears to be correlated with somewhat slower end-to-end distance dynamics, as observed previously in quenching experiments²³.

To obtain more mechanistic insight into ssNA dynamics, we quantified the contribution of internal friction. Internal friction in biomolecules describes the dissipative force resisting conformational changes or molecular motion that is not caused by friction against the solvent but by the motion of parts of the molecules with respect to each other⁴⁴⁻⁴⁶. A commonly used operational definition of internal friction is based on measurements of the dynamics as a function of solvent viscosity, and the frictional contribution independent of the solvent is obtained by extrapolating to zero viscosity^{24, 46}. This concept is particularly well justified for polymers, where, in the context of the Rouse and Zimm models of chain dynamics with internal friction⁴⁶⁻ ⁴⁸, the total reconfiguration time of the chain, τ_r , can be decomposed into two additive terms, the contribution from internal friction, τ_i , which is independent of solvent viscosity, η , and the solvent viscosity-dependent term, τ_s :

$$\tau_r \approx \tau_i + \frac{\eta}{\eta_0} \tau_s(\eta_0)$$

where η_0 is the viscosity of water. τ_i thus corresponds to the value of τ_r extrapolated to $\eta = 0$ (Fig. 2d).

To quantify internal friction, we varied the solvent viscosity by changing the glycerol concentration (Fig. 2d, S2). Strikingly, the resulting values of τ_i are zero within experimental uncertainty for all ssNAs investigated (Fig. 2d), suggesting that internal friction makes a negligible contribution to their end-to-end distance dynamics. In sequences with little or no base stacking, such as dT_{19} , this observation may not be surprising and is reminiscent of highly expanded unfolded and disordered proteins with little intrachain interactions that could slow down the dynamics⁹, but it may be more surprising for sequences with pronounced base stacking, such as d/rA₁₉. A possible interpretation that emerges from the dominant configurations from the HCG approach (Fig. 1) and previous simulations²⁹ is that the end-to-end dynamics in such sequences are dominated by the rapid motions of relatively long stacked segments rotating about a few nucleotides where stacking is absent. The motion of those stacked segments through the solvent would hardly be impeded by interactions within the chains and would be dominated by solvent friction.

In contrast to our results, Uzawa et al.23, 24 observed a small but significant contribution of internal friction for the rates of end-toend contact formation in single-stranded oligodeoxynucleotides with lengths similar to those used here. However, the two experiments probe very different parts of the end-to-end distance distribution: While FRET with the Förster radii used here is dominated by distance fluctuations about the center of the distributions, contact quenching probes their short-distance tails. It is conceivable that forming contacts between the chain termini requires conformational rearrangements that involve more pronounced barriers, corresponding to higher internal friction. With the distance distributions and effective diffusion coefficients from our results, we estimate end-to-end collision rates^{26, 36} (see Methods) between $0.2 \cdot 10^6 \text{ s}^{-1}$ and $2 \cdot 10^6 \text{ s}^{-1}$ for dT_{19} , depending on the distance distribution used (Table S5). Despite the pronounced dependence on the detailed shape of the distribution, the contact rate of 1.25.10⁶ reported by Uzawa et al.²³ for dT₂₀ is within this range.

In summary, with single-molecule FRET and nsFCS aided by nanophotonic enhancement, we observe very rapid end-to-end distance dynamics of ssNAs. In combination with conformational

ensembles generated by hierarchical chain growth, simple polymer models, and reweighting based on experimental restraints, we obtain reconfiguration times in the 10-ns range, and we observe no detectable contribution of internal friction. These dynamics are much faster than for most unfolded or disordered proteins with similar average end-to-end distances. Especially for unfolded proteins under native solution conditions, reconfiguration times up to several hundred nanoseconds have been observed^{9, 26}, often with a pronounced contribution from internal friction dominated by intrachain interactions^{26, 49}. The more rapid reconfiguration in ssNAs may be linked to their larger persistence lengths (Table S3) and the hinge-like motions of partially stacked segments relative to each other, which are expected to be dominated by solvent friction. It will be interesting to relate the fast dynamics of singlestranded nucleic acids to processes such as structure formation and binding.

Author information

Corresponding Authors

*erik.d.holmstrom@ku.edu, gerhard.hummer@biophys.mpg.de, schuler@bioc.uzh.ch

Author Contributions

MN, LP, EDH, GH, and BS designed research; DN developed data analysis tools; MN performed measurements; DN helped with instrumentation; LP performed and analyzed HCG; MN, EDH, VvR and DN analyzed measurements and simulations; MN and RKT fabricated ZMWs; EDH, OM, GH, and BS supervised research; MN, DN and BS prepared the manuscript with the help of all authors.

Notes

The authors declare no competing financial interests.

Acknowledgments

We thank Jérôme Wenger for advice on ZMW fabrication, Gianluca Galeno for assistance with purification and labeling, José María Mateos Melero for assistance with focus ion beam milling, and the Center for Microscopy and Image Analysis at the University of Zurich for access to instrumentation. This work was supported by the Swiss National Science Foundation (B.S.), the University of Kansas (E.D.H.), and the Max Planck Society (G.H.).

References

1. Antonik, M.; Felekyan, S.; Gaiduk, A.; Seidel, C. A., Separating structural heterogeneities from stochastic variations in fluorescence resonance energy transfer distributions via photon distribution analysis. *J Phys Chem B* **2006**, *110* (13), 6970-8.

2. Haas, E.; Katchalskikatzir, E.; Steinberg, I. Z., Brownian-Motion of Ends of Oligopeptide Chains in Solution as Estimated by Energy-Transfer between Chain Ends. *Biopolymers* **1978**, *17* (1), 11-31.

3. Bieri, O.; Wirz, J.; Hellrung, B.; Schutkowski, M.; Drewello, M.; Kiefhaber, T., The speed limit for protein folding measured by triplet-triplet energy transfer. *Proc Natl Acad Sci U S A* **1999**, *96* (17), 9597-601.

4. Lapidus, L. J.; Eaton, W. A.; Hofrichter, J., Measuring the rate of intramolecular contact formation in polypeptides. *Proc Natl Acad Sci U S A* **2000**, *97* (13), 7220-5.

5. Wang, X.; Nau, W. M., Kinetics of end-to-end collision in short singlestranded nucleic acids. *J Am Chem Soc* **2004**, *126* (3), 808-13.

6. Nettels, D.; Gopich, I. V.; Hoffmann, A.; Schuler, B., Ultrafast dynamics of protein collapse from single-molecule photon statistics. *Proc Natl Acad Sci U S A* **2007**, *104* (8), 2655-60.

7. Doose, S.; Neuweiler, H.; Sauer, M., Fluorescence quenching by photoinduced electron transfer: a reporter for conformational dynamics of macromolecules. *Chemphyschem* **2009**, *10* (9-10), 1389-98.

8. Biehl, R.; Richter, D., Slow internal protein dynamics in solution. *Journal of physics. Condensed matter : an Institute of Physics journal* **2014**, *26* (50), 503103.

9. Schuler, B.; Soranno, A.; Hofmann, H.; Nettels, D., Single-Molecule FRET Spectroscopy and the Polymer Physics of Unfolded and Intrinsically Disordered Proteins. *Annu Rev Biophys* **2016**, *45*, 207-31.

10. Eaton, W. A., Searching for "downhill scenarios" in protein folding. *Proc Natl Acad Sci U S A* **1999**, *96* (11), 5897-9.

11. Kubelka, J.; Hofrichter, J.; Eaton, W. A., The protein folding 'speed limit'. *Curr Opin Struct Biol* **2004**, *14* (1), 76-88.

12. Chowdhury, A.; Nettels, D.; Schuler, B., Interaction Dynamics of Intrinsically Disordered Proteins from Single-Molecule Spectroscopy. *Annu. Rev. Biophys.* **2023**.

13. Woodson, S. A., Compact intermediates in RNA folding. *Annu. Rev. Biophys.* **2010**, *39*, 61-77.

14. Zheng, W.; Zerze, G. H.; Borgia, A.; Mittal, J.; Schuler, B.; Best, R. B., Inferring properties of disordered chains from FRET transfer efficiencies. *J Chem Phys* **2018**, *148* (12), 123329.

15. Gross, P.; Laurens, N.; Oddershede, L.; Bockelmann, U.; Wuite, G. J.; Peterman, E. J., Twist, Stretch and Melt: Quantifying How DNA Complies to Tension. *Biophys J* **2011**, *100* (3), 74-75.

16. Chen, H.; Meisburger, S. P.; Pabit, S. A.; Sutton, J. L.; Webb, W. W.; Pollack, L., Ionic strength-dependent persistence lengths of single-stranded RNA and DNA. *Proc Natl Acad Sci U S A* **2012**, *109* (3), 799-804.

17. Plumridge, A.; Meisburger, S. P.; Pollack, L., Visualizing singlestranded nucleic acids in solution. *Nucleic Acids Res* **2017**, *45* (9), e66.

