Tethered particle analysis of supercoiled circular DNA using peptide nucleic acid handles

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This protocol describes how to monitor individual naturally supercoiled circular DNA plasmids bound via peptide nucleic acid (PNA) handles between a bead and a surface. The protocol was developed for single-molecule investigation of the dynamics of supercoiled DNA, and it allows the investigation of both the dynamics of the molecule itself and of its interactions with a regulatory protein. Two bis-PNA clamps designed to bind with extremely high affinity to predetermined homopurine sequence sites in supercoiled DNA are prepared: one conjugated with digoxigenin for attachment to an anti-digoxigenin-coated glass cover slide, and one conjugated with biotin for attachment to a submicron-sized streptavidin-coated polystyrene bead. Plasmids are constructed, purified and incubated with the PNA handles. The dynamics of the construct is analyzed by tracking the tethered bead using video microscopy: less supercoiling results in more movement, and more supercoiling results in less movement. In contrast to other single-molecule methodologies, the current methodology allows for studying DNA in its naturally supercoiled state with constant linking number and constant writhe. The protocol has potential for use in studying the influence of supercoils on the dynamics of DNA and its associated proteins, e.g., topoisomerase. The procedure takes ~4 weeks.

INTRODUCTION

Single-molecule investigations have proven to be successful for unraveling spatial and temporal information hidden in classical ensemble studies, for instance, the typical stepping size of a single molecular motor and its instantaneous velocities[1]. By using force-manipulation techniques[2], the force required to stall a molecular motor, as well as the mechanical properties of a biopolymer undergoing rupture, stretching or twisting[3], have also been investigated.

Despite the fact that the natural state of DNA inside a living cell is supercoiled, single-molecule investigations of DNA are typically carried out on linear DNA; that is, on a DNA tether with two ends that can be free or attached to a bead or a molecule, or on linear DNA on which supercoils were externally imposed using magnetic tweezers. This is true, e.g., for investigations of DNA's elasticity[4,5] and interaction with DNA-associating proteins[6–11], as well as of the DNA looping caused by regulatory proteins[12–15]. Before regulatory proteins, such as λ repressor protein (CI), attach to their specific DNA sites, they search the DNA to find the attachment sequence. The search mechanisms of proteins along the DNA may differ between linear and supercoiled DNA: on a supercoiled DNA the juxtaposition of any two sites markedly further apart than the dsDNA persistence length (which is defined as the length over which the polymer appears straight with respect to thermal fluctuations) is more frequent than on relaxed DNA[16,17]. This is because the dsDNA appears floppy and easily folds on length scales larger than the dsDNA persistence length. At a juxtaposition site, a protein bound to the DNA backbone can jump more quickly to the juxtaposed site than if the protein had to stroll along the entire backbone[18]. As the natural state of DNA is the supercoiled state, this is the most relevant state to study when probing the physical, chemical and dynamical properties of DNA itself, as well as its interaction with associated molecules.

Development of the protocol

Nielsen and co-workers[19,20] developed a method to attach supercoiled circular DNA to a streptavidin-coated polystyrene bead via a biotin-PNA conjugate. Recently, we exploited this method to analyze supercoiled circular DNA by tethered particle motion (TPM; see an overview of the main steps involved in **Fig. 1**). In our approach, the DNA is tethered with two PNA handles: one site of the plasmid was bound via the biotin-PNA to a streptavidin-coated polystyrene bead, and another site of the plasmid was bound via digoxigenin-PNA to an anti-digoxigenin-coated surface[16] (**Fig. 2**). The PNA handles bound specifically at their designated targets on the circular DNA, as determined by both electrophoretic mobility shift and single-molecule analysis. By using this construct and recording the thermal fluctuations of a reporter bead, we compared naturally supercoiled circular DNA with circular relaxed DNA (DNA with the identical sequence, which had been 'relaxed' by treatment with a nicking enzyme). The internal dynamics of the two forms of DNA plasmids were determined by calculating the autocorrelation between the positions visited by the reporter bead in the TPM time series. The supercoiled DNA showed a faster juxtaposition rate (the frequency with which two sites on the DNA are in vicinity of each other) than the relaxed DNA. We interpret the faster juxtaposition rate as the supercoiled form having a smaller number of accessible states; hence, two separated sites along the DNA contour are more often juxtaposed.

