

Single-molecule dynamic structural biology with vertically arranged DNA on a fluorescence microscope

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1 Supplementary Discussion

1.1 Single-Molecule vs. Ensemble Precision

When comparing Fig. 1d and Fig. 1e, the reader will note a significant difference in the obtained axial precision. This leads to two questions: i) Why is the precision/uncertainty of the distribution in Fig. 1e substantially larger than the one in the respective case in Fig. 1d? ii) While Fig. 1d shows that the precision worsens for longer dsDNA segments, Fig. 1e depicts the contrary, with an improving precision for longer dsDNA segments.

The discrepancy underlying i) can be explained by the fact that in a single trace exhibiting one stable state the photon count is solely shot-noise limited, while the histogram of an ensemble of single molecules as in Fig. 1e accounts for the local variations in graphene quality. This heterogeneity comprises, *e.g.* holes and defects in the crystal structure, which can hinder the quenching properties of graphene, thereby reducing the effective d_0 , as well as small bilayer areas. Each result could be reliably reproduced with independent graphene samples, showing that these heterogeneities do not significantly affect the final population-averaged result. Though, it is reasonable to assume that they can increase the variability between different analyzed single molecules. For ii), two scenarios must be distinguished: the resulting fluorescence lifetime from a short-noise limited trace is estimated more accurately, *i.e.* the respective uncertainty is smaller, the smaller the respective lifetime value. The uncertainty in lifetime propagates for a photon count of 1000 and a lifetime of 3.0 ns almost linearly with the distance z . For this, shorter dsDNA segments mean higher quenching of the attached fluorophores, lower fluorescence lifetimes and, consequently, a smaller uncertainty on the resulting distance z . In contrast, when a population of single molecules is considered, one has to take into account that fluorophores attached to shorter dsDNA segments, and hence closer to graphene, sense a comparatively smaller area of the underlying graphene sheet. Therefore, the effective d_0 -value for this smaller area reflects more strongly local variations of the graphene sheet leading to an increased uncertainty compared to longer dsDNA segments. More precisely, the area of quenching A_q of the graphene sheet sensed by a molecule scales quadratically with its distance z from the surface, as shown in the following formula: $A_q = \pi \left(\sqrt{\frac{1}{1-\beta}} - 1 \right) z^2 = \pi r_q^2$. Here, β represents the part of the total graphene area contributing to the quenching. For reference, a dye located at 14.0 nm above the surface (as for the 36 bp dsDNA segment) will receive 99% (*i.e.* $\beta = 0.99$) of the total quenching effect from an area of 5,542 nm². In contrast, with $z = 19.3$ nm (as for the 66 bp dsDNA segment) this value is 10,532 nm². This screening effect of a defined area of graphene is visualized in Fig. S6.

1.2 Comparison of Accuracy Obtained by GET and FRET

As outlined in the data analysis part of the methods section of the main text, a measurement of a distance z is converted into a bending angle θ using a purely one-dimensional model for dsDNA. The two dsDNA segments separated by the kink can be modeled as two rigid cylinders allowing each cylinder to have an independent torsion angle (φ and ψ respectively) with respect to its axis, as well as a relative displacement of the two cylinders in the three directions (x_0, y_0 and z_0). In this case, the whole construct has six degrees of freedom, as depicted in Extended Data Fig. 4. In order to estimate θ with FRET, both dsDNA segments have to be labeled with dye molecules. Thus, the bending angle θ is estimated from the measured distance between acceptor and donor dye molecule. This distance depends on each one of the aforementioned degrees of freedom, and hence parallel experiments are required in order to estimate their values, increasing the complexity of both the measurements and the model.

In order to estimate θ with graphene energy transfer (GET), instead, only the upper dsDNA segment has to be labeled. Thanks to the planar geometry of graphene, its distance from the dye depends only on two degrees of freedom (z_0 and ψ - the torsion angle of the upper dsDNA segment). Additionally, given that the typical donor-acceptor distance is far greater in GET than in FRET, every variation in the distance between them due to the uncertainty in the additional degrees of freedom has a smaller relative effect. This is summarized in Extended Data Fig. 4, where one can see that the inaccuracy introduced by using a one-dimensional model for dsDNA is roughly ten times bigger in the case of FRET compared to GET. The small value of the inaccuracy for GET, of the same order of magnitude as the experimental uncertainty, justifies the use of a one-dimensional model, allowing to estimate bending angles with single measurements on a single molecule, which is crucial, especially, when measuring dynamics (*e.g.* in Endo IV experiments).

1.3 Translocation of AGT - Filtering of Steps

To quantify the steps of AGT monomers and clusters diffusing along dsDNA we performed a filtering step to the height time traces. This filter allowed us to consider only initial and final states (before and after the protein step) with heights that can be accurately measured. The steps were detected as follows:

- (i) Each time bin was compared with the following bin. If the difference between them was above a given threshold, then a “state change” was computed (threshold was set to 2.2 Å in Fig. 4; in Fig. S25 we show results using different values)
- (ii) We defined a “stable state” every time at least five consecutive bins were found between “state change” events.

- (iii) To estimate the height of the stable state, we calculated the mean height value of the internal bins, leaving out the first and last bins, since their height might be an average between the height of the analyzed stable state and the following (or previous) states.

Since both clusters and monomers showed slow and fast diffusive components, the spatio-temporal resolution achieved in our GETvNA measurements did not always allow for detecting single base-pair displacements. For example, in some fast events, as the one shown in the zoomed-in region from Fig. 4c, the protein has moved more than 5 nm (> 14 bp) in the axial direction within the duration of a single time bin (50 ms). In such scenarios, a fixed time bin of 50 ms impaired the possibilities of studying whether the protein made a stepwise movement or not. In other words, when the protein has moved too fast, the height corresponding to adjacent bins differs significantly, changes constantly, and therefore the measured position in each time bin only represents an average. During the slow diffusion of the protein, it is possible to observe displacements that correspond to < 1 nm (< 3 bp) jumps, as highlighted in Fig. 4c, and also visible in Fig. 4g. Thus, our temporal resolution demonstrated to be good enough to track the AGT translocation with single-base pair precision in the slow mode. To calculate the height associated with a protein step in the dsDNA axis direction, we performed the filtering step described above. We chose a threshold of $2.2 \text{ \AA}$ for $|z_{t_{i+1}} - z_{t_i}|$, *i.e.* for the absolute value of the difference in height between consecutive bins. As observed in the examples from Fig. 4g (and 4h), every time the protein remained at a stable height, the standard deviation of the axial position was around $0.8\text{-}1 \text{ \AA}$. Therefore, $2.2 \text{ \AA}$ represents a value that is above the maximum achievable precision and will therefore not detect artificial jumps when the protein stays on a stable height. On the other hand, it is smaller than the height of a single base pair and will thus allow detecting single base pair jumps. We chose stable states that lasted at least five bins, to have a minimum number of three points to determine the height of each state. Expectedly, only a small fraction of bins from every analyzed time trace were included in the analysis after this filter was applied, enabling us to obtain an accurate measure of the protein displacement. To increase the statistics, we allowed a temporal window between stable states having a maximum width of 200 bins, *i.e.* 10 s. This also enabled us to detect steps of 0 bp whenever the protein returned to the same base pair after visiting for a short period a neighboring one, as evidenced by the central peak of the histogram from Fig. 4i.

In Fig. S25, we show the effect of varying the threshold to detect jumps by AGT. The shown range for the lower, spatial threshold is limited to $1.8 \text{ \AA} - 2.4 \text{ \AA}$, reflecting the values with a physical meaning, since smaller values overlap with the localization precision and would give rise to the detection of many false jumps. In turn, with a larger spatial threshold, the number of detected stable states lasting more than three bins drastically dropped. Additionally, larger values would decrease the probability of detecting jumps of one base pair, therefore distorting the shape of the ± 1 bp populations in the protein step histogram. In Fig. S25, the effect of varying the time binning of the protein steps histogram, is also shown. Within this range, the presence of peaks matching multiple integers of single bp is observed in all cases. A smaller bin size would lead to the appearance of

artificial peaks due to the insufficient number of events per bin, while a bin size larger than 1.0 Å would lead to the resolution becoming limited by binning and not by the capability of GETvNA. In Fig. S27, we depict the protein step histogram for the data obtained with dsDNA lacking an A-tract, where the peaks observed in Fig. 4i are not visible anymore, evidencing that the A-tract plays a role in slowing down AGT diffusion, enabling the detection of single base pair steps.

2 Supplementary Figures

Fig. S1.

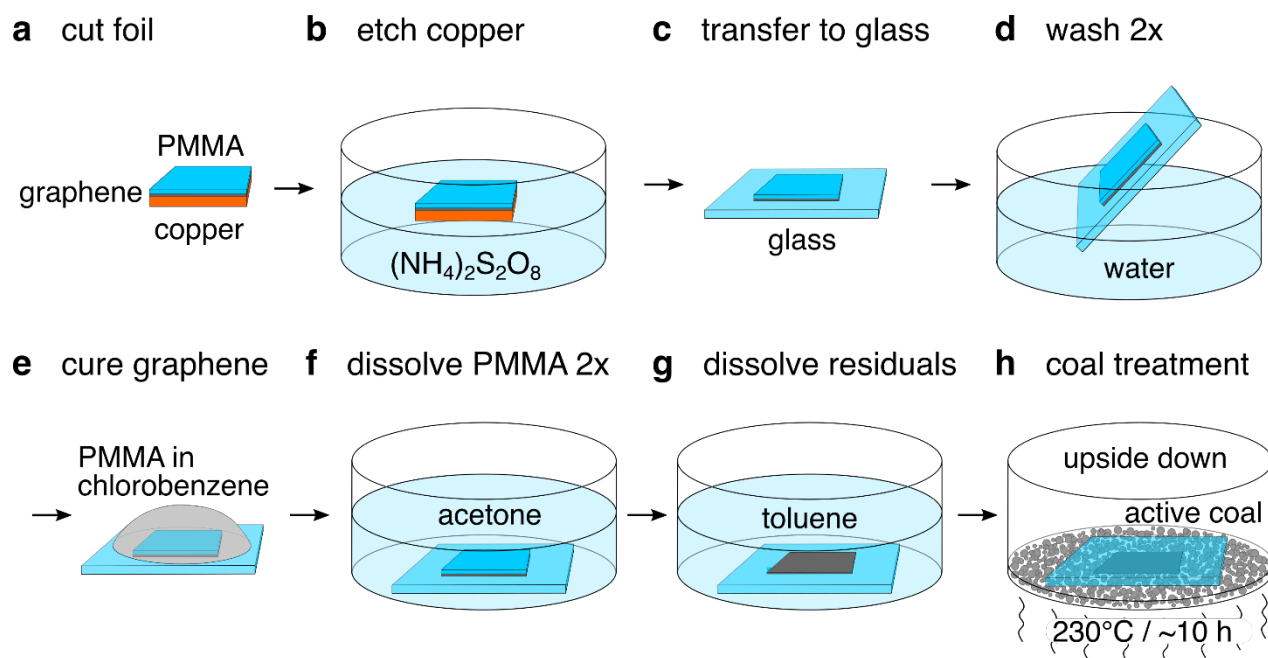


Fig. S1. Workflow for graphene-on-glass transfer. The sketches show the preparation of graphene-on-glass coverslips with the single steps denoted by the letters **a)** to **h)**. Adapted from Krause *et al.*²¹.

Fig. S2.