18. Eichhorn, C. D.; Feng, J.; Suddala, K. C.; Walter, N. G.; Brooks, C. L., 3rd; Al-Hashimi, H. M., Unraveling the structural complexity in a singlestranded RNA tail: implications for efficient ligand binding in the prequeuosine riboswitch. *Nucleic Acids Res.* **2012**, *40* (3), 1345-55.

19. Murphy, M. C.; Rasnik, I.; Cheng, W.; Lohman, T. M.; Ha, T. J., Probing single-stranded DNA conformational flexibility using fluorescence spectroscopy. *Biophys J* **2004**, *86* (4), 2530-2537.

20. Meisburger, S. P.; Sutton, J. L.; Chen, H.; Pabit, S. A.; Kirmizialtin, S.; Elber, R.; Pollack, L., Polyelectrolyte properties of single stranded DNA measured using SAXS and single-molecule FRET: Beyond the wormlike chain model. *Biopolymers* **2013**, *99* (12), 1032-45.

21. Eichhorn, C. D.; Al-Hashimi, H. M., Structural dynamics of a singlestranded RNA-helix junction using NMR. *RNA* **2014**, *20* (6), 782-91.

22. Shusterman, R.; Alon, S.; Gavrinyov, T.; Krichevsky, O., Monomer dynamics in double- and single-stranded DNA polymers. *Phys. Rev. Lett.* **2004**, *92* (4), 048303.

23. Uzawa, T.; Isoshima, T.; Ito, Y.; Ishimori, K.; Makarov, D. E.; Plaxco, K. W., Sequence and temperature dependence of the end-to-end collision dynamics of single-stranded DNA. *Biophys J* **2013**, *104* (11), 2485-92.

24. Uzawa, T.; Cheng, R. R.; Cash, K. J.; Makarov, D. E.; Plaxco, K. W., The length and viscosity dependence of end-to-end collision rates in single-stranded DNA. *Biophys. J.* **2009**, *97* (1), 205-10.

25. Kawai, K.; Yoshida, H.; Sugimoto, A.; Fujitsuka, M.; Majima, T., Kinetics of transient end-to-end contact of single-stranded DNAs. J. Am. Chem. Soc. **2005**, *127* (38), 13232-7.

26. Soranno, A.; Holla, A.; Dingfelder, F.; Nettels, D.; Makarov, D. E.; Schuler, B., Integrated view of internal friction in unfolded proteins from single-molecule FRET, contact quenching, theory, and simulations. *Proc. Natl. Acad. Sci. USA* **2017**, *114* (10), E1833-E1839.

27. Buscaglia, M.; Lapidus, L. J.; Eaton, W. A.; Hofrichter, J., Effects of denaturants on the dynamics of loop formation in polypeptides. *Biophys. J.* **2006**, *91* (1), 276-288.

28. Toan, N. M.; Morrison, G.; Hyeon, C.; Thirumalai, D., Kinetics of loop formation in polymer chains. *J Phys Chem B* **2008**, *112* (19), 6094-106.

29. Grotz, K. K.; Nueesch, M. F.; Holmstrom, E. D.; Heinz, M.; Stelzl, L. S.; Schuler, B.; Hummer, G., Dispersion Correction Alleviates Dye Stacking of Single-Stranded DNA and RNA in Simulations of Single-Molecule Fluorescence Experiments. *J Phys Chem B* **2018**, *122* (49), 11626-11639.

30. Tan, D.; Piana, S.; Dirks, R. M.; Shaw, D. E., RNA force field with accuracy comparable to state-of-the-art protein force fields. *Proc Natl Acad Sci U S A* **2018**, *115* (7), E1346-E1355.

31. Pietrek, L. M.; Stelzl, L. S.; Hummer, G., Structural ensembles of disordered proteins from hierarchical chain growth and simulation. *Curr. Opin. Struct. Biol.* **2023**, *78*, 102501.

32. Pietrek, L. M.; Stelzl, L. S.; Hummer, G., Hierarchical assembly of single-stranded RNA. *bioRxiv* **2023**, 2023.08.01.551474.

33. Burge, S.; Parkinson, G. N.; Hazel, P.; Todd, A. K.; Neidle, S., Quadruplex DNA: sequence, topology and structure. *Nucleic Acids Res.* **2006**, *34* (19), 5402-15.

34. de Torres, J.; Ghenuche, P.; Moparthi, S. B.; Grigoriev, V.; Wenger, J., FRET enhancement in aluminum zero-mode waveguides. *Chemphyschem* **2015**, *16* (4), 782-8.

35. Nüesch, M. F.; Ivanovic, M. T.; Claude, J. B.; Nettels, D.; Best, R. B.; Wenger, J.; Schuler, B., Single-molecule Detection of Ultrafast Biomolecular Dynamics with Nanophotonics. *J. Am. Chem. Soc.* **2022**, *144* (1), 52-56.

36. Gopich, I. V.; Nettels, D.; Schuler, B.; Szabo, A., Protein dynamics from single-molecule fluorescence intensity correlation functions. *The Journal of chemical physics* **2009**, *131* (9), 095102.

37. Bustamante, C.; Marko, J. F.; Siggia, E. D.; Smith, S., Entropic Elasticity of Lambda-Phage DNA. *Science* **1994**, *265* (5178), 1599-1600.

38. O'Brien, E. P.; Morrison, G.; Brooks, B. R.; Thirumalai, D., How accurate are polymer models in the analysis of Förster resonance energy transfer experiments on proteins? *The Journal of chemical physics* **2009**, *130* (12), 124903.

39. Barth, A.; Opanasyuk, O.; Peulen, T. O.; Felekyan, S.; Kalinin, S.; Sanabria, H.; Seidel, C. A. M., Unraveling multi-state molecular dynamics in single-molecule FRET experiments. I. Theory of FRET-lines. *J Chem Phys* **2022**, *156* (14), 141501.

40. Gopich, I. V.; Szabo, A., Theory of the energy transfer efficiency and fluorescence lifetime distribution in single-molecule FRET. *Proc Natl Acad Sci U S A* **2012**, *109* (20), 7747-52.

41. Hummer, G.; Köfinger, J., Bayesian ensemble refinement by replica simulations and reweighting. *The Journal of chemical physics* **2015**, *143* (24), 243150.

42. Plumridge, A.; Meisburger, S. P.; Andresen, K.; Pollack, L., The impact of base stacking on the conformations and electrostatics of single-stranded DNA. *Nucleic. Acids. Res.* **2017**, *45* (7), 3932-3943.

43. Goddard, N. L.; Bonnet, G.; Krichevsky, O.; Libchaber, A., Sequence dependent rigidity of single stranded DNA. *Phys. Rev. Lett.* **2000**, *85* (11), 2400-3.

44. Ansari, A.; Jones, C. M.; Henry, E. R.; Hofrichter, J.; Eaton, W. A., The role of solvent viscosity in the dynamics of protein conformational changes. *Science* **1992**, *256* (5065), 1796-8.

45. Hagen, S. J.; Qiu, L. L.; Pabit, S. A., Diffusional limits to the speed of protein folding: fact or friction? *J. Phys. Cond. Mat.* **2005**, *17* (18), S1503-S1514.

46. Soranno, A.; Buchli, B.; Nettels, D.; Müller-Späth, S.; Cheng, R. R.; Pfeil, S. H.; Hoffmann, A.; Lipman, E. A.; Makarov, D. E.; Schuler, B., Quantifying internal friction in unfolded and intrinsically disordered proteins with single molecule spectroscopy. *Proc. Natl. Acad. Sci. USA* **2012**, *109* (44), 17800-17806.

47. Khatri, B. S.; McLeish, T. C. B., Rouse model with internal friction: A coarse grained framework for single biopolymer dynamics. *Macromolecules* **2007**, *40* (18), 6770-6777.

48. Cheng, R. R.; Hawk, A. T.; Makarov, D. E., Exploring the role of internal friction in the dynamics of unfolded proteins using simple polymer models. *The Journal of chemical physics* **2013**, *138* (7).

49. Zheng, W.; Hofmann, H.; Schuler, B.; Best, R. B., Origin of Internal Friction in Disordered Proteins Depends on Solvent Quality. *J. Phys. Chem. B* **2018**, *122* (49), 11478-11487.