By using this assay, we determined the probability of λ repressor CI–mediated looping in the model system of bacteriophage λ, in which CI binds cooperatively to the λ operators and clamps them together. The plasmid contained the entire λ immunity region (~2.3 kbp) flanked by the λ operators (**Fig. 3**). The PNA target sites were placed as closely as possible to the two λ operators to enhance the ability to observe CI clamping. We found that the

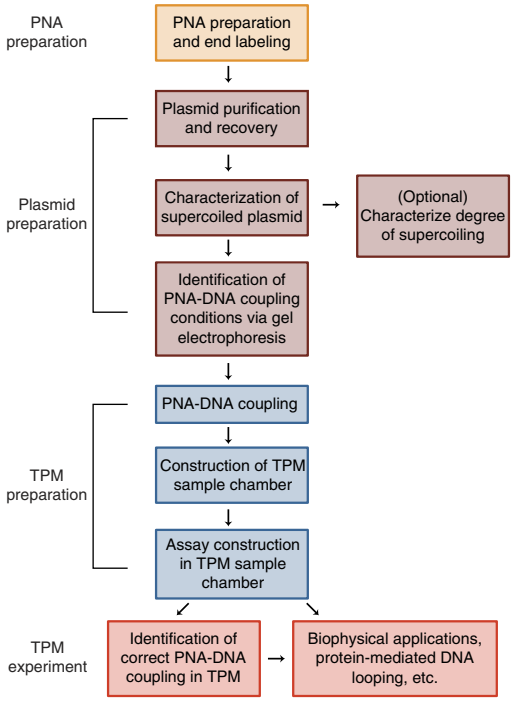

Figure 1 | Flowchart of the entire procedure providing an overview of the main steps involved in developing the assay and performing tethered particle motion experiments.

efficiency of the so-called λ switch substantially increases and the Hill coefficient rises on supercoiled DNA as compared with linear DNA or relaxed plasmids[16]. These findings infer that the transition between the CI-mediated looped and unlooped states occurs exactly at the CI concentration corresponding to the minimum number of CI molecules capable of maintaining repression of *cI* transcription[21]. Hence, as CI concentration declines during induction, the supercoiled state prevents autoregulation of *cI* from interfering with induction[21].

Future applications

This method is immediately applicable to examine the interactions between DNA in its natural supercoiled state and proteins that associate with DNA. So far, essentially all single-molecule DNA-protein studies have characterized protein interactions with linear DNA molecules or with linear DNA molecules that were mechanically twisted to impose supercoils[12–15,22,23]. Similar studies could be conducted using naturally supercoiled DNA. There is reason to believe that the topology and tertiary structure of the DNA in itself has a role in regulating protein interactions. Hence, the behavior observed with supercoiled DNA may differ from the behavior observed with linear DNA. One interesting possibility could be to address the effective nonspecific DNA binding by site-specific DNA-binding molecules. To this end, DNA lacking the binding site could be introduced into the prepared sample chambers together with the binding protein; the nonspecific DNA could be random linear fragments or purified supercoiled DNA, and the results could be compared with an experiment in which the nonspecific DNA was omitted. Similarly, the effect of potential cofactors such as general DNA-binding proteins and metabolites could be examined[23]. In addition, with few modifications, the assay could examine the behavior of topoisomerases on single molecules of circular DNA[24]. The assay can also be used to explore the physical properties of the supercoiled plasmid itself, e.g., the stiffness of the system[25]. As PNAs can also bind to plasmid DNA that can be transported into the cytoplasm and even into the nucleus[26], it is also possible to bring the PNA handles into the cytoplasm. Furthermore, micro- or nanoparticles can be brought inside the cell[27], and via the PNA handles they can be attached to chromosomal DNA. By such a construct one can use, e.g., optical or magnetic tweezers to study the forces acting on chromosomal DNA during cell division.