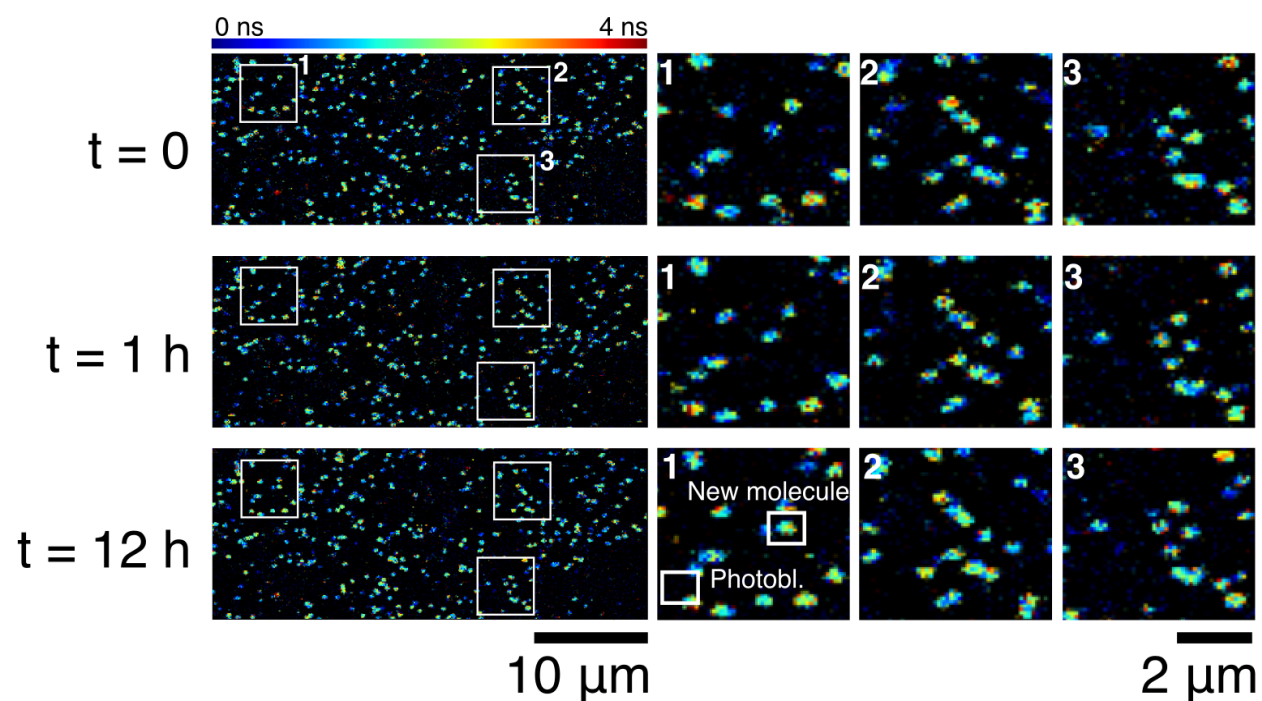


Fig. S2. Long-term stability of GETvNA. Exemplary FLIM images with the fluorescence lifetime being color-coded. The images were acquired as indicated at three different time points. The three columns to the right show zoom-ins (1-3) of the original FLIM image depicted on the left. Here, the 51mer sample was used, imaged with 3 μW excitation power, 0.6 ms dwell time and 50 nm pixel size. We repeated this experiment three times and obtained similar results.

Fig. S3.

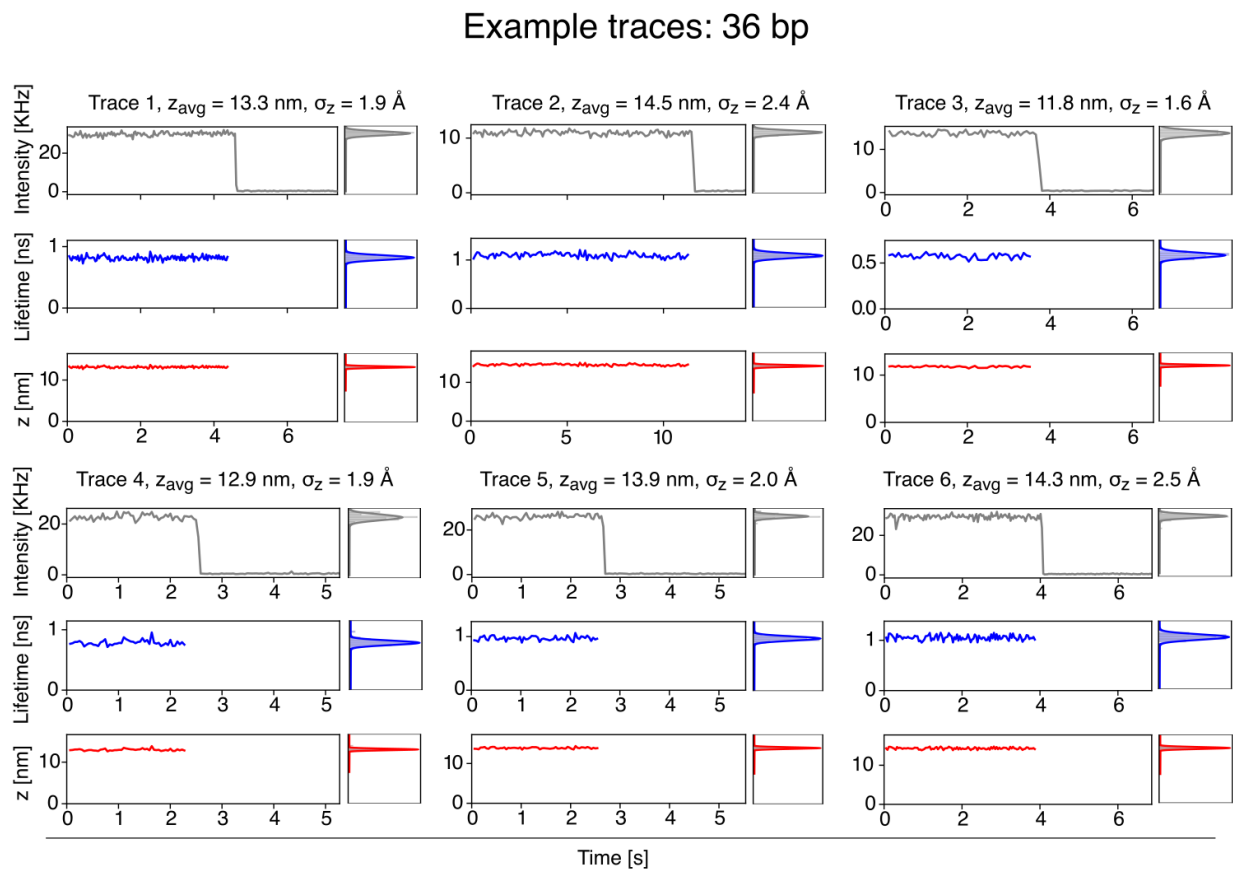


Fig. S3. Exemplary time traces of systems containing a 36 bp-long dsDNA segment. Six exemplary time traces are shown. For each case, the fluorescence intensity, the fluorescence lifetime, and the distance to graphene z are plotted. On the right of each plot, the corresponding histogram is shown. The average distance to graphene, as well as the axial localization precision, are presented on top of each trace.

Fig. S4.

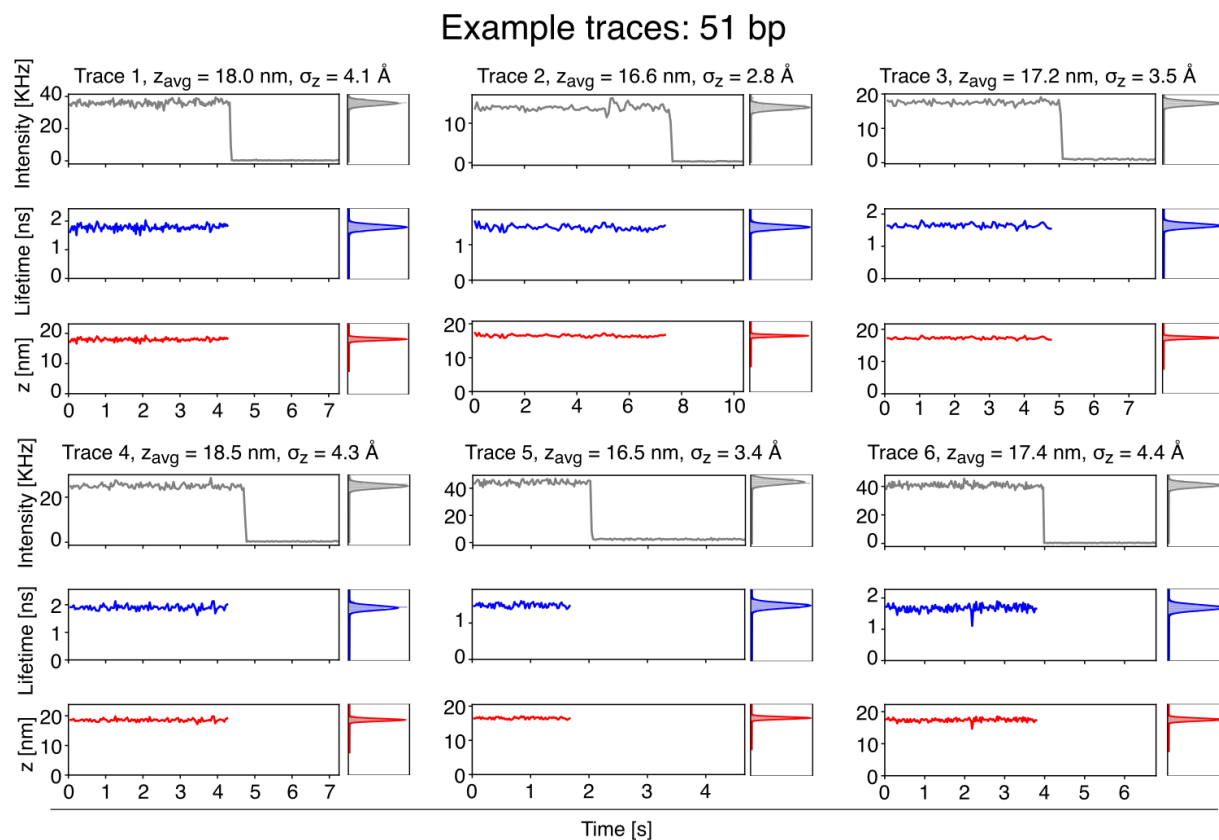


Fig. S4. Exemplary time traces of systems containing a 51 bp-long dsDNA segment. Six exemplary time traces are shown. For each case, the fluorescence intensity, the fluorescence lifetime, and the distance to graphene z are plotted. On the right of each plot, the corresponding histogram is shown. The average distance to graphene, as well as the axial localization precision, are presented on top of each trace.

Fig. S5.

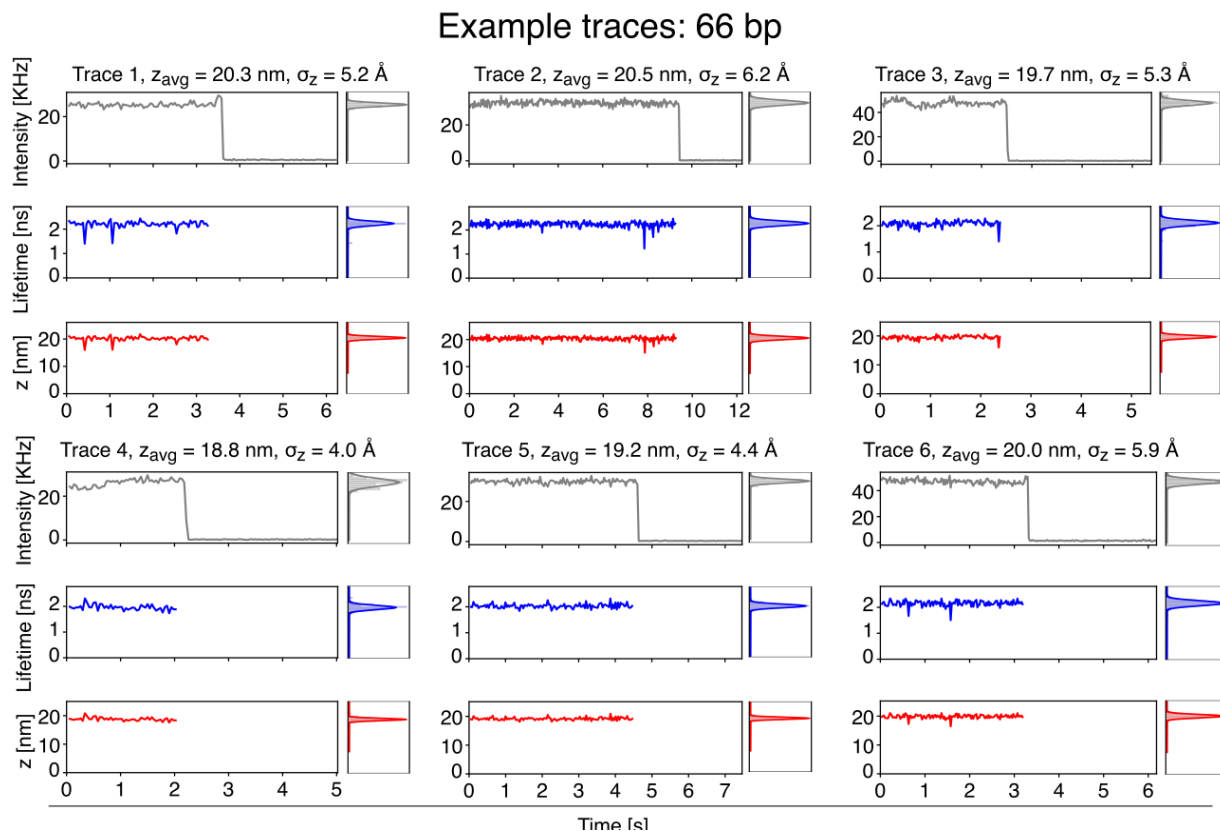


Fig. S5. Exemplary time traces of systems containing a 66 bp-long dsDNA segment. Six exemplary time traces are shown. For each case, the fluorescence intensity, the fluorescence lifetime, and the distance to graphene z are plotted. On the right of each plot, the corresponding histogram is shown. The average distance to graphene, as well as the axial localization precision, are presented on top of each trace.