Supporting Information

Nanosecond chain dynamics of single-stranded nucleic acids

Mark F. Nüesch^a, Lisa Pietrek^b, Erik D. Holmstrom^{a,c,d*}, Daniel Nettels^a, Valentin von Roten^a, Rafael Kronenberg-Tenga^a, Ohad Medalia^a, Gerhard Hummer^{b,e*}, Benjamin Schuler^{a,f*}

^aDepartment of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland ^bDepartment of Theoretical Biophysics, Max Planck Institute of Biophysics, Max-von-Laue Straße 3, 60438 Frankfurt am Main, Germany

^cDepartment of Chemistry, University of Kansas, Lawrence, KS, USA

^dDepartment of Molecular Biosciences, University of Kansas, Lawrence, KS, USA

^eInstitute for Biophysics, Goethe University Frankfurt, 60438 Frankfurt am Main, Germany

^fDepartment of Physics, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Materials and Methods

Purification and labeling of nucleic acids. Terminally functionalized homopolymeric oligonucleotides with a 5'-end dithiol and a 3'-end primary amine for labeling $(dA_{19}, dC_{19}, dT_{19}, dT_{19}, dT_{38}, rA_{19}, rC_{19})$ and rU₁₉; Extended Data Table 1) were synthesized and purified by high pressure liquid chromatography (HPLC) by Integrated DNA Technologies. Prior to labeling, the oligonucleotides were dissolved in 10 mM sodium phosphate buffer pH 7, and filtered and concentrated (Amicon Ultra-0.5 mL, MWCO 3 KDa) to remove free primary amines that would interfere with downstream reactions. After this step, each oligonucleotide was site-specifically labeled at the 5'-end with thiol-reactive Alexa Fluor 594 maleimide, and at the 3'-end with amine-reactive Alexa Fluor 488 succinimidyl ester according to the following procedure. The synthetically incorporated thiol groups at the 5'-ends were reduced with 100 mM tris(2carboxyethyl)phosphine (TCEP) at oligonucleotide concentrations of \sim 10 μ M. After \sim 1 h, the buffer of the samples was exchanged to 10 mM sodium phosphate pH 7, and the samples were concentrated (Amicon Ultra-0.5 mL Centrifugal Filters MWCO 3 KDa) to ~10 μ M. The acceptor dye (dissolved in 5 μ L dimethyl sulphoxide (DMSO) and vortexed) was added to the sample at a ratio of 1:10 (oligonucleotide:dye) and incubated for 60 min. For the reaction of the amine-reactive donor dye with the 3'-end of the oligonucleotides, the pH of the acceptor labeling reaction mixtures was increased to pH 8 by addition of 1 M sodium phosphate buffer pH 8. The donor dye (dissolved in 5 μ l DMSO and vortexed) was added to the corresponding reaction mix in a tenfold excess over oligonucleotide and incubated for 60 minutes. Unreacted dye was removed with a desalting spin column (Zeba, Pierce, MWCO 7 kDa), and the labeled constructs were purified on a reversed-phase column (Dr. Maisch ReprosilPur 200 C18-AQ, 5 µm) using HPLC (Agilent 1100 series). Samples were lyophilized over night, then dissolved in H_2O , and stored at -80 °C until use.

Production of ZMWs. Using borosilicate glass coverslips coated with a 100 nm aluminum layer (Deposition Research Laboratory, St. Charles, MO), ZMWs with a diameter of 120 nm were milled into the aluminum layer at a 90° angle using a gallium focused ion beam (FIB-SEM Zeiss Auriga 40 CrossBeam) with a voltage of 30 kV and a 10 pA beam current at room temperature. The passivation of the aluminum and glass surfaces of the ZMW was performed as described previously^{1, 2}.

Single-molecule spectroscopy. Single-molecule fluorescence experiments with and without ZMWs were performed on a four-channel MicroTime 200 confocal instrument (PicoQuant) equipped with either an Olympus UplanApo 60x/1.20 Water objective for measurements without ZMW or an Olympus UplanSapo 100x/1.4 Oil objective for measurements with ZMWs. Alexa488 was excited with a diode laser (LDH-D-C-485, PicoQuant) at an average power of 100 μ W (measured at the back aperture of the objective). The laser was operated in continuous-wave mode for nsFCS experiments and in pulsed mode with interleaved excitation (PIE)³ for fluorescence lifetime measurements. The wavelength range used for acceptor excitation was selected with two band pass filters (z582/15 and z580/23, Chroma) from the emission of a supercontinuum laser (EXW-12 SuperK Extreme, NKT Photonics) operating at pulse repetition rate of 20 MHz (45 µW average laser power after the band pass filters). The SYNC out of the SuperK Extreme triggered interleaved pulses from the 488-nm diode laser. Sample fluorescence was collected by the microscope objective, separated from scattered light with a triple band pass filter (r405/488/594, Chroma) and focused on a 100-µm pinhole. After the pinhole, fluorescence emission was separated into two channels, either with a polarizing beam splitter for fluorescence lifetime measurements, or with a 50/50 beam splitter for nsFCS measurements to avoid the effects of detector deadtimes and afterpulsing on the correlation functions⁴. Finally, the fluorescence photons were distributed by wavelength into four channels by dichroic mirrors (585DCXR, Chroma), additionally filtered by bandpass filters (ET525/50M and HQ650/100, Chroma), and focused onto one of four singlephoton avalanche detectors (SPCM-AQRH-14-TR, Excelitas). The arrival times of the detected photons were recorded with a HydraHarp 400 counting module (PicoQuant, Berlin, Germany).

All free-diffusion single-molecule experiments were conducted with labeled oligonucleotide concentrations between 100 and 250 pM without ZMWs or between 50 and 300 nM with ZMWs in 10 mM HEPES buffer pH 7.0 (adjusted with 35 mM NaOH), 0.01% Tween 20, 143 mM β -mercaptoethanol (BME), 150 mM NaCl, and for the viscosity dependence with appropriately chosen concentrations of glycerol (without ZMW) in 18-well plastic slides (ibidi) or in ZMWs at 22°C.

Single-molecule FRET data analysis. Data analysis was carried out using the Mathematica (Wolfram Research) package Fretica (<u>https://github.com/SchulerLab</u>). For the identification of photon bursts, the photon recordings were time-binned (1 ms binning for measurements without ZMWs, 0.2 ms for measurements with ZMWs). Photon numbers per bin were corrected for background, crosstalk, differences in detection efficiencies and quantum yields of the fluorophores, and for direct excitation of the acceptor⁵. Bins with more than 50 photons were identified as photon bursts. Ratiometric transfer efficiencies were obtained for each burst from $E = n_A/(n_A + n_D)$, where n_A and n_D are the corrected numbers of donor and acceptor photons in the photon burst, respectively. The *E* values were histogrammed. The subpopulation corresponding to the FRET-labeled species was fitted with a Gaussian peak function or analyzed by photon distribution analysis taking into account the experimentally observed burst size distribution⁶⁻⁸ (Fig. S1). Bursts from experiments in PIE mode were further selected according to the fluorescence stoichiometry ratio⁹⁻¹¹, *S* (0.2 < *S* < 0.8) (Fig. 2, S3).

End-to-end distance distributions. For analyzing the single-molecule FRET data of the ssNA variants, we employed end-to-end distance distributions of analytical polymer models as well as the distance distributions obtained by the hierarchical chain growth (HCG) approach¹² (see hierarchical chain growth). The three polymer models used and the corresponding end-to-end distance probability density functions were:

1. Gaussian chain (GC)¹³:

$$P_{GC}(r) = 4 \pi r^2 \left[\frac{3}{2 \pi \langle r^2 \rangle}\right]^{\frac{3}{2}} e^{-\frac{3 r^2}{2 \langle r^2 \rangle}}.$$
 (1)

2. Worm-like chain (WLC)^{13, 14}:

$$P_{\text{WLC}}(r) = \begin{cases} C (r/l_c)^2 (1 - (r/l_c)^2)^{-9/2} e^{-3l_c/4l_p} (1 - (r/l_c)^2)^{-1}, & r \le l_c \\ 0, & r > l_c \end{cases}$$
(2)

where l_c and l_p are the contour- and persistence lengths of the chain, respectively (Table S3). C is a normalization constant.

3. Self-avoiding walk polymer (SAW-v)¹⁵:

$$P_{\text{SAW}-\nu}(r) = A \frac{4\pi}{R} \left(\frac{r}{R}\right)^{2+g} e^{-\alpha \left(\frac{r}{R}\right)^{\delta}},$$
(3)
with $R = \langle r^2 \rangle^{\frac{1}{2}}, \ g = \frac{\gamma-1}{\nu}, \ \delta = \frac{1}{1-\nu}, \ \gamma \approx 1.1615 \text{ and } \nu = \frac{\ln(\frac{R}{b})}{\ln(n)},$

where b and n are the segment length and the number of segments of the polymer, respectively (Table S3). A is a normalization constant.

If the rotational correlation time, τ_{rot} (Table S2), of the chromophores is short relative to the fluorescence lifetime, τ_D , of the donor (such that orientational factor $\kappa^2 \approx 2/3$)¹⁶, and the end-to-end distance dynamics of the polypeptide chain (with relaxation time τ_r) are slow relative to τ_D , the experimentally determined mean transfer efficiency, $\langle E \rangle$, can be related to the distance distribution, P(r), by¹⁷:

$$\langle E \rangle = \langle \varepsilon \rangle \equiv \int_0^\infty \varepsilon(r) P(r) dr, \tag{4}$$

where $\varepsilon(r) = R_0^6/(R_0^6 + r^6)$, and R_0 is the Förster radius (5.4 nm for Alexa 488/594)^{2, 18-20}. See Table S3 for the values of the parameters used (l_c, b, n) and inferred (R, l_p, v) by solving Eq. 4 numerically for the corresponding variable. Time-resolved fluorescence anisotropy measurements (Fig. S4) indicate high mobility of the fluorophores (Table S2), suggesting $\kappa^2 \approx 2/3$.