Comparison with other methods

The most commonly used geometry for studying DNA elasticity, protein association, supercoiling or protein-mediated looping at the single molecule level has been the linear assay, in which a linear DNA tether has been attached at one end to a surface and at the other end to a bead. The bead is then manipulated by optical or magnetic tweezers[15,24], or its thermal fluctuations are studied by TPM[12,28]. In such tethered linear DNA assays, the dynamics of the molecule are found by monitoring the position of the bead with a camera. TPM, in which supercoiling has been introduced on a linear tether using magnetic tweezers, presents a challenge in that it can be difficult to distinguish two different events, e.g., change in writhe and protein-mediated DNA looping, which both cause an overall change in the length of the tether. The assay described in the current protocol has the advantage that the linking number of the circular plasmid is 'locked,' as there are no free ends to rotate. In addition, we never observed a writhe change of the plasmid alone, possibly owing to the fact that the bead is too large to go through the plasmid circle. Hence, the plasmid stays in its natural supercoiled conformation during the experiment. As the writhe number remains constant, any changes in measured tether length can be attributed to protein-DNA interactions rather than to changes in supercoiled state. Of course, if one studies a catalytic protein such as topoisomerase with the plasmid assay,

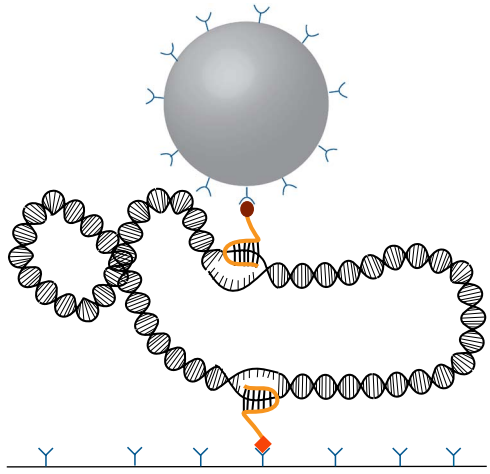

Figure 2 | Sketch of the naturally supercoiled DNA to which two PNA handles (yellow) are specifically attached. One PNA is biotinylated (red oval) and it can specifically attach to a streptavidin (blue cups)-coated bead. The other PNA is digoxigenin-labeled (orange square) and it specifically attaches to an anti-digoxigenin (blue Y)-coated surface.