Fig. S6.

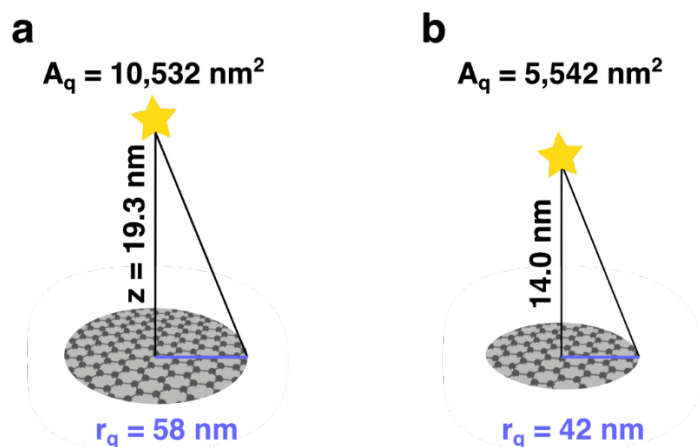


Fig. S6. Comparison of the graphene area sensed by single molecules located at different distances z . The sketches show how quenched fluorescent dyes positioned at larger distances to graphene are sensitive to larger graphene areas. In **a**), $z = 19.3 \text{ nm}$ and in **b**) $z = 14.0 \text{ nm}$. The respective quenching area A_q as well as the radius r_q of the corresponding circular area of graphene are depicted. Here, both quantities are related by $A_q = \pi r_q^2$. In both cases, A_q represents the area of graphene responsible for 99% of the fluorescence quenching experienced by the dye as introduced in section 1.1.

Fig. S7.

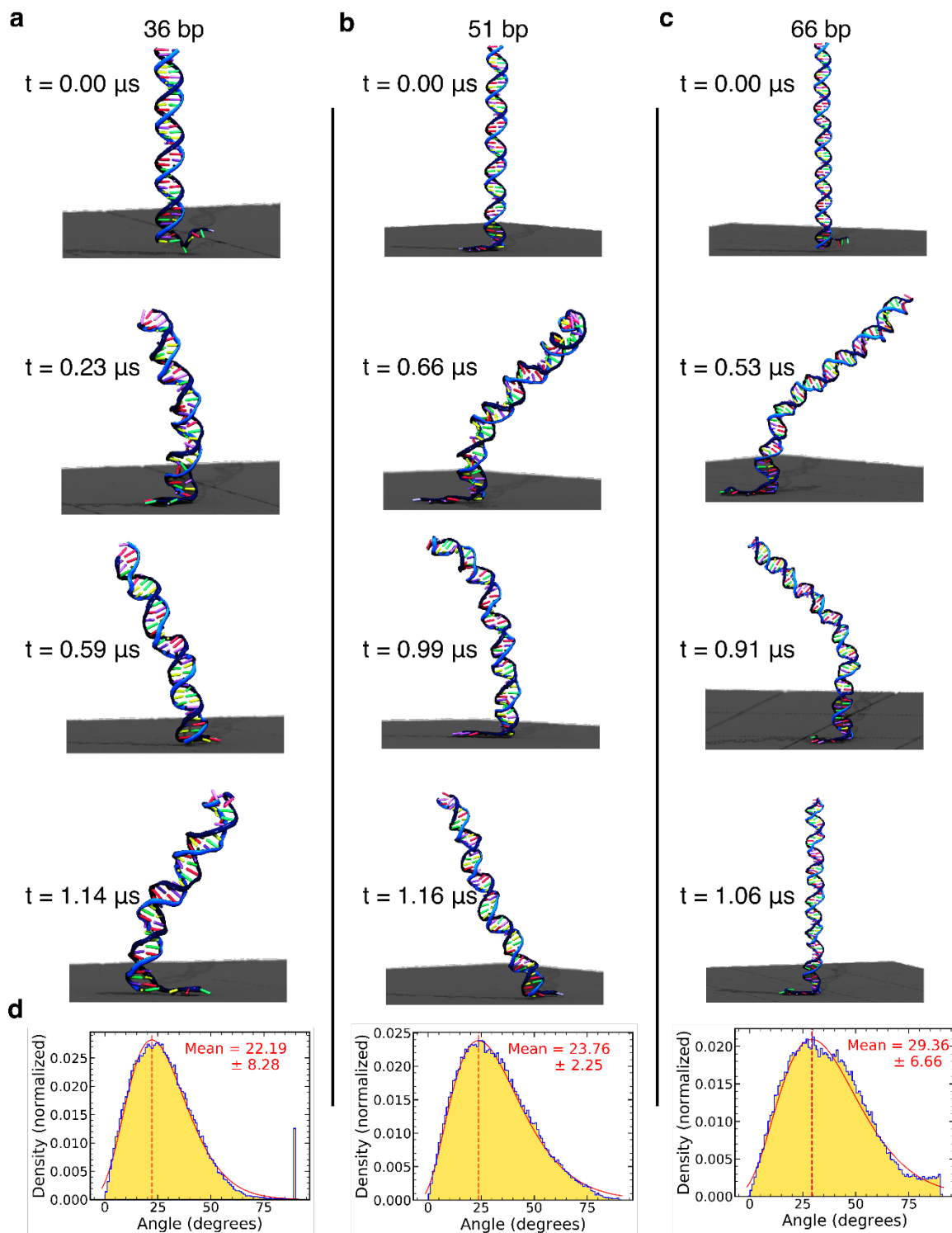


Fig. S7. MD simulations of DNA constructs. a-c) Snapshots illustrating MD simulations of the 36, 51 and 66 base pair DNA constructs at the indicated simulation times as in supplementary movies S1-S3. For visual clarity, water and ions are not shown. The DNA backbone is shown as a

ribbon (blue) and the bases as sticks colored by the base type. The graphene sheet is shown as a smooth surface (gray). **d)** Distribution of the angle that a DNA duplex at the end of each construct forms with the z-axis. To compute the orientation of the duplex, coordinates of N1 atoms of purine and N3 atoms of pyrimidine bases were recorded every 9.6 ps for a six base pair fragment of the constructs located two base pairs away from the construct's end. For every frame of the MD trajectory, the direction was identified from the covariance matrix of the atomic coordinates, as the eigenvector of the largest eigenvalue. A gamma distribution (red) was fitted to the distributions to obtain the mean values (red dashed line).

Fig. S8.

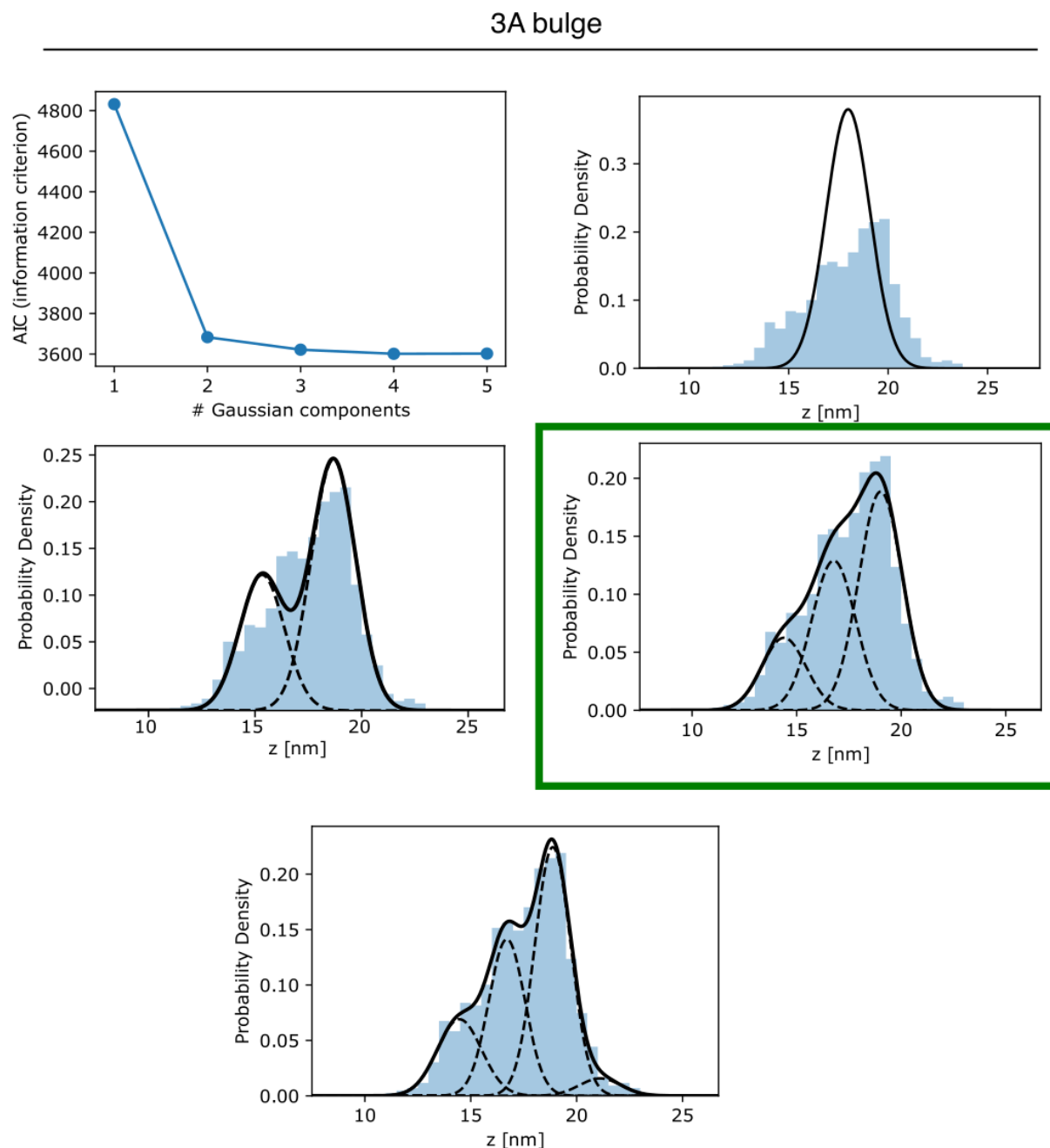


Fig. S8. Selection of the number of fitted Gaussian functions for 3A bulge. For the selection of the number of fitted Gaussian functions a Gaussian mixture model with a varying number of Gaussian components was used to fit the data (blue histogram). The underlying Akaike-information criterium (top left) was computed for each scenario for judgement. From top to bottom the number of Gaussian components in increased incrementally by one. The green frame highlights the scenario that is applied to the data in Fig. 2 of the main text.

Fig. S9.