Effect of glycerol on conformational free energy. Viscogens can affect intramolecular interactions and thus lead to changes in conformational free energy. The changes in transfer efficiency upon addition of glycerol were small under the conditions used here; we estimated the order of magnitude of the effect based on a simple approximation. Distance distributions, P(r), can be converted into potentials of mean force, F(r), through Boltzmann inversion (Fig 1f, S5):

$$F(r) = -k_B T \ln P(r), \tag{5}$$

where k_B is the Boltzmann constant and T the temperature. To estimate the change in conformational free energy upon addition of glycerol, we utilized Eq. 5 for the ssNA that exhibited the largest influence of glycerol on transfer efficiency, dT_{19} ($\Delta E = 0.07$), assuming a Gaussian chain distance distribution, with R_0 corrected for the refractive index change due to glycerol. The free energy change was estimated from

$$\frac{\Delta F}{k_B T} = \int_0^\infty P_{\rm GC}^{(35\%)}(r) \ln P_{\rm GC}^{(35\%)}(r) dr - \int_0^\infty P_{\rm GC}^{(0\%)}(r) \ln P_{\rm GC}^{(0\%)}(r) dr = \ln \sqrt{\frac{\langle r_{35\%}^2 \rangle}{\langle r_{0\%}^2 \rangle}}, \quad (6)$$

where $P_{GC}^{(0\%)}(r)$ and $P_{GC}^{(35\%)}(r)$ are the probability density functions of the chain at 0% and 35% glycerol, respectively, and $\langle r_{0\%}^2 \rangle$ and $\langle r_{35\%}^2 \rangle$ are the corresponding mean squared end-to-end distances. The resulting conformational free energy change is $\Delta F \approx 0.1 k_B T$, corresponding to a change in $R = \langle r^2 \rangle^{1/2}$ by ~0.7 nm. We thus conclude that the energetic changes within the chain upon glycerol addition are unlikely to affect internal friction.

Single-molecule fluorescence lifetime analysis. From PIE experiments, the donor and acceptor fluorescence lifetimes, $\tau_{\rm D}$ and $\tau_{\rm A}$, for each burst were determined as previously described²¹. The distributions of relative lifetimes, $\tau_{\rm D}/\tau_{\rm D0}$ and $(\tau_{\rm A}-\tau_{\rm A0})/\tau_{\rm D0}$, versus transfer efficiency for the FRET-active population are shown in Figs. 1e, 2a and S3. $\tau_{\rm D0}$ and $\tau_{\rm A0}$ are the fluorescence lifetimes of donor and acceptor in the absence of FRET, respectively (see Table S2). Fig. S3 shows the distributions of relative donor lifetime versus transfer efficiency including the donor-only population. $\tau_{\rm A0}$ and $\tau_{\rm D0}$ were obtained from independent ensemble lifetime measurements as described below. The figures show dynamic FRET lines²² that were calculated assuming end-to-end distance distributions, P(r), for a Gaussian chain¹³ (GC), a worm-like chain¹³ (WLC), and for the SAW- ν polymer¹⁵ (SAW- ν) models. For the case that P(r) is sampled faster than the interphoton time (~ 10 µs) but slowly compared to $\tau_{\rm D}$ (3.5 – 4 ns; Table S2), it has been shown that²³:

$$\frac{\tau_{\rm D}}{\tau_{\rm D0}} = 1 - \langle \varepsilon \rangle + \frac{\sigma_{\varepsilon}^2}{1 - \langle \varepsilon \rangle}$$

and

$$\frac{\tau_{\rm A} - \tau_{\rm A0}}{\tau_{\rm D0}} = 1 - \langle \varepsilon \rangle - \frac{\sigma_{\varepsilon}^2}{\langle \varepsilon \rangle} , \qquad (7)$$

where $\sigma_{\varepsilon}^2 = \int_0^{\infty} (\varepsilon(r) - \langle \varepsilon \rangle)^2 P(r) dr$ is the variance of the transfer efficiency distribution corresponding to P(r). The dynamic FRET lines were obtained by varying the model parameters of the corresponding distributions, $\langle r^2 \rangle$ for the GC; the persistence length, l_p , for the WLC; and the scaling exponent, ν , for the SAW- ν model, respectively. The static FRET line, $\tau_D/\tau_{D0} = (\tau_A - \tau_{A0})/\tau_{D0} = 1 - \langle \varepsilon \rangle$, corresponds to fixed interdye distances. Note that this type of fluorescence lifetime analysis is only valid for the regime where τ_{rot} is short relative to τ_D , and τ_D is short relative to τ_r , i.e., $\tau_{rot} < \tau_{D0} < \tau_r$ (Table S2, S4).

Hierarchical chain growth and fluorophore modeling. To carry out hierarchical chain growth (HCG), we created a molecular dynamics (MD) fragment library. Subsequently, we built heterotetramers with sequence d/rGXYZ. G served as a fixed head group at the 5' end. For the other nucleotides "XYZ", we used all 4^3 combinations of thymine, uracil, cytosine and adenine. The heteromeric fragment library was extensively sampled via temperature replica exchange MD simulations, utilizing the panBSC1 force-field²⁴ for DNA and the DESRES force-field²⁵ for RNA. For both DNA and RNA, the TIP4P-D water model²⁶ was used. Fragments were placed in a dodecahedral box, solvated with 150 mM NaCl and neutralized, resulting in a system comprising ~6600 atoms. Depending on the fragment sequence, the total number varied by about 50 atoms. The fragment with the abasic site was parameterized as described by Heinz *et al.*²⁷. For each system, we ran 24 replicas over a temperature range of 300 – 420 K for 100 ns as described before²⁸. Afterwards, we randomly selected fragment conformations from the MD fragment library at 300 K to assemble disordered ssNAs with HCG in a hierarchical manner²⁸. Fragment assembly was performed as previously described²⁸.

We also used HCG to build libraries of dye-labeled DNA and RNA 4-mer fragments. As inputs, we used the 4-mer libraries built here and the libraries built by Grotz et al.²⁹ for the dyes Alexa Fluor 594 and Alexa Fluor 488 attached to dideoxyadenosinemonophosphate (dA_2) and dideoxythimidinemonophosphate (dT_2) at the 5' and 3' ends, respectively. The use of dA_2 - and dT_2 -dye fragments to model fluorophores attached to DNA and RNA chains has been validated by Grotz et al.²⁹ We used the dA₂ library for purines (A, G) at the respective end and the dT₂ library for pyrimidines (U, C). Pairs of random structures were repeatedly drawn from the library of DNA or RNA 4-mer fragments and from library of dA_2 or dT_2 labeled with Alexa 594 or Alexa 488. For each pair, we performed a rigid body superposition of the heavy atoms of the terminal sugar moiety and nucleobase, leaving out nonmatching atoms of the base. If the RMSD of the superposition was below 0.8 Å, we searched for clashing heavy atoms within a pair distance of 2.0 Å. If no clashing atoms were detected, the dye was attached to the DNA or RNA 4-mer fragment according to the superposition, excluding the terminal oxygen atoms of the 4-mer. The resulting libraries of DNA and RNA 4-mer structures with the FRET dyes Alexa Fluor 594 and Alexa Fluor 488 attached at their 5' or 3' ends were subsequently used to build dye-labeled DNA and RNA chains by HCG.

Bayesian ensemble refinement. To optimize the agreement of the HCG ensembles with the experimental data, we reweighted the ensembles of configurations based on two experimental observables from the single-molecule measurements: the mean transfer efficiency, $\langle E \rangle$, and the variance of the underlying transfer efficiency distribution, σ_{ε}^2 , as described in *Single-molecule fluorescence lifetime analysis*. The transfer efficiency was calculated for each of the *N* ensemble members, and uniform weights $w_{\alpha}^0 = 1/N$ were initially assigned to all of them. Optimal weights were found using Bayesian inference of ensembles³⁰ (BioEn) by minimizing