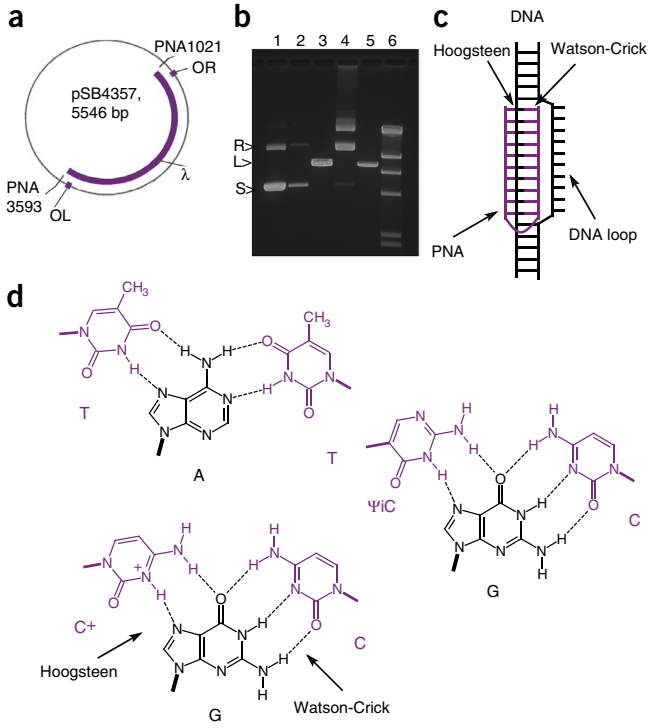

Figure 3 | Constructed plasmids. (**a**) 117 base pairs reside between the edges of the target of PNA1021 (biotin-labeled) and OR. 79 base pairs reside between the edges of the target of PNA3593 (digoxigenin-labeled) and OL. The purple part corresponds to the λ immunity region. The operators OL and OR can be clamped by the CI protein. (**b**) Illustration of the need to make the plasmid in a *recA*⁻ strain. Shown is an ethidium bromide–stained agarose gel of DNA electrophoresed in TAE buffer. Lane 1: a 7.6-kb plasmid prepared from a *recA*⁻ strain. Lane 2: same as Lane 1, but diluted tenfold. Lane 3: The 7.6-kb plasmid used in lanes 1 and 2 linearized with a restriction enzyme that cuts the plasmid once. Lane 4: A 7.6-kb plasmid >99.9% identical in sequence to the plasmid used in lanes 1–3 but prepared from a *recA*⁺ strain. Lane 5: The 7.6-kb plasmid used in lane 4 linearized with a restriction enzyme that cut the plasmid once. Lane 6: 0.5 μg of λ DNA cut with HindIII. R and S indicate, respectively, the mobility of relaxed and supercoiled plasmid in lanes 1 and 2. L indicates the mobility of linearized DNA in lanes 3 and 5. The plasmid preparations had been stored at 4 °C for 5 years. (**c**) Schematic drawing of the bis-PNA dsDNA triplex invasion complex (purple), showing how one PNA (Watson-Crick) strand replaces the sequence identical DNA strand (now forming a single-stranded loop), whereas the other (Hoogsteen) PNA strand stabilizes the internally formed PNA-DNA duplex via binding the major groove of this, forming a very stable PNA-DNA-PNA triplex. (**d**) The base triplets that are responsible for this binding (purple). Because such stable triplexes are only formed with adenine and guanine in the DNA, this type of PNA triplex invasive binding requires a homopurine DNA target. Furthermore, the C-G-C triplet requires an N-3 protonated cytosine, and because the pKa of cytosine is 4.5 (in solution) a triplex with cytosine has markedly reduced the stability at neutral pH. However, by using the pseudoisocytosine isomer, the pH dependence is minimized.

the linking number, as well as twist and writhe, would be expected to change upon enzymatic action. Furthermore, the assay allows for investigation of circular DNA, as the attachment of the PNA handles does not require single-stranded overhangs at the end of the tether. TPM studies on linear relaxed DNA have provided great insights into processes such as protein-mediated DNA looping. However, the thermodynamic parameters identified in these studies found that additional features must have a role (possibly tertiary structures as caused by supercoiling) in order for the interactions to be as efficient as those observed *in vivo*[12,14].

Experimental design

Plasmid design. The plasmids used in the study presented in Norregaard *et al.*[16] were designed to contain the λ operators, OR and OL (operator right and operator left), and the entire immunity region of bacteriophage λ of ~2.3 kbp. The target sites for the PNAs flanked the λ operator sites. The total length of the plasmid was 5,546 bp (**Fig. 3a**). A chloroquine gel (shown in **Fig. 4**) examination of the purified supercoiled plasmid found that the preparation was composed of a population of supercoiled species varying in at least ten states of supercoiling. This variation was reflected in our TPM experiments, in which many different overall lengths of the supercoiled plasmid were observed. In addition to the naturally supercoiled DNA plasmid, we prepared a plasmid that was relaxed by nicking with a single-stranded endonuclease.

Strain choice. As the plasmids must be monomer circles for simple interpretation of the TPM data, the host strain must be recombination-deficient (*recA*⁻). We chose a strain bearing a reporter that would allow us to genetically characterize our constructs[16], but any *recA*⁻ strain repressing any toxic genes on the plasmid and producing the desired DNA modifications should be suitable[29]. Once introduced into a *recA*⁻ strain, the multimeric state of the plasmid is 'locked'. That is, if introduced as a monomer, it propagates as a monomer. If introduced as a dimer, it propagates as a dimer and so on. In a *Rec*⁺ strain, the plasmid exists in an array of multimeric states (**Fig. 3b**). If the plasmid after purification is not a monomer, it can easily be converted to a monomer by digestion with a restriction endonuclease that cuts only once in the plasmid, ligation under dilute conditions to favor intramolecular ligation and transformation into a *recA*⁻ strain. The multimeric state of the plasmid can be determined by partial digestion with the same restriction endonuclease, followed by agarose gel electrophoresis and staining with ethidium bromide.