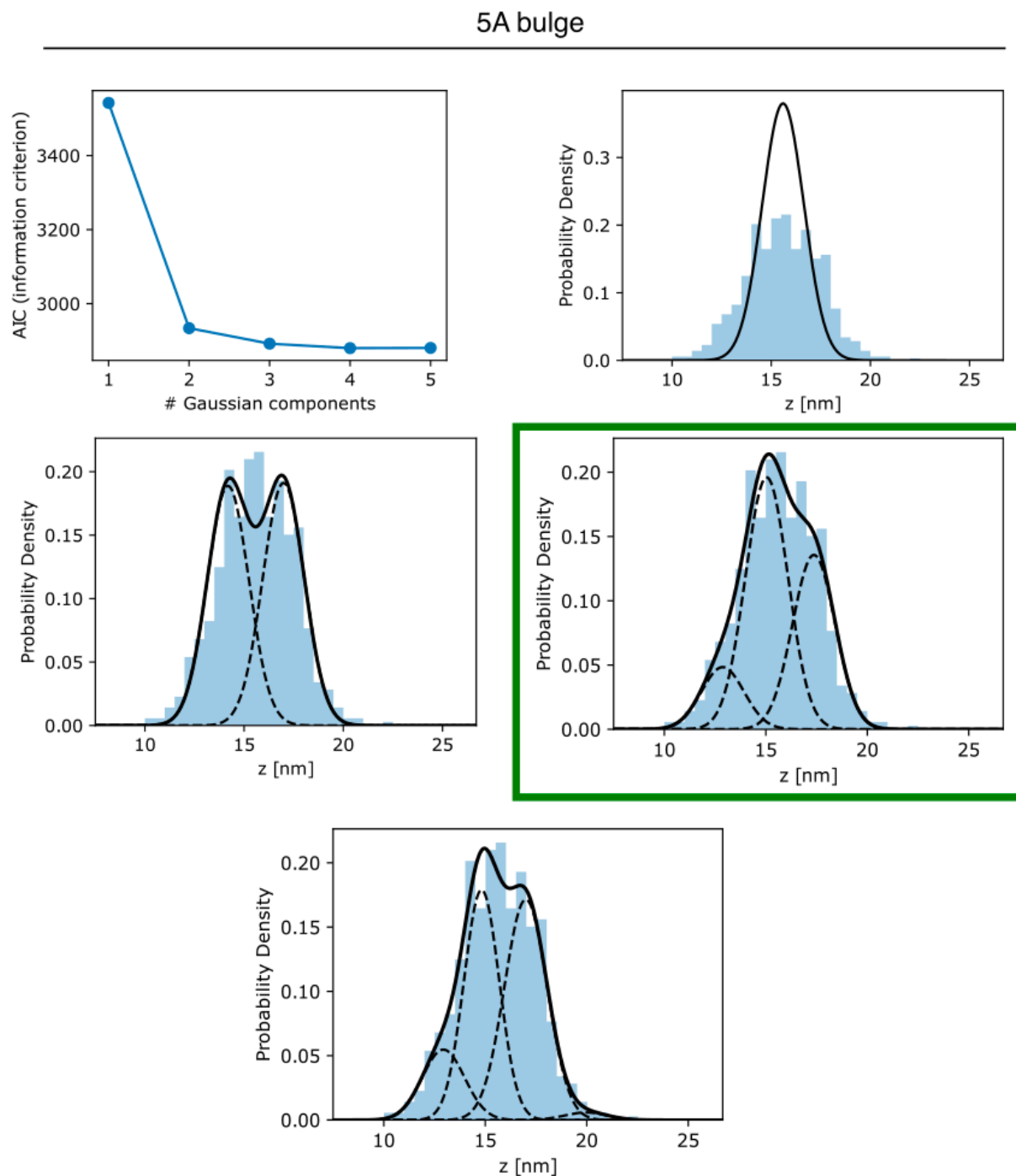


Fig. S9. Selection of the number of fitted Gaussian functions for 5A bulge. For the selection of the number of fitted Gaussian functions a Gaussian mixture model with a varying number of Gaussian components was used to fit the data (blue histogram). The underlying Akaike-information criterium (top left) was computed for each scenario for judgement. From top to bottom the number

of Gaussian components in increased incrementally by one. The green frame highlights the scenario that is applied to the data in Fig. 2 of the main text.

Fig. S10.

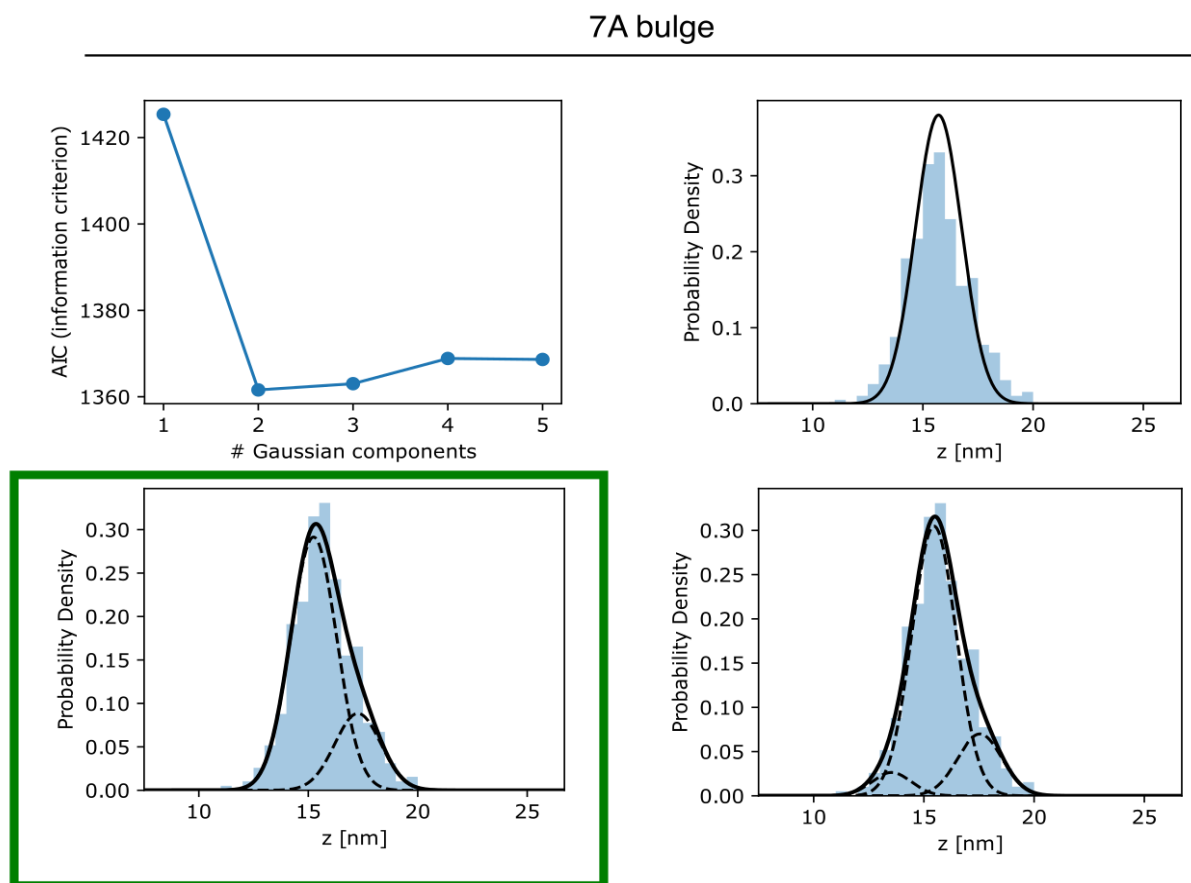


Fig. S10. Selection of the number of fitted Gaussian functions for 7A bulge. For the selection of the number of fitted Gaussian functions a Gaussian mixture model with a varying number of Gaussian components was used to fit the data (blue histogram). The underlying Akaike-information criterium (top left) was computed for each scenario for judgement. From top to bottom the number of Gaussian components in increased incrementally by one. The green frame highlights the scenario that is applied to the data in Fig. 2 of the main text.

Fig. S11.

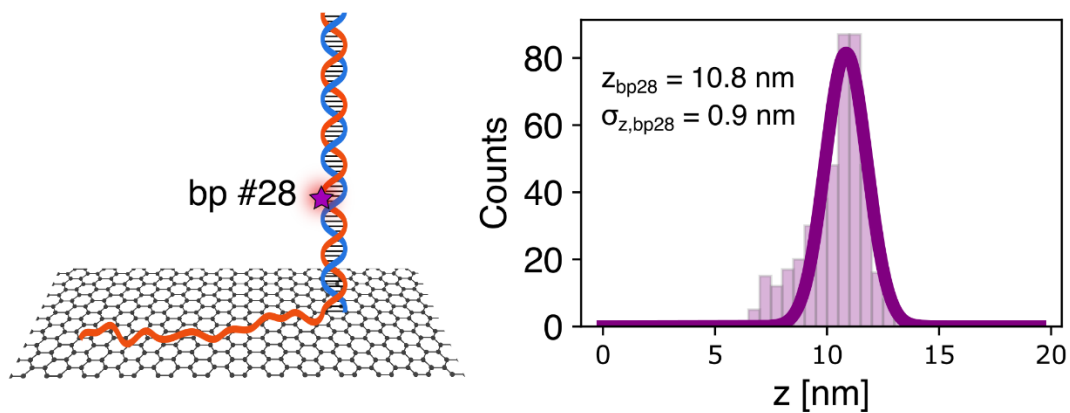


Fig. S11. A-tract height determination. Left: sketch of the internally labeled construct. The dsDNA segment was functionalized with ATTO 647N at one of the bases of bp #28. Right: height histogram for bp #28 obtained from 436 single-molecule traces.

Fig. S12.

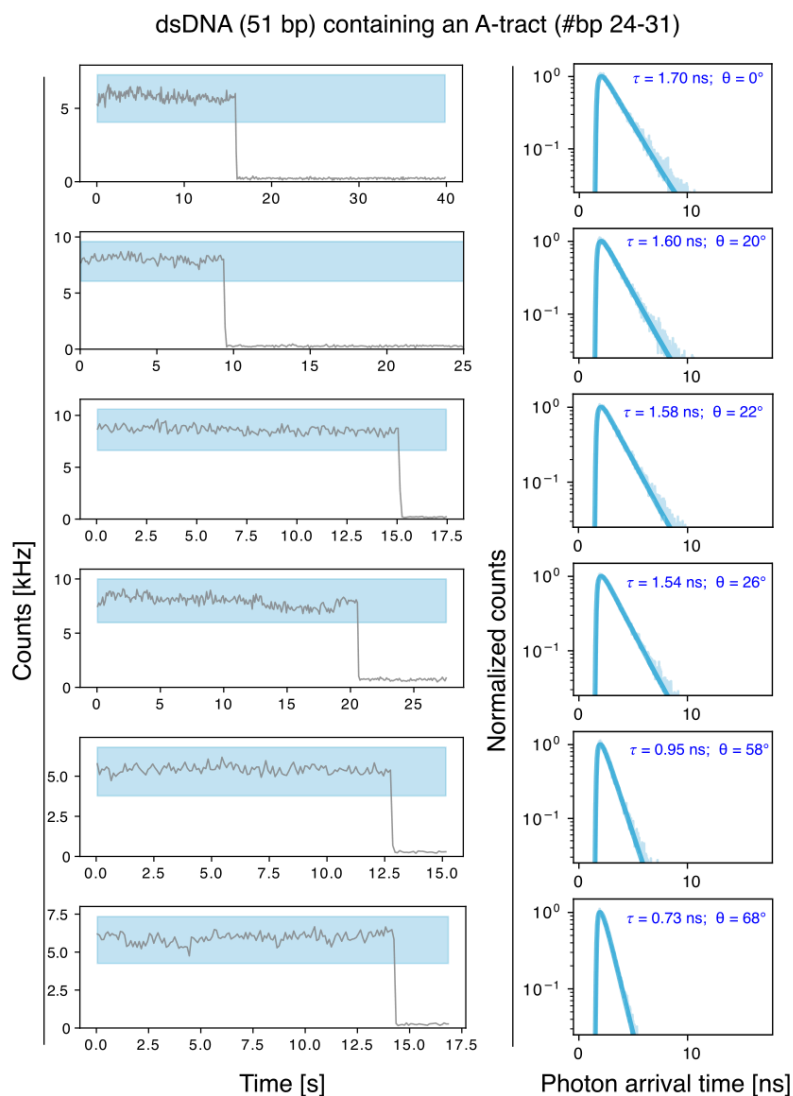


Fig. S12. Exemplary time traces of systems where the dsDNA segment contained an A-tract. The A-tract was located between bp #24 and #31 (counting from the dsDNA end facing graphene). Six example time traces are shown. The fluorescence intensity time traces are depicted on the left, and the fluorescence decays and corresponding monoexponential fits on the right. For each case, the fitted fluorescence lifetime and the corresponding bending angle are shown within the fluorescence decay plots. The rectangles from the intensity time traces highlight the photons used to obtain the fluorescence lifetime decay curves.

Fig. S13.