٧

$$\Delta G(w_1, \dots, w_N) = \frac{1}{2}\chi^2 - \theta \Delta S$$
(8)
with $\chi^2 = \frac{(\langle \varepsilon \rangle_{\text{BioEn}} - \langle \varepsilon \rangle)^2}{Var(\langle \varepsilon \rangle)} + \frac{(\sigma_{\varepsilon \text{BioEn}}^2 - \sigma_{\varepsilon}^2)^2}{Var(\sigma_{\varepsilon}^2)}$
and $\Delta S = -\sum_{\alpha=1}^N w_\alpha \ln \frac{w_\alpha}{w_\alpha^0}$

by simulated annealing for different values of θ . Reweighted values are given by $\langle \epsilon \rangle_{\text{BioEn}} = \sum_{\alpha=1}^{N} w_{\alpha} \varepsilon_{\alpha}$ and $\sigma_{\varepsilon}^2_{\text{BioEn}} = \sum_{\alpha=1}^N w_\alpha \, \varepsilon_\alpha^2 - \langle \varepsilon \rangle_{\text{BioEn}}^2$, where w_α and ε_α are the weight and the transfer efficiency of the α th ensemble member, respectively, with $\sum_{\alpha=1}^{N} w_{\alpha} = 1$. For each ensemble, we chose the largest value of θ for which $\langle \varepsilon \rangle_{BioEn}$ and $\sigma_{\varepsilon BioEn}^2$ agreed with the measured values within the experimental uncertainties of $Var(\langle E \rangle)^{1/2} = 0.03$ and $Var(\sigma_{\varepsilon}^2)^{1/2} = 0.003$, respectively. The end-to-end distance distributions of initial and reweighted ensembles for all ssRNAs and dT_{19}^{ab} are depicted in Fig. S5. A useful quantity to estimate the quality of the prior distribution is the effective fraction of configurations used from the initial ensemble, $\phi_{\rm eff}=e^{\Delta S}$. For the 19mer ssRNA ensembles and ${
m dT}_{19}^{\rm ab}$, $\phi_{\rm eff}$ was between 77% and 89%, for the 19mer ssDNA ensembles, $\phi_{
m eff}$ was between 39% and 52%, indicating that the prior distributions for ssRNA were in better agreement with the experimental data than for ssDNA. In view of the strong HCG ensemble reweighting required for ssDNA, which in the case of dT_{38} resulted in a bimodal end-to-end distance distribution, we instead reweighted the transfer efficiency distributions obtained from the analytical polymer models for ssDNA. To achieve this, we discretize the transfer efficiency range uniformly between zero and one, $arepsilon_lpha=(lpha-1)\Deltaarepsilon$, with $\Deltaarepsilon=0.03$, and lpha ranging from 1 to N = 33. We used as priors the distance distributions, P(r), from the analytical polymer models (Eqs. 1-3) to obtain the initial weights $w_{\alpha}^0 = a P(r(\varepsilon_{\alpha})) \left| \frac{dr}{d\varepsilon_{\alpha}} \right|$, where $r(\varepsilon_{\alpha}) = R_0 (1/\varepsilon_{\alpha} - 1)^{1/6}$ and ais a normalization constant ensuring $\sum_{\alpha=1}^{N} w_{\alpha}^{0} = 1$. We then minimized ΔG with respect to $w_{1}, ..., w_{N}$

as described above. For all three different prior distributions, we found very similar reweighted distributions (i.e., values of w_{α}). The end-to-end distance distributions of prior and reweighted polymer models for all ssDNAs are depicted in Fig. S5. We used the reweighted HCG distributions (HCG_{BioEn}) for ssRNA and the reweighted polymer model distributions (PM_{BioEn}) for ssDNA to convert τ_{cd} to τ_r (see below, Eqs. 13/14, Fig. 2, Table S4). We note, however, that even with the reweighted distance distributions from HCG for ssDNA, the resulting values of τ_r are very similar to those from the alternative analyses (Table S4).

Fluorescence correlation spectroscopy (FCS). FCS measurements were performed on freely diffusing Alexa 488- and Alexa 595-labeled oligonucleotides at concentrations and buffer conditions as described in "Free diffusion single molecule spectroscopy". Additionally, we included appropriate concentrations of glycerol to increase the solvent viscosity (measurements performed without ZMWs). The correlation between two time-dependent signal intensities, $I_i(t)$ and $I_j(t)$, measured on two detectors i and j, is defined as:

$$G_{ij}(\tau) = \frac{\langle I_i(t)I_j(t+\tau)\rangle}{\langle I_i(t)\rangle\langle I_j(t)\rangle} - 1,$$
(9)

where the pointed brackets indicate averaging over t. In our experiments, we use two acceptor and two donor detection channels, resulting in the autocorrelations $G_{AA}(\tau)$ and $G_{DD}(\tau)$, and cross-correlations $G_{AD}(\tau)$ and $G_{DA}(\tau)$. By correlating detector pairs, and not the signal from a detector with itself, contributions to the correlations from dead times and afterpulsing of the detectors are eliminated^{4, 31}. Full FCS curves with logarithmically spaced lag times ranging from nanoseconds to seconds (Fig. 1c) were fitted with^{32, 33}

$$G_{ij}(\tau) = a_{ij} \frac{\left(1 - c_{ab}^{ij} e^{-\frac{|\tau|}{\tau_{ab}^{ij}}}\right) \left(1 + c_{cd}^{ij} e^{-\frac{|\tau|}{\tau_{cd}}}\right) \left(1 + c_T^{ij} e^{-\frac{|\tau|}{\tau_T}}\right)}{\left(1 + \frac{|\tau|}{\tau_D}\right) \left(1 + \frac{|\tau|}{s^2 \tau_D}\right)^{1/2}}.$$
(10)

The three terms in the numerator with amplitudes c_{ab} , c_{cd} , c_T and timescales τ_{ab} , τ_{cd} , τ_T describe photon antibunching, chain dynamics, and triplet blinking, respectively. τ_D is the translational diffusion time of the labeled molecules through the confocal volume; a point spread function (PSF) of 3-dimensional Gaussian shape is assumed, with a ratio of axial over lateral radii of $s = \omega_z/\omega_{xy}$ (s = 5.3 without and s = 1.0 with ZMW; note that this PSF is not expected to be a good approximation for the confocal volume in the ZMWs but has been commonly used owing to a lack of suitable alternatives^{1, 34}), and a_{ij} is the amplitude of the correlation functions. Parameters without indices *ij* are treated as shared parameters in the global fits of the auto- and crosscorrelation functions. To study the dynamics in more detail, donor and acceptor fluorescence auto- and crosscorrelation curves were computed and analyzed over a linearly spaced range of lag times, τ , up to a maximum, τ_{max} , that exceeds τ_c by an order of magnitude (Fig. S2). For the subpopulation-specific analysis, we used only photons of bursts with *E* in the range of ±0.2 of the mean transfer efficiency of the FRET-active population, which reduces the contribution of donor-only and acceptor-only signal to the correlation. For direct comparison, correlation curves were normalized to unity at τ_{max} . After normalization and in the limit of $|\tau| \ll \tau_T^{ij}$ and $|\tau| \ll \tau_D^{ij}$, Eq. 10 reduces to:

$$g_{ij}(\tau) = b_{ij} \left(1 - c_{ab} \ e^{-\frac{|\tau|}{\tau_{ab}}} \right) \left(1 + c_{cd} \ e^{-\frac{|\tau|}{\tau_{cd}}} \right), \tag{11}$$

where $b_{ij} = 1/G_{ij}(\tau_{max})$ is the normalization constant.

For quantifying the solvent viscosity directly in the samples as a function of glycerol concentration, we used the information available from the FCS measurements. The average diffusion time of the labeled oligonucleotides through the confocal volume is directly proportional to the solvent viscosity, η , so η can be estimated from an FCS-based calibration curve. Calibration curves were obtained by measuring the diffusion time by means of acceptor and donor autocorrelations and acceptor-donor cross correlations of double-labeled dT_{19}^{ab} at five different known solvent viscosities adjusted with glycerol. The viscosity of each solution was determined using a cone/plate viscometer (DV-I+, Brookfield Engineering Laboratories, Middleboro, MA, USA). Diffusion times were normalized to the diffusion time in buffer and their dependence on viscosity fitted linearly. The solvent viscosity of all other solutions was obtained based on this calibration from the diffusion times of the samples. The values and uncertainties plotted in Fig. 2d represent the resulting means and standard deviations.

Polarization-resolved ensemble fluorescence lifetime measurements. To determine the relevant timescales for fluorescence lifetime analysis, we performed polarization-resolved ensemble lifetime measurements of all ssNAs on a custom-built fluorescence lifetime spectrometer³³, which allowed us to determine the fluorescence lifetimes of Alexa Fluor 488 and 594 as well as the fluorescence anisotropy decays of the dyes conjugated to the different ssNAs. Fluorescence decays of the donor fluorophore were measured on constructs labeled only with Alexa 488. The acceptor fluorescence lifetime decays and corresponding anisotropy decays were measured upon acceptor excitation of double-labeled constructs. All measurements were performed at 150 mM NaCl, 0.01% Tween 20, 0.143 mM BME in 10 mM HEPES buffer with sample concentrations of 50-200 nM. Alexa 488 was excited by a picosecond diode laser (LDH DC 485) at 488 nm with a pulse repetition rate of 40 MHz. Alexa 594 was excited by a supercontinuum light source (SC450-4, Fianium, Southampton, UK), with the wavelength selected using a z582/15 band-pass filter and a pulse frequency of 40 MHz. The emitted donor fluorescence was filtered with an ET 525/50 filter (Chroma Technology), and the acceptor fluorescence with an HQ 650/100 filter (Chroma Technology). The emitted photons were detected with a microchannel plate photomultiplier tube (R3809U-50; Hamamatsu City, Japan) and the arrival times recorded with a PicoHarp 300 photon-counting module (PicoQuant). Intensity decays, $I_{VH}(t)$ and $I_{VV}(t)$, with horizontal and vertical polarizer orientation, respectively, were measured with vertically polarized excitation (Fig. S4). The decays were fitted globally with

$$I_{VH}(t) = \beta \left(1 - r_0 \left(\alpha \, e^{-t/\tau_{rot}} + (1 - \alpha) e^{-t/\tau_M} \right) \right) e^{-t/\tau_{fl}} + c_{VH}$$

$$I_{VV}(t) = G \,\beta \left(1 + 2 \, r_0 \left(\alpha e^{-t/\tau_{rot}} + (1 - \alpha) e^{-t/\tau_M} \right) \right) e^{-t/\tau_{fl}} + c_{VV}, \tag{12}$$

convolved with the instrument response function (IRF, measured with scattered light). $r_0 = 0.38$ is the limiting anisotropy of the dyes³⁵; *G* accounts for the different detection efficiencies of vertically and horizontally polarized light and was obtained for the donor and acceptor intensities from the ratio of the vertical and horizontal emission after horizontal excitation, $G = I_{HV}/I_{HH}$. The offsets c_{VV} and c_{VH} account for background signal. The two rotational correlation times, τ_{rot} and τ_M , account for fast fluorophore rotation and slower tumbling of the entire labeled molecule, respectively. α represents the fractional amplitude of the fast component; β and τ_{fl} represent the amplitude and relaxation time of the fluorescence decay, respectively (Table S2).

Conversion of the intensity correlation time, τ_{cd} , to the chain reconfiguration time τ_r . For any *r*-dependent observable, f(r), the correlation time, τ_f , is defined as

$$\tau_f \equiv \int_0^\infty \frac{\langle \delta f(r(t)) \delta f(r(0)) \rangle_r}{\langle \delta f(r)^2 \rangle_r} dt,$$
(13)

where $\delta f(r) = f(r) - \langle f(r) \rangle_r$, and $\langle \cdot \rangle_r$ denotes $\langle \cdot \rangle_r = \int P(r) dr$. The numerator is defined using the joint probability, $P(r_0, r_t)$, of populating at an arbitrary time zero the distance r_0 and at a later time t the distance r_t . With these definitions, we have $\langle \delta f(r(t)) \delta f(r(0)) \rangle_r = \iint \delta f(r_t) \delta f(r_0) P(r_0, r_t) dr_0 dr_t$. If the dynamics of r(t) are well described as diffusive motion in a potential of mean force, $F(r) = -k_B T ln P(r)$, then τ_f can be calculated from³⁶

$$\tau_f = \frac{\int_0^\infty P(r)^{-1} \left[\int_0^r \delta f(\rho) P(\rho) \, d\rho \right]^2 dr}{D \int_0^\infty \delta f(\rho)^2 P(r) \, dr},\tag{14}$$

where *D* is the effective end-to-end diffusion coefficient. From fitting the nsFCS curves, we get the intensity correlation time $\tau_{cd} = \tau_{\epsilon}$, where $f(r) = \epsilon(r)$ is the transfer efficiency. We can use Eq. 14 to convert τ_{cd} to the physically more interesting chain reconfiguration time, τ_r , where f(r) = r. We calculated conversion ratios $\vartheta = \tau_{cd}/\tau_r$ for all distance distributions used. ϑ as a function of R/R_0 was calculated for the GC, WLC and the SAW-v polymer models, as well as for the HCG_{BioEn} ensembles by varying R_0 (Fig. 1g). Note that ϑ is independent of *D*. The resulting values of τ_r are given in Table S4.

End-to-end contact rates. For comparing end-to-end distance dynamics measured here with published values of end-to-end contact formation rates³⁷, we used *D* obtained using Eq. 14 for all polymer models and all ssNA variants to estimate end-to-end contact rates, k_{ee} (see Table S5), according to³⁸

$$\frac{1}{k_{ee}} = \frac{1}{k_{\rm R}} + \frac{1}{D} \int_{a}^{\infty} \frac{1}{P(r)} \left[\int_{r}^{\infty} P(\rho) \, d\rho \right]^{2} dr, \tag{15}$$

where $k_{\rm R} = q P(a)$ is the reaction-limited rate, with a quenching rate upon contact of $q = 10^{12} s^{-1}$ and a quenching distance of a = 0.4 nm.^{20, 39}



Figure S1. Transfer efficiency histograms of ssNAs.

Transfer efficiency histograms (gray) of ssNAs at 150 mM NaCl in 10 mM HEPES buffer pH 7 fitted with shot noise-limited photon distribution analysis (PDA)⁶⁻⁸ (black solid line). The donor-only populations obtained from PDA ($E \approx 0$) are shown in dark gray and the FRET populations in orange. The similarity in width of measured and PDA histograms indicates the absence of dynamics on a timescale of the diffusion time (300 µs - 1 ms) or slower. dC₁₉ shows a second FRET-active population (pale green), which was excluded from the lifetime and nsFCS analyses. This population increases at low salt, low pH, and low temperatures, and is likely to correspond to the formation of an i-motif⁴⁰.



Figure S2. Example data from nsFCS of ssNAs at different solvent viscosities.

Normalized donor (green) and acceptor (red) fluorescence autoand crosscorrelation (blue) curves of ssNAs at different solvent viscosities, η , with fits (Eq. 11, black solid lines) and timescale of chain dynamics (τ_{cd}) indicated. The uncertainty of au_{cd} in the absence of viscogen (η = 0.9 ± 0.2) represents the standard deviation from three measurements, two without and one with ZMW. See Methods for information on the uncertainty in solvent viscosity. The small negative amplitudes crosscorrelations in the measured for dA_{19} and rA_{19} indicate a low amplitude of long-range distance dynamics.



Figure S3. 3D histograms of relative donor fluorescence lifetime versus transfer efficiency.

Joint distributions of relative lifetime and transfer efficiency of ssNA variants measured at 150 mM NaCl in HEPES buffer pH 7. Indicated are the variances, σ_{ε}^2 (colored) and $\sigma_{\varepsilon BioEn}^2$ (black), donor lifetimes in the absence of FRET, τ_{D0} (see SI/Methods, Table S2), and differences of mean transfer efficiency, $\Delta E = \langle \varepsilon \rangle - \langle E \rangle$, between experimental values and values expected from the HCG_{BioEn} ensembles (for calculation of $\langle \varepsilon \rangle$ from HCG_{BioEn} ensembles, see *Bayesian ensemble refinement*). The black solid curves represent the dynamic lines calculated for the WLC polymer model by varying the persistence length (Eq. 2, Table S3), and the black dashed lines represent the relation expected for static inter-dye distances. In the case of dC₁₉, a second population at $E \approx 0.9$ is visible, which was excluded from further analysis. For dT₃₈, the donor-only population was excluded for clarity by excluding photon burst with PIE stoichiometry ratios above 0.7.



Figure S4. Fluorescence lifetime and anisotropy decays of Alexa Fluor 488 and 594 conjugated to ssNAs.

Normalized parallel (||) and perpendicular (\perp) polarized fluorescence intensity, *l*(*t*), and anisotropy decays, *r*(t) (points), of single-donor labeled (top, green) and double-labeled (bottom, red) ssNAs (150 mM NaCl in HEPES buffer pH7) after donor (top) and acceptor excitation (bottom), respectively, with fits (solid and dashed black lines: Eq. 12, see Table S2) and instrument response function (gray). The rapid anisotropy decays of both donor and acceptor indicate high mobility of the fluorophores.



Figure S5. Dye-to-dye distance distributions and corresponding potentials of mean force from polymer models and hierarchical chain growth.

Probability density functions, P(r), of the dye-to-dye distances and corresponding potentials of mean force, F(r), from polymer models (GC: blue, WLC: orange, SAW-v: red) and from HCG (gray). The reweighted distributions (PM_{BioEn}, HCG_{BioEn}) are shown in light blue and cyan, respectively (reweighted according to the experimentally obtained means and variances of the transfer efficiency distributions of ssNAs, see Methods). Root mean squared dye-to-dye distance are indicated as vertical lines.

Table S1. Sequences of oligoribonucleotides $(rA_{19}, rC_{19}, rU_{19})$ and oligodeoxyribonucleotides $(dA_{19}, dC_{19}, dT_{19}, dT_{38}, dT_{19}^{ab})$.

dA	$5'-5ThioMC6-D/\DeltaAAA/3\DeltamMO/-3'$
un ₁₉	
ac ₁₉	5'-5ThioMC6-D/CCCCCCCCCCCCCC/3AmMO/-3
dT ₁₉	5'-5ThioMC6-D/TTTTTTTTTTTTTTTTT/3AmMO/-3'
dT ₁₉	5'-5ThioMC6-D/TidSpTidSpTidSpTidSpTidSpTidSpTidSpTidSp
dT ₃₈	5'-5ThioMC6-D/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
rA ₁₉	5'-5ThioMC6-D/rArArArArArArArArArArArArArArArArArArA
rC ₁₉	5'-5ThioMC6-D/rCrCrCrCrCrCrCrCrCrCrCrCrCrCrCrCrC/3AmMO/-3'
rU ₁₉	5'-5ThioMC6-D/rUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrU

5'-5ThioMC6-D/	dithiol with a 6-carbon spacer for maleimide labeling
/idSp/	1,2'-Dideoxyribose modification used to insert single base space
/3AmMO/-3'	amino modifier for succinimidyl ester labeling

	$ au_{fl(A488)}$ (ns)	$ au_{fl(A594)}$ (ns)	$\tau_{rot(A488)}(ns) \alpha$ (%)	$\tau_{rot(A594)}(\mathrm{ns}) \alpha$ (%)	$ au_M({ m ns})$
dA ₁₉	3.5	4.0	0.46 89	0.57 76	1.55
dC ₁₉	3.9	4.0	0.40 87	0.65 79	1.97
dT ₁₉	4.0	4.1	0.44 79	0.59 86	1.38
rA ₁₉	3.7	3.9	0.41 88	0.62 72	2.01
rC ₁₉	3.9	4.0	0.39 69	0.51 80	1.42
rU ₁₉	4.0	4.0	0.43 82	0.69 77	1.56

 Table S2. Results from polarization-resolved fluorescence lifetime analysis.