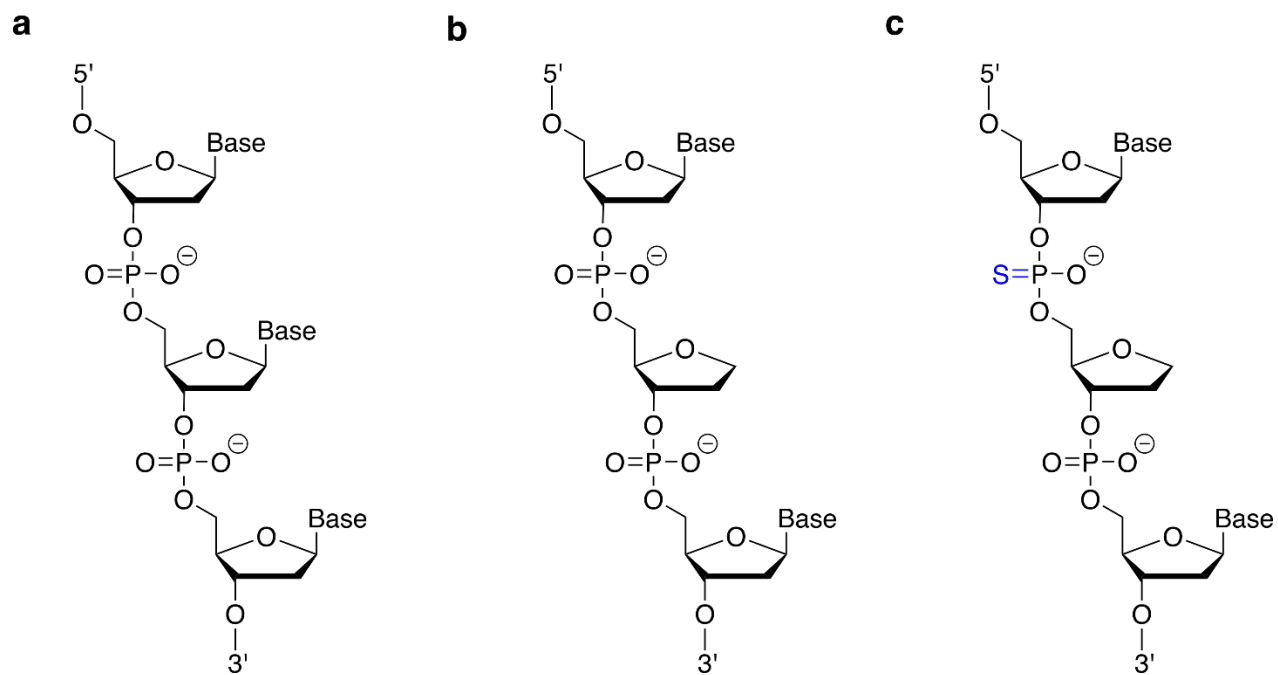


Fig. S13. Chemical structures of DNA modifications. **a)** Unmodified ssDNA. **b)** DNA strand with an AP site as used within this study. **c)** Additional PTO modification indicated in blue creating a DNA strand with AP site as well as a PTO modification.

Fig. S14.

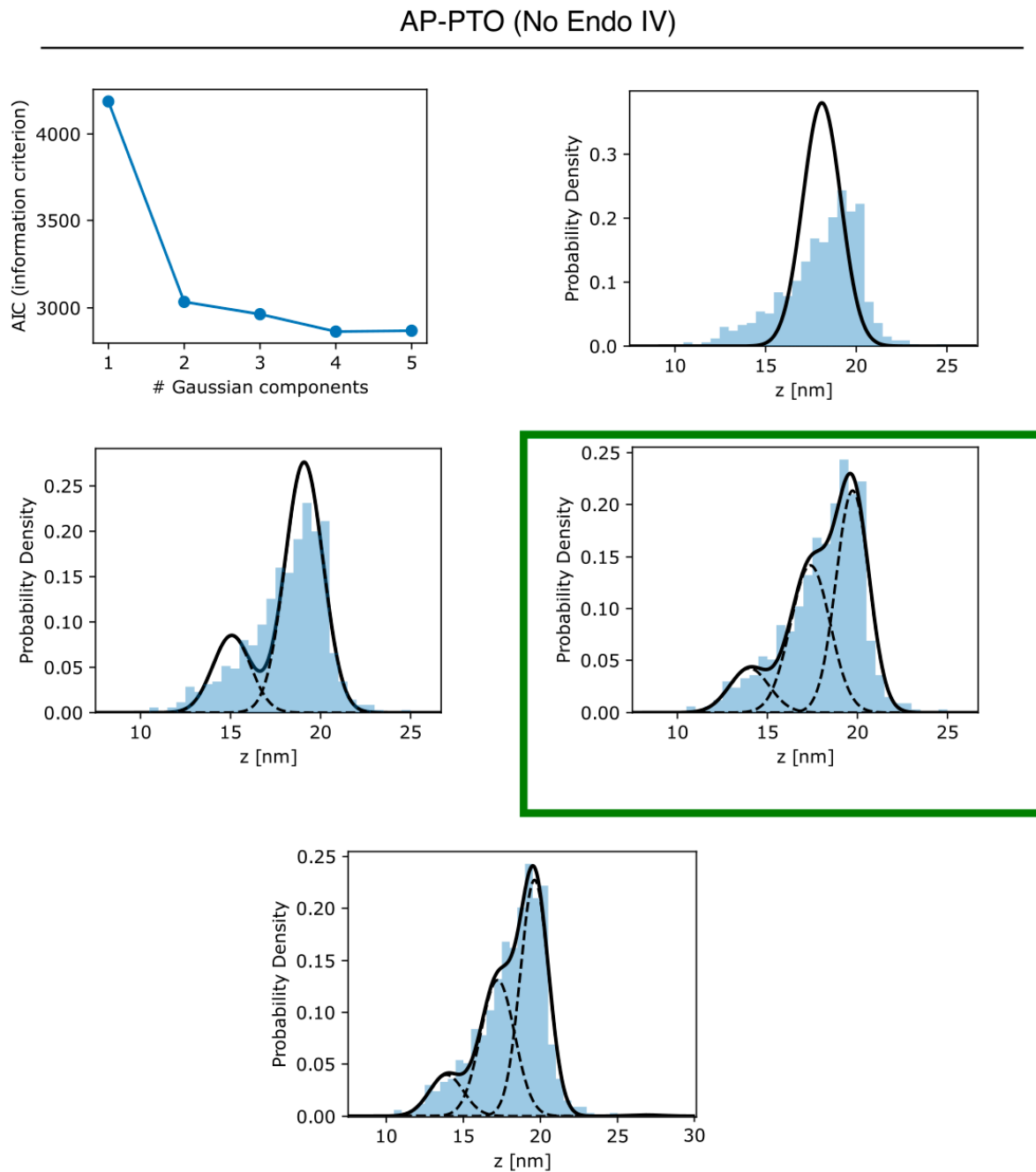


Fig. S14. Selection of the number of fitted Gaussian functions for dsDNA with AP-site and PTO modification, but no added Endo IV. For the selection of the number of fitted Gaussian functions a Gaussian mixture model with a varying number of Gaussian components was used to fit the data (blue histogram). The underlying Akaike-information criterium (top left) was computed for each scenario for judgement. From top to bottom the number of Gaussian components in

increased incrementally by one. The green frame highlights the scenario that is applied to the data in Fig. 2 of the main text.

Fig. S15.

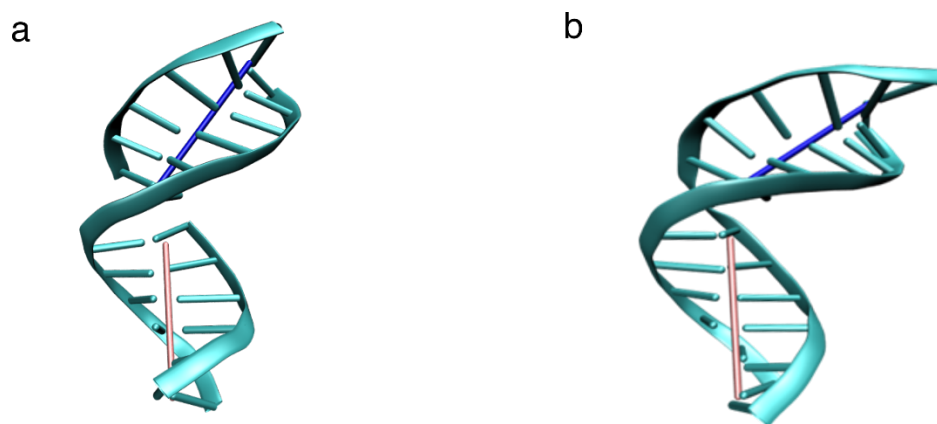


Fig. S15. Structures of the alpha and beta anomers of dsDNA with an AP site. The shown average structures were reconstructed from NMR studies. A cartoon representation of the DNA in the PDB structures 2HSL in **a)** and 2HSS in **b)** is shown in pale blue. Superimposed are the two axes of the helix (dark blue and red) of the two segments divided by the AP site. The angle between both axes is 42.3° for the alpha anomer and 53.3° for the beta anomer.

Fig. S16.

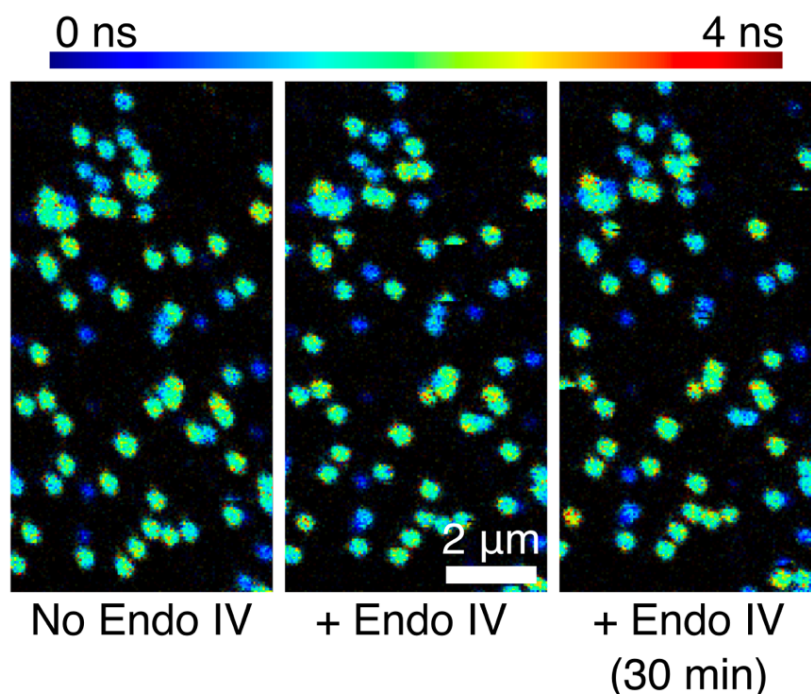


Fig. S16. Change of the fluorescence lifetime due to the addition of Endo IV. Here, the 51mer sample was used, imaged with 4 μ W excitation power, 1 ms dwell time and 50 nm pixel size. We repeated this experiment twice and obtained similar results.

Fig. S17.