To exclude the influence of the FRET process on the fluorescence lifetime and anisotropy decay analysis of the donor, we measured donor fluorescence decays of ssNAs labeled only with the donor. Note the reduction in fluorescence lifetime of the donor in the presence of purine bases (dA_{19} and rA_{19}), indicating slight dynamic quenching of Alexa Fluor 488 by adenine. For dT_{38} and dT_{19}^{ab} , we used the lifetimes measured for dT_{19} .

	<i>R_{HCG}</i> (nm)	R _{GC} (nm)	$R_{WLC}\mid l_p$ (nm)	$R_{SAW u}$ (nm) $ u$	l_c (nm)	<i>b</i> (nm) <i>n</i>
dA ₁₉	6.4	7.3	6.8 1.3	6.7 0.71	19.1	0.55 34
dC ₁₉	5.6	6.1	5.9 0.9	5.8 0.67	19.1	0.55 34
dT ₁₉	5.5	5.8	5.6 0.9	5.6 0.66	19.1	0.55 34
dT ₁₉	5.4	6.1	5.9 1.1	5.8 0.67	19.1	0.55 34
dT ₃₈	10.0	10.9	10.2 1.4	9.1 0.69	32.4	0.55 59
rA ₁₉	5.5	6.1	5.9 0.9	5.8 0.67	19.1	0.55 34
rC ₁₉	5.9	6.7	6.4 1.1	6.2 0.69	19.1	0.55 34
rU ₁₉	5.1	5.6	5.5 0.8	5.4 0.65	19.1	0.55 34

Table S3. Fit results (R, l_p, ν) obtained by relating the experimentally determined transfer efficiencies to the analytical polymer models and the values used for l_c , b, and n.

The contour length, l_c , used for the analysis of the WLC model was calculated assuming an interphosphate distance of 0.7 nm for both ssRNA and ssDNA⁴¹. The linkers including donor and acceptor dye were modeled in PyMol⁴² in an all-trans conformation, resulting in a total length of 5.8 nm for all constructs. For the SAW- ν polymer model, a segment length of *b* = 0.55 nm was assumed¹⁵.

Table S4. Measured fluorescence correlation times, τ_{cd} , and resulting chain reconfiguration times, τ_r , obtained from Eq. 14 for the different polymer models (GC, WLC, SAW-v) and the HCG_{BioEn} ensembles of ssDNAs and ssRNAs (150mM NaCl in HEPES buffer pH7). Reconfiguration times in gray are only shown for completeness (see Bayesian ensemble refinement for details).

	dA ₁₉	dC ₁₉	dT ₁₉	dT ₁₉	rA ₁₉	rC ₁₉	rU ₁₉	dT ₃₈
τ_{cd} (ns)	14 ±2	12 ±2	10 ±1	8 ±1	17 ±3	10 ±2	10 ±2	21 ±3
$ au_r^{GC}$ (ns)	17 ±2	13 ±2	11 ±2	9 ±1	19 ±2	12 ±2	11 ±2	37±5
$ au_r^{WLC}$ (ns)	15 ±2	12 ±2	10 ±1	8 ±1	18 ±3	11±2	10±2	29±4
$\tau_r^{SAW\nu}$ (ns)	15 ±2	12 ±2	10 ±1	8 ±1	17 ±3	10 ±2	10 ±2	21 ±3
$ au_r^{HCG_{BioEn}}$ (ns)	15 ±2	13 ±2	11 ± 1	9 ±1	17 ±3	10 ±2	11 ±2	31±4
$ au_r^{PM_{BioEn}}$ (ns)	16 ±2	13 ±2	11 ± 1	9 ±1	18 ±4	11 ±2	11 ±2	30 ± 4

Table S5. End-to-end contact rates (k_{ee}) obtained with Eq. 15 for the different polymer models (GC, WLC, SAW-v) and the HCG_{BioEn} ensembles.

$k_{ee} (10^6 s^{-1})$	dA ₁₉	dC ₁₉	dT ₁₉	dT ₁₉ ^{ab}	rA ₁₉	rC ₁₉	rU ₁₉	dT ₃₈
k_{ee}^{GC}	2.37	3.64	4.23	5.33	2.42	3.41	4.88	0.69
k_{ee}^{WLC}	1.48	2.39	2.80	3.51	1.56	2.20	3.27	0.53
$k_{ee}^{SAW\nu}$	0.76	1.26	1.52	1.86	0.84	1.13	1.76	0.33
$k_{ee}^{HCG_{BioEn}}$	0.27	0.31	0.40	0.40	0.20	0.28	0.33	0.04
$k_{ee}^{PM_{BioEn}}$	0.37	0.67	0.97	0.50	0.93	0.61	0.69	0.15

References:

1. Patra, S.; Baibakov, M.; Claude, J. B.; Wenger, J., Surface passivation of zero-mode waveguide nanostructures: benchmarking protocols and fluorescent labels. *Sci Rep* **2020**, *10* (1), 5235.

2. Nüesch, M. F.; Ivanovic, M. T.; Claude, J. B.; Nettels, D.; Best, R. B.; Wenger, J.; Schuler, B., Single-molecule Detection of Ultrafast Biomolecular Dynamics with Nanophotonics. *J. Am. Chem. Soc.* **2022**, *144* (1), 52-56.

3. Muller, B. K.; Zaychikov, E.; Brauchle, C.; Lamb, D. C., Pulsed interleaved excitation. *Biophys. J.* **2005**, *89* (5), 3508-22.

4. Nettels, D.; Gopich, I. V.; Hoffmann, A.; Schuler, B., Ultrafast dynamics of protein collapse from single-molecule photon statistics. *Proc Natl Acad Sci U S A* **2007**, *104* (8), 2655-60.

5. Schuler, B., Application of single molecule Forster resonance energy transfer to protein folding. *Methods Mol Biol* **2007**, *350*, 115-38.

6. Antonik, M.; Felekyan, S.; Gaiduk, A.; Seidel, C. A., Separating structural heterogeneities from stochastic variations in fluorescence resonance energy transfer distributions via photon distribution analysis. *J Phys Chem B* **2006**, *110* (13), 6970-8.

7. Nir, E.; Michalet, X.; Hamadani, K. M.; Laurence, T. A.; Neuhauser, D.; Kovchegov, Y.; Weiss, S., Shot-noise limited single-molecule FRET histograms: comparison between theory and experiments. *J Phys Chem B* **2006**, *110* (44), 22103-24.

8. Kalinin, S.; Felekyan, S.; Valeri, A.; Seidel, C. A., Characterizing multiple molecular states in single-molecule multiparameter fluorescence detection by probability distribution analysis. *J Phys Chem B* **2008**, *112* (28), 8361-74.

9. Kudryavtsev, V.; Sikor, M.; Kalinin, S.; Mokranjac, D.; Seidel, C. A.; Lamb, D. C., Combining MFD and PIE for accurate single-pair Forster resonance energy transfer measurements. *Chemphyschem* **2012**, *13* (4), 1060-78.

10. Lee, N. K.; Kapanidis, A. N.; Wang, Y.; Michalet, X.; Mukhopadhyay, J.; Ebright, R. H.; Weiss, S., Accurate FRET measurements within single diffusing biomolecules using alternating-laser excitation. *Biophys J* **2005**, *88* (4), 2939-53.

11. Holmstrom, E. D.; Holla, A.; Zheng, W.; Nettels, D.; Best, R. B.; Schuler, B., Accurate Transfer Efficiencies, Distance Distributions, and Ensembles of Unfolded and Intrinsically Disordered Proteins From Single-Molecule FRET. *Methods Enzymol* **2018**, *611*, 287-325.

12. Pietrek, L. M.; Stelzl, L. S.; Hummer, G., Structural ensembles of disordered proteins from hierarchical chain growth and simulation. *Curr. Opin. Struct. Biol.* **2023**, *78*, 102501.

13. Schuler, B.; Soranno, A.; Hofmann, H.; Nettels, D., Single-Molecule FRET Spectroscopy and the Polymer Physics of Unfolded and Intrinsically Disordered Proteins. *Annu Rev Biophys* **2016**, *45*, 207-31.

14. O'Brien, E. P.; Morrison, G.; Brooks, B. R.; Thirumalai, D., How accurate are polymer models in the analysis of Förster resonance energy transfer experiments on proteins? *The Journal of chemical physics* **2009**, *130* (12), 124903.

15. Zheng, W.; Zerze, G. H.; Borgia, A.; Mittal, J.; Schuler, B.; Best, R. B., Inferring properties of disordered chains from FRET transfer efficiencies. *J Chem Phys* **2018**, *148* (12), 123329.

16. Hellenkamp, B.; Schmid, S.; Doroshenko, O.; Opanasyuk, O.; Kuhnemuth, R.; Adariani, S. R.; Ambrose, B.; Aznauryan, M.; Barth, A.; Birkedal, V.; Bowen, M. E.; Chen, H.; Cordes, T.; Eilert, T.; Fijen, C.; Gebhardt, C.; Gotz, M.; Gouridis, G.; Gratton, E.; Ha, T.; Hao, P.; Hanke, C. A.; Hartmann, A.; Hendrix, J.; Hildebrandt, L. L.; Hirschfeld, V.; Hohlbein, J.; Hua, B.; Hubner, C. G.; Kallis, E.; Kapanidis, A. N.; Kim, J. Y.; Krainer, G.; Lamb, D. C.; Lee, N. K.; Lemke, E. A.; Levesque, B.; Levitus, M.; McCann, J. J.; Naredi-Rainer, N.; Nettels, D.; Ngo, T.; Qiu, R.; Robb, N. C.; Rocker, C.; Sanabria, H.; Schlierf, M.; Schroder, T.; Schuler, B.; Seidel, H.; Streit, L.; Thurn, J.; Tinnefeld, P.; Tyagi, S.; Vandenberk, N.; Vera, A. M.; Weninger, K. R.; Wunsch, B.; Yanez-Orozco, I. S.; Michaelis, J.; Seidel, C. A. M.; Craggs, T. D.; Hugel, T., Publisher Correction: Precision and accuracy of single-molecule FRET measurements-a multi-laboratory benchmark study. *Nat Methods* **2018**, *15* (11), 984.

17. Schuler, B., Single-molecule FRET of protein structure and dynamics - a primer. *J Nanobiotechnology* **2013**, *11 Suppl 1* (Suppl 1), S2.

18. Aznauryan, M.; Delgado, L.; Soranno, A.; Nettels, D.; Huang, J. R.; Labhardt, A. M.; Grzesiek, S.; Schuler, B., Comprehensive structural and dynamical view of an unfolded protein from the combination of single-molecule FRET, NMR, and SAXS. *Proc Natl Acad Sci U S A* **2016**, *113* (37), E5389-98.

19. Schuler, B.; Lipman, E. A.; Eaton, W. A., Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy. *Nature* **2002**, *419* (6908), 743-7.

20. Soranno, A.; Holla, A.; Dingfelder, F.; Nettels, D.; Makarov, D. E.; Schuler, B., Integrated view of internal friction in unfolded proteins from single-molecule FRET, contact quenching, theory, and simulations. *Proc. Natl. Acad. Sci. USA* **2017**, *114* (10), E1833-E1839.

Galvanetto, N.; Ivanovic, M. T.; Chowdhury, A.; Sottini, A.; Nuesch, M. F.; Nettels, D.; Best,
 R. B.; Schuler, B., Extreme dynamics in a biomolecular condensate. *Nature* 2023, *619* (7971), 876-883.
 Barth, A.; Opanasyuk, O.; Peulen, T. O.; Felekyan, S.; Kalinin, S.; Sanabria, H.; Seidel, C. A.
 M., Unraveling multi-state molecular dynamics in single-molecule FRET experiments. I. Theory of
 FRET-lines. *J Chem Phys* 2022, *156* (14), 141501.

23. Gopich, I. V.; Szabo, A., Theory of the energy transfer efficiency and fluorescence lifetime distribution in single-molecule FRET. *Proc Natl Acad Sci U S A* **2012**, *109* (20), 7747-52.

24. Ivani, I.; Dans, P. D.; Noy, A.; Perez, A.; Faustino, I.; Hospital, A.; Walther, J.; Andrio, P.; Goni, R.; Balaceanu, A.; Portella, G.; Battistini, F.; Gelpi, J. L.; Gonzalez, C.; Vendruscolo, M.; Laughton, C. A.; Harris, S. A.; Case, D. A.; Orozco, M., Parmbsc1: a refined force field for DNA simulations. *Nat Methods* **2016**, *13* (1), 55-8.

25. Tan, D.; Piana, S.; Dirks, R. M.; Shaw, D. E., RNA force field with accuracy comparable to state-of-the-art protein force fields. *Proc Natl Acad Sci U S A* **2018**, *115* (7), E1346-E1355.

26. Piana, S.; Donchev, A. G.; Robustelli, P.; Shaw, D. E., Water dispersion interactions strongly influence simulated structural properties of disordered protein states. *J Phys Chem B* **2015**, *119* (16), 5113-23.

27. Heinz, M.; Erlenbach, N.; Stelzl, L. S.; Thierolf, G.; Kamble, N. R.; Sigurdsson, S. T.; Prisner, T. F.; Hummer, G., High-resolution EPR distance measurements on RNA and DNA with the non-covalent G spin label. *Nucleic Acids Res.* **2020**, *48* (2), 924-933.

28. Pietrek, L. M.; Stelzl, L. S.; Hummer, G., Hierarchical assembly of single-stranded RNA. *bioRxiv* **2023**, 2023.08.01.551474.

29. Grotz, K. K.; Nueesch, M. F.; Holmstrom, E. D.; Heinz, M.; Stelzl, L. S.; Schuler, B.; Hummer, G., Dispersion Correction Alleviates Dye Stacking of Single-Stranded DNA and RNA in Simulations of Single-Molecule Fluorescence Experiments. *J Phys Chem B* **2018**, *122* (49), 11626-11639.

30. Hummer, G.; Köfinger, J., Bayesian ensemble refinement by replica simulations and reweighting. *The Journal of chemical physics* **2015**, *143* (24), 243150.

31. Hanbury Brown, R.; Twiss, R. Q., Correlation between Photons in Two Coherent Beams of Light. *Nature* **1956**, *177* (4497), 27-29.

32. Zander, C.; Enderlein, J.; Keller, R. A., *Single Molecule Detection in Solution, Methods and Applications*. Wiley-VCH: Berlin, 2002.

33. Nettels, D.; Hoffmann, A.; Schuler, B., Unfolded protein and peptide dynamics investigated with single-molecule FRET and correlation spectroscopy from picoseconds to seconds. *J Phys Chem B* **2008**, *112* (19), 6137-46.

34. Baibakov, M.; Barulin, A.; Roy, P.; Claude, J. B.; Patra, S.; Wenger, J., Zero-mode waveguides can be made better: fluorescence enhancement with rectangular aluminum nanoapertures from the visible to the deep ultraviolet. *Nanoscale Adv* **2020**, *2* (9), 4153-4160.

35. Hillger, F.; Hanni, D.; Nettels, D.; Geister, S.; Grandin, M.; Textor, M.; Schuler, B., Probing protein-chaperone interactions with single-molecule fluorescence spectroscopy. *Angew Chem Int Ed Engl* **2008**, *47* (33), 6184-8.

36. Gopich, I. V.; Nettels, D.; Schuler, B.; Szabo, A., Protein dynamics from single-molecule fluorescence intensity correlation functions. *The Journal of chemical physics* **2009**, *131* (9), 095102.

37. Uzawa, T.; Isoshima, T.; Ito, Y.; Ishimori, K.; Makarov, D. E.; Plaxco, K. W., Sequence and temperature dependence of the end-to-end collision dynamics of single-stranded DNA. *Biophys J* **2013**, *104* (11), 2485-92.

38. Lapidus, L. J.; Steinbach, P. J.; Eaton, W. A.; Szabo, A.; Hofrichter, J., Effects of Chain Stiffness on the Dynamics of Loop Formation in Polypeptides. Appendix: Testing a 1-Dimensional Diffusion Model for Peptide Dynamics. *The Journal of Physical Chemistry B* **2002**, *106* (44), 11628-11640.

39. Lapidus, L. J.; Eaton, W. A.; Hofrichter, J., Measuring the rate of intramolecular contact formation in polypeptides. *Proc Natl Acad Sci U S A* **2000**, *97* (13), 7220-5.

40. Abou Assi, H.; Garavís, M.; González, C.; Damha, M. J., i-Motif DNA: structural features and significance to cell biology. *Nucleic Acids Res.* **2018**, *46* (16), 8038-8056.

41. Ho, P. S.; Megan, C., DNA Structure: Alphabet Soup for the Cellular Soul. In *DNA Replication*, Herve, S., Ed. IntechOpen: Rijeka, 2011; p Ch. 1.

42. DeLano, W. L., The PyMOL molecular graphics system. <u>http://www.pymol.org</u> 2002.