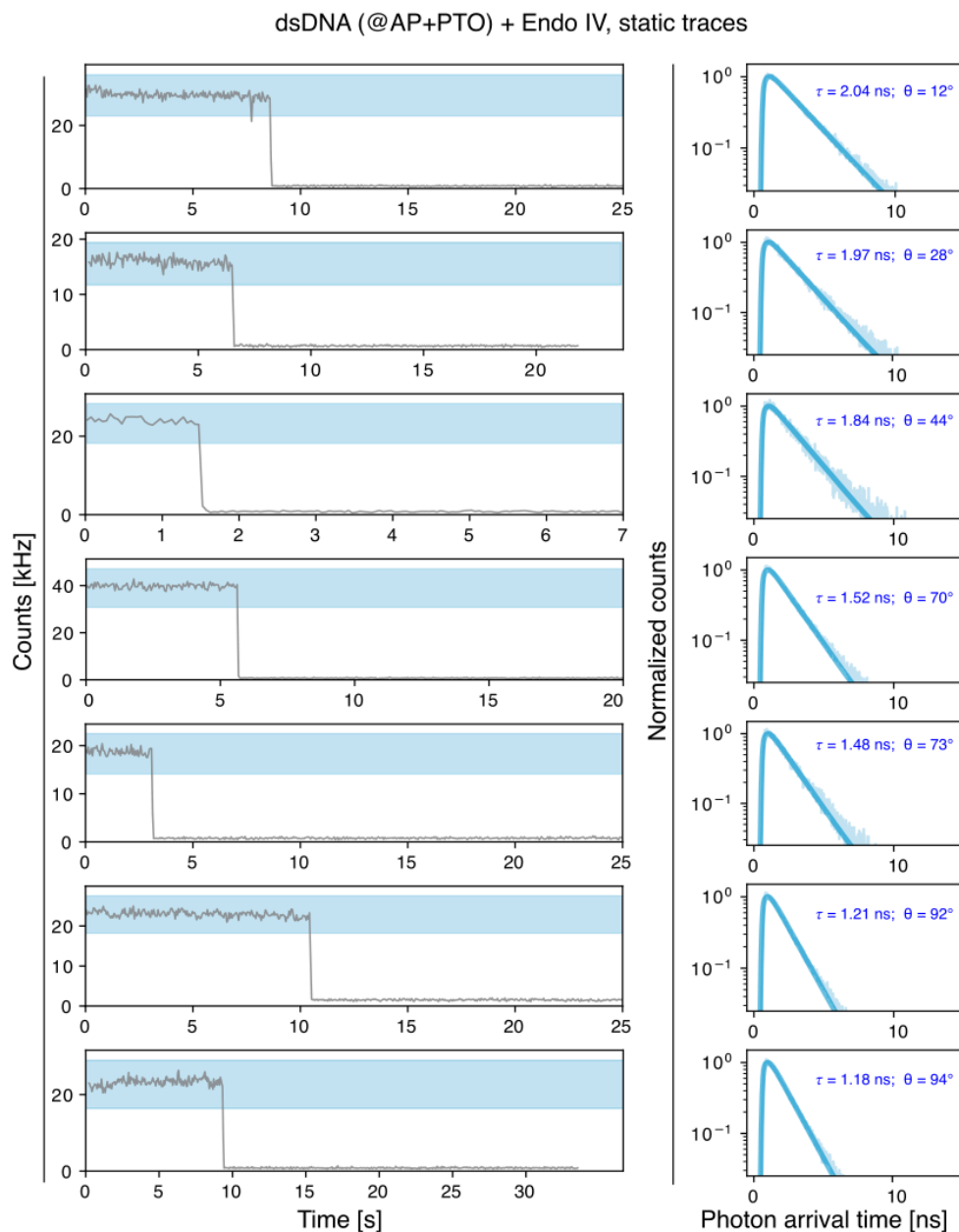


Fig. S17. Exemplary time traces not exhibiting a state switching in the presence of Endo IV.

Here, the measurements correspond to systems where the dsDNA segment contained an AP site and a PTO modification. Seven example time traces are shown. The fluorescence intensity time traces are depicted on the left, and the fluorescence decays and corresponding monoexponential fits on the right. The rectangles from the intensity time traces highlight the photons used to obtain the fluorescence lifetime decay curves. The fitted fluorescence lifetime and the corresponding bending angle are shown next to the fluorescence decays.

Fig. S18.

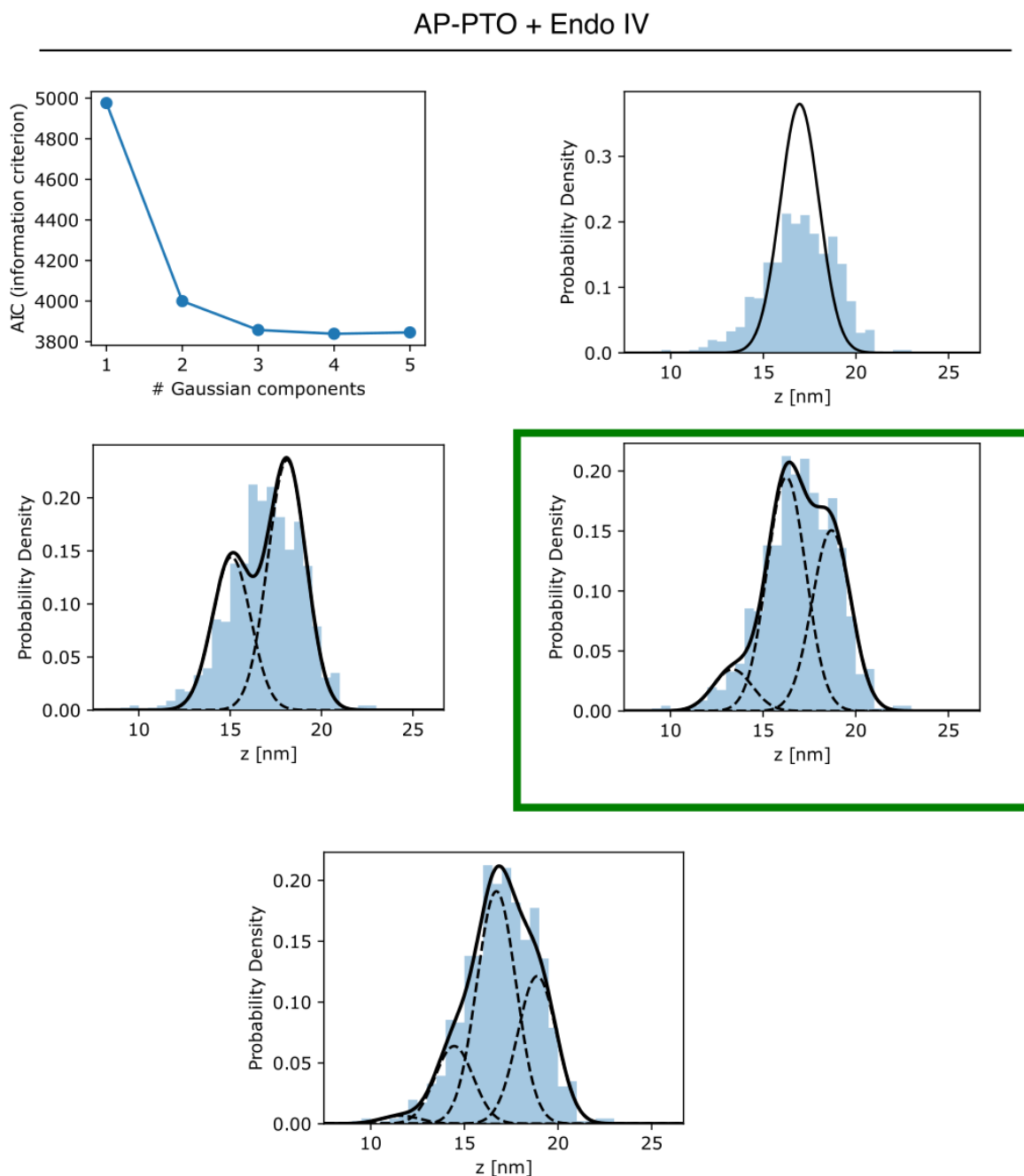


Fig. S18. Selection of the number of fitted Gaussian functions for dsDNA with AP-site and PTO modification and added Endo IV. For the selection of the number of fitted Gaussian functions a Gaussian mixture model with a varying number of Gaussian components was used to fit the data (blue histogram). The underlying Akaike-information criterium (top left) was computed for each scenario for judgement. From top to bottom the number of Gaussian components in

increased incrementally by one. The green frame highlights the scenario that is applied to the data in Fig. 2 of the main text.

Fig. S19.

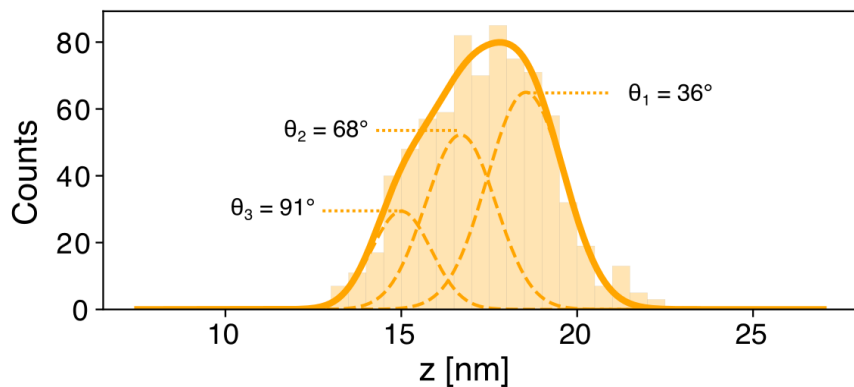


Fig. S19. Histogram of heights obtained by GETvNA for dsDNA containing an AP site and lacking a PTO modification, with and without Endo IV. For static traces, a single height was computed, obtained from the fluorescence lifetime fitted using all the photons detected before photobleaching. For the dynamic two-state switching, two fluorescence lifetimes corresponding to the two states were computed. A three-peak Gaussian distribution was used to fit the experimental distribution. The bending angles corresponding to each subpopulation are also shown.

Fig. S20.

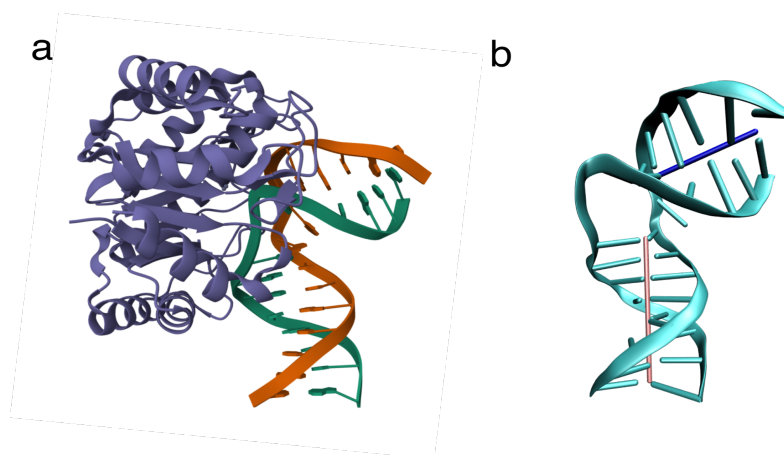


Fig. S20. Crystal structure of Endo IV bending dsDNA. a) PDB structure 2NQJ³⁸ showing Endo IV (purple) recognizing an AP site and introducing subsequent bending of the dsDNA segment. **b)** Helical axis of the Endo IV-bent DNA. A cartoon representation of the DNA in the PDB structure 2NQJ is shown in pale blue. Here, the protein is not shown for visual clarity. Superimposed are the two axes of the helix (dark blue and red) of the two segments divided by the AP site. The angle between both axes of the helix is 74.4°.

Fig. S21.

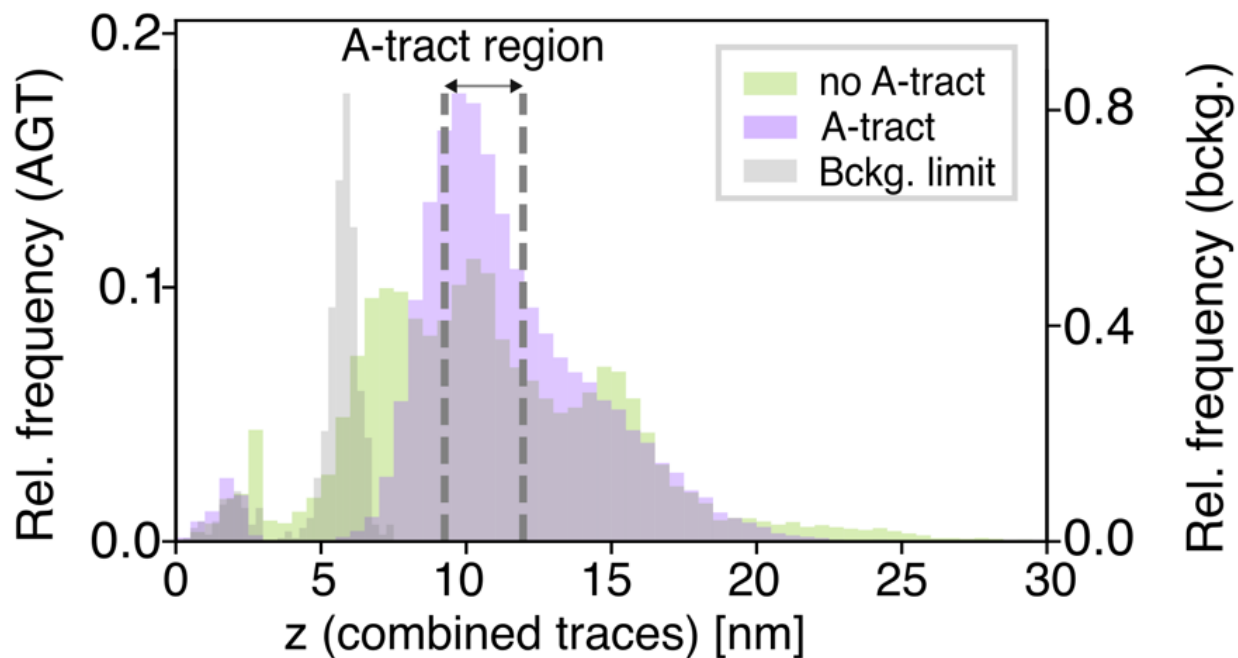


Fig. S21. Height histograms for AGT cluster dependent on the presence of an A-tract. For each distribution, twelve traces lasting 180 seconds were used. Here, it should be noticed that cluster growth was produced by the binding of unlabeled AGT to fluorescently labeled AGT already bound to DNA. This explains why the labeled proteins remained around the A-tract region and were not axially shifted.

Fig. S22.

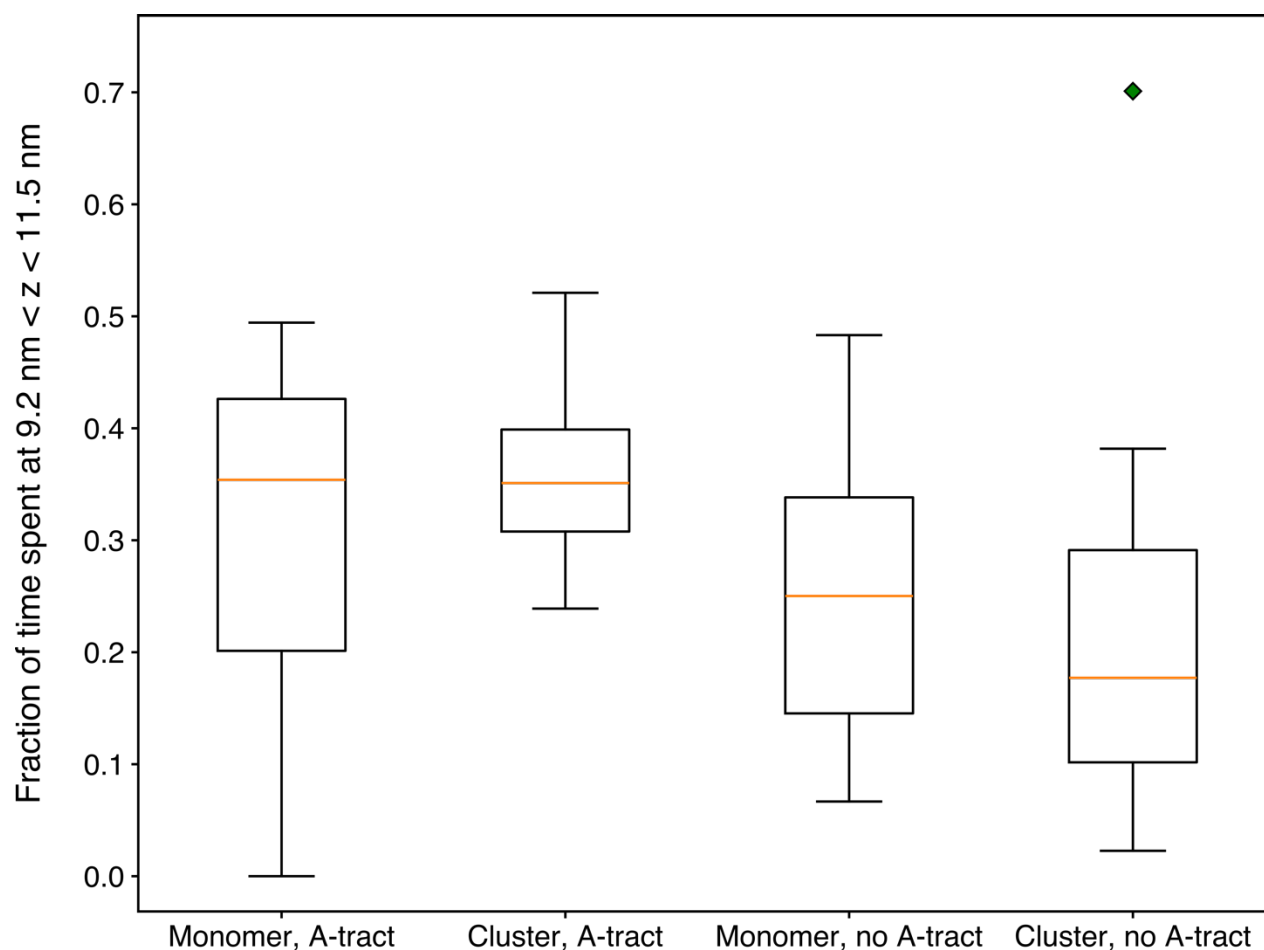


Fig. S22. Boxplot showing the fraction of time spent within the region where the A-tract is located (in samples containing an A-tract). For each condition, twelve traces lasting 180 seconds were used (except for Monomer, A-tract, where we used twenty-two traces), and from each of them the time spent between 9.2 nm and 11.5 nm was computed. The shown distributions contain traces from two independent measurements corresponding to technical replicates (we prepared different graphene surfaces, but using the same batch of AGT mutant). The box extends from the first quartile to the third quartile of the data, and the median is marked with a line. The whiskers extend from the box to the farthest data point lying within $1.5\times$ the inter-quartile range from the box, and points extending farther from that range are marked as outliers.

Fig. S23.

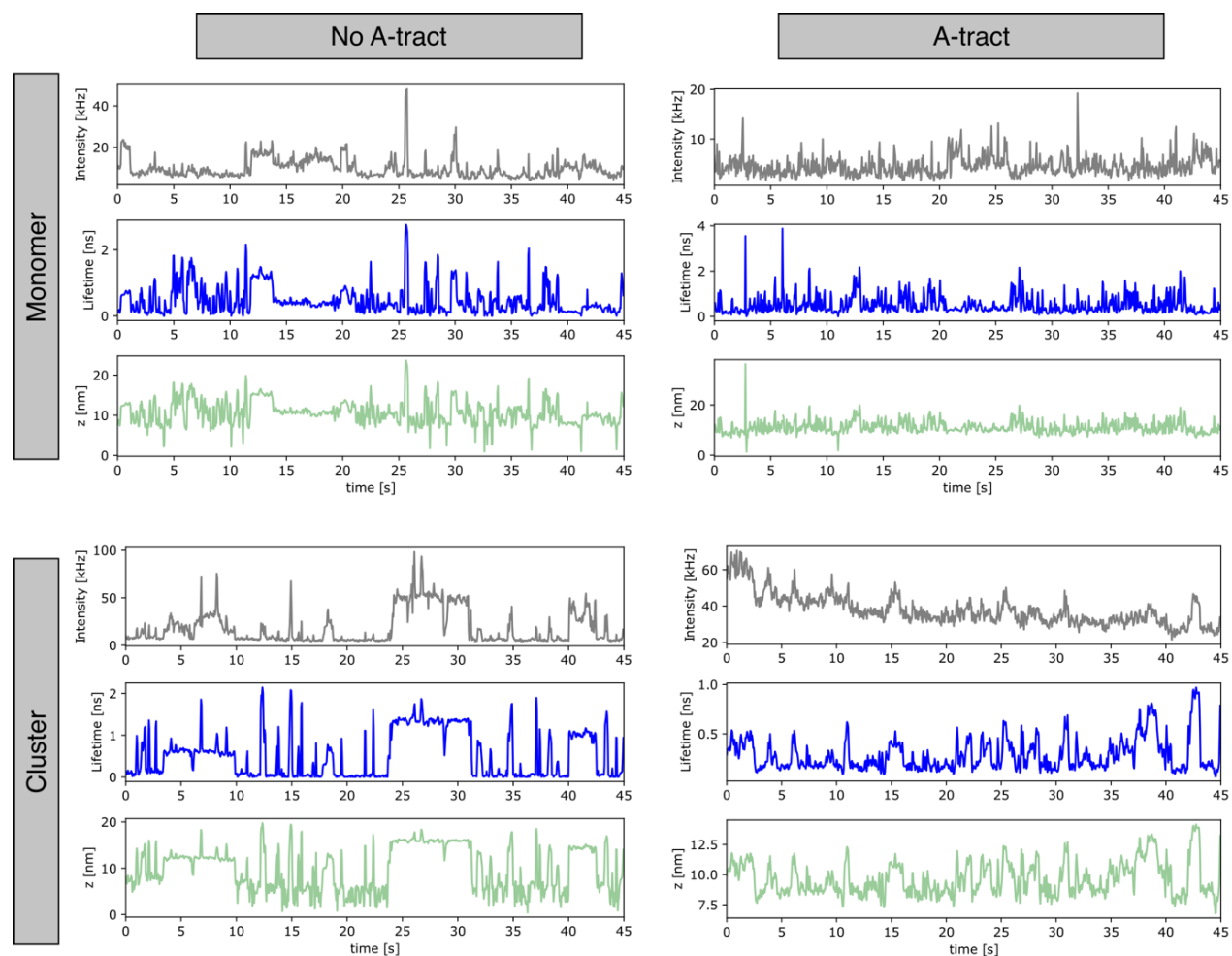


Fig. S23. Exemplary time traces of AGT clusters and monomers, diffusing along dsDNA containing or lacking the A-tract. For all cases, the fluorescence intensity, fluorescence lifetime and height time traces are depicted.

Fig. S24.

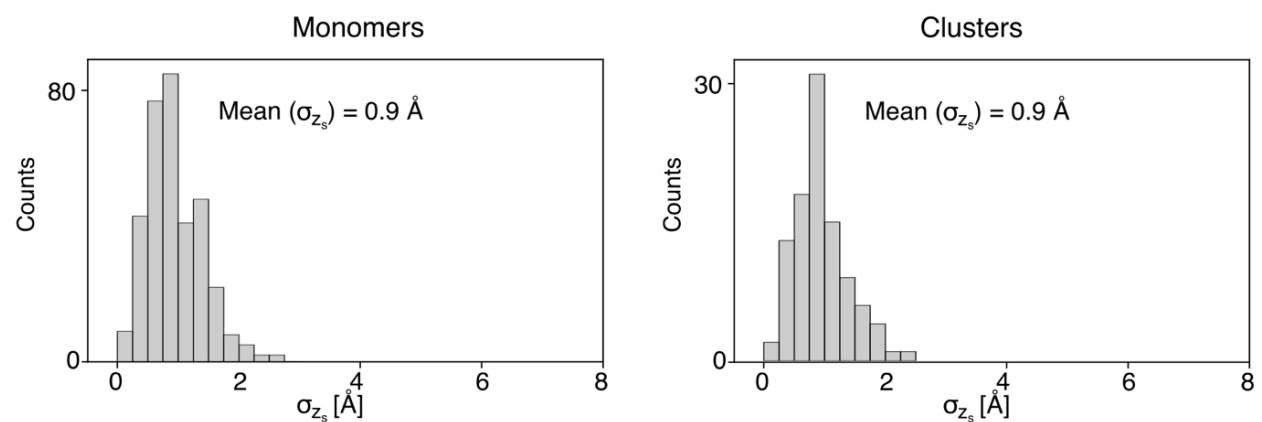


Fig. S24. Histogram of the precision of AGT steps for monomers and clusters. The data are obtained from stable states found in traces of AGT diffusing on DNA containing an A-tract, corresponding to monomers (left) and clusters (right), respectively.

Fig. S25.

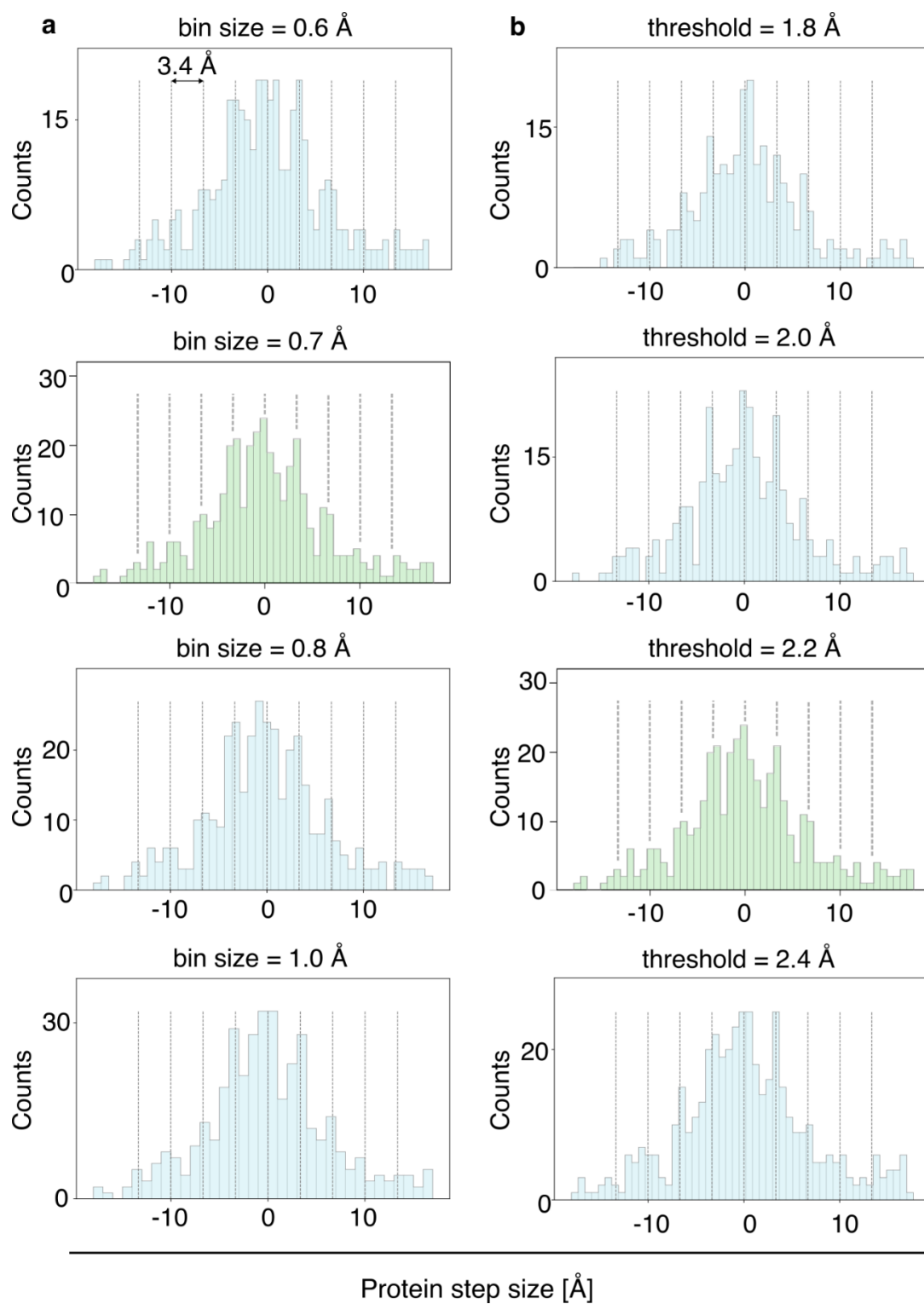


Fig. S25. Influence of the parameters used to filter and study AGT steps when diffusing on dsDNA containing an A-tract. The histograms of the protein step size obtained for all the data with DNA after performing the filtering step with different parameters are shown. On the left, the influence of the chosen time bin is depicted, while on the right we present how the height threshold used to detect a protein step affects the step size distribution. Whereas the purple plots show the tested combination of parameters, the two green plots represent the final values used in Fig. 4 in the main text.

Fig. S26.

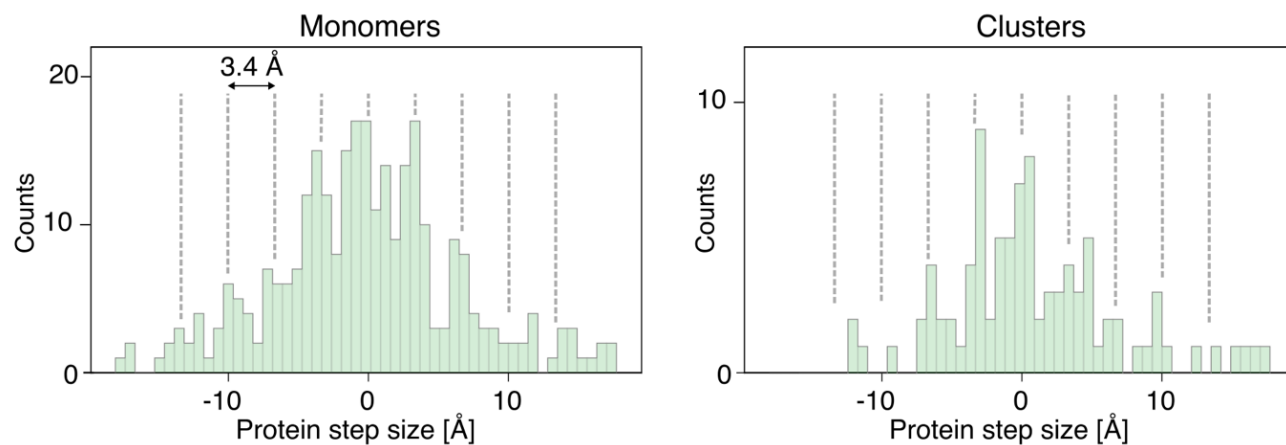


Fig. S26. Histogram of AGT step sizes for monomers and clusters. The data are obtained from stable states found in traces of AGT diffusing on DNA containing an A-tract, corresponding to monomers (left) and clusters (right), respectively.

Fig. S27.

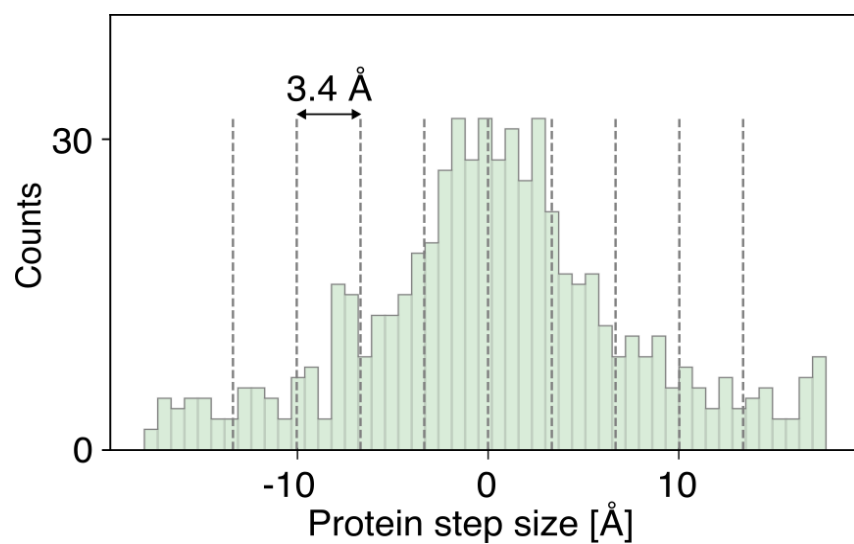


Fig. S27. Histogram of protein step size, obtained for all the data acquired on DNA lacking an A-tract after performing the filtering step described in section 1.3.

Fig. S28.

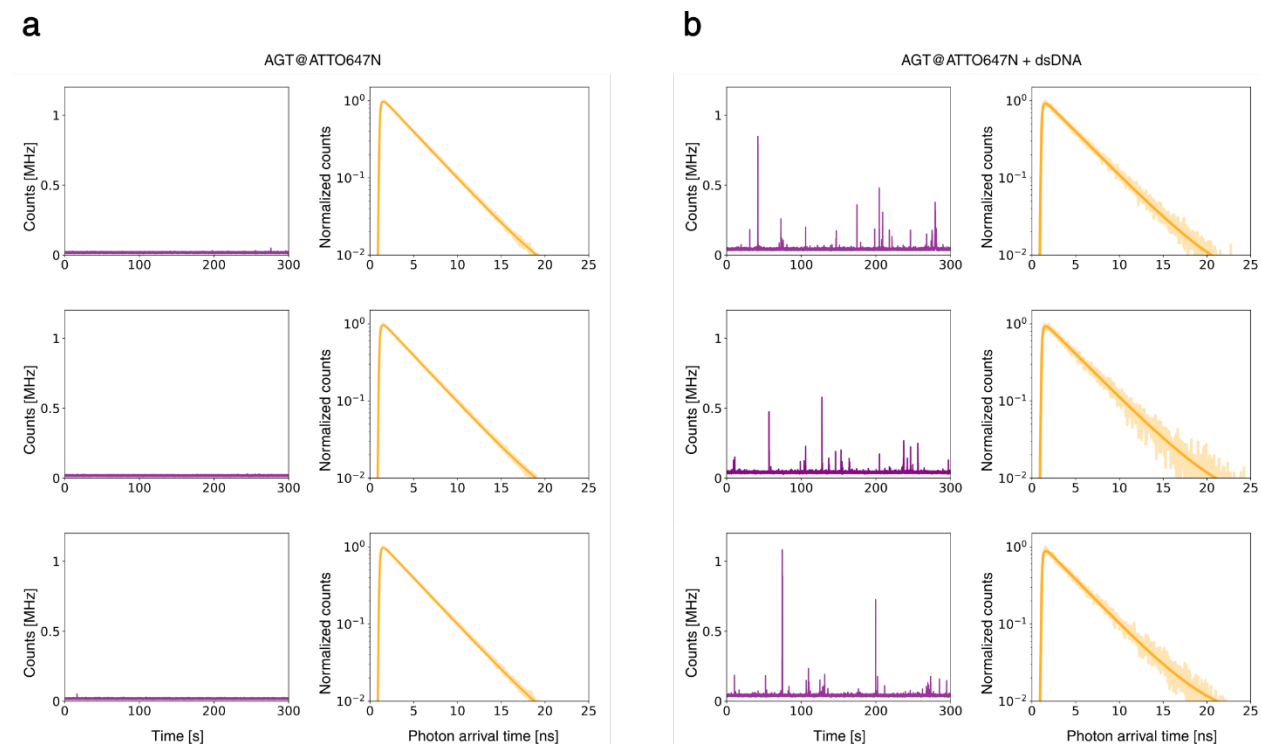


Fig. S28. Measurement of the unquenched reference lifetime for ATTO 647N-labeled AGT enzyme bound to dsDNA. **a)** Time traces and decay curves relative to 300 nM ATTO 647N-labeled AGT and 3.7 μ M unlabeled AGT diluted in AGT experiment buffer, measured in solution, and excited with a 640 nm laser. The decay curves are obtained by considering all photons and correspond to an average fluorescent lifetime of 3.58 ns. **b)** Time traces and decay curves relative to 300 nM ATTO 647N-labeled AGT, 3.7 μ M unlabeled AGT and 10 μ M dsDNA (unlabeled 66mer A-tract + 136mer A-tract) diluted in AGT experiment buffer and measured in solution. Intensity spikes appear with the addition of dsDNA and are hence interpreted as DNA-protein complexes diffusing in solution. The decay curves are obtained considering only the photons in the intensity spikes with a 120 kHz lower threshold in order to have a minimum signal-to-background ratio of 2. Those events exhibit an average fluorescent lifetime of 3.78 ns, which is then used as the unquenched reference lifetime for AGT measurements.

3 Supplementary Tables

Table S1. Reference parameters

List of used reference parameters for computing the distance from graphene for a measured fluorescence lifetime and a given fluorophore. The listed fluorescence lifetimes are deconvoluted.

Quantity	Value
d_0 , ATTO 542 ¹²	17.7 nm
d_0 , ATTO 647N ¹²	18.5 nm
τ_0 , ATTO 542 in Tx-Tq-PCA with 1× PCD	3.51 ns
τ_0 , ATTO 542 in NEB3-Tx-Tq-PCA with 1× PCD	3.52 ns
τ_0 , ATTO 542 internally-labeled in Tx-Tq-PCA with 1× PCD	3.40 ns
τ_0 , ATTO 647N with AGT bound to DNA in AGT experiment buffer	3.78 ns
τ_0 , ATTO 647N internally-labeled in Tx-Tq-PCA with 1× PCD	3.74 ns