Although plasmids will not recombine in a *recA*⁻ strain, they can form catenates. That is, two circles can be interlocked. As much as 1% of our plasmids appear to be catenates, as determined by agarose gel electrophoresis after relaxing the plasmid preparation with a single-strand nicking endonuclease. The anisotropic and diminished diffusion of the tethered bead (as detailed in the PROCEDURE) permits elimination of catenates with appropriate selection criteria.

Choice of PNA targets and design of bis-PNAs. Binding of bis-PNAs[30] to double-stranded DNA via PNA triplex invasion requires opening of the DNA duplex and formation of a PNA-DNA:PNA triplex clamp on the homopurine strand[31]. In this triplex, one PNA strand binds the DNA in an antiparallel orientation (PNA C terminus facing the 5′-DNA end) target by Watson-Crick base pairing, whereas the other PNA strand binds the thus-formed PNA-DNA duplex in a parallel orientation by Hoogsteen base pairing. A homopurine target is necessary for the formation of Hoogsteen base pairing in the PNA_2DNA triplex (**Fig. 3c**). PNA targets must be homopurine tracts of no fewer than eight and optimally ten bases to allow effective and stable triplex invasion with optimal stability and sequence discrimination. Furthermore, the target should not be too G-rich (maximum 50–60%), and the

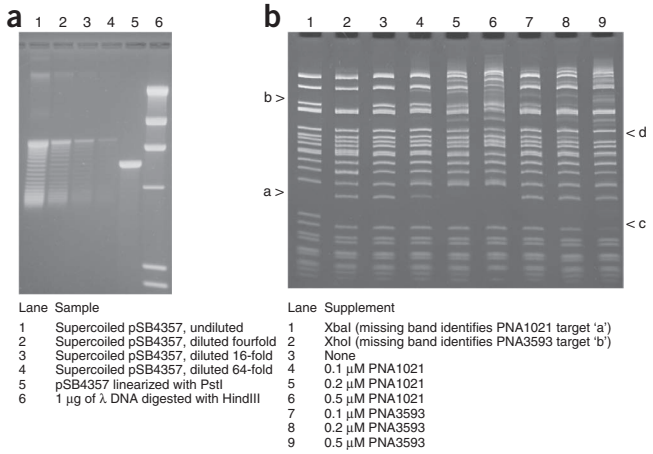

Figure 4 | Electrophoretic analysis of materials. (**a**) Distribution of supercoils assessed by a chloroquine gel. Lanes 1–4 are serial fourfold dilutions of the plasmid preparation pSB4357. Lane 5 is linearized plasmid DNA, and lane 6 contains size standards. A simple gel band quantification analysis using ImageJ of lane 2 shows that there are at least ten different supercoiled conformations in our sample. This gel is reproduced from Norregaard *et al.*[16] with permission. (**b**) Initial identification of PNA concentrations. The target of PNA1021, AAGAAGAAAA, is adjacent to an XbaI restriction recognition site and resides in the 188-bp HinfI fragment. This fragment migrates as the band marked a, and it is present in lane 3 but absent in lane 1. Lane 4 shows that the lowest concentration of PNA1021, 0.1 µM, reduces the amount of target fragment migrating at the same mobility as the naked DNA. The target of PNA3593, AGAGAAAGAA, is adjacent to an XhoI restriction recognition site. It resides within the band marked b and is present in lane 3 but absent in lane 2. Lane 7 shows that the lowest concentration of PNA3593, 0.1 µM, reduces the concentration of this fragment migrating at the same mobility as the naked DNA. At higher concentrations of PNA3593, the mobility of other fragments is altered. It can be seen in lane 9 that the bands marked c and d are depleted compared with lane 7. This gel is reproduced from Norregaard *et al.*[16] with permission.

two or more targets required should both differ from each other and any other sequence in the plasmid by at least two bases. The bis-PNA is designed with Watson-Crick binding and a Hoogsteen PNA oligomer connected with a flexible linker typically composed of three (or four) 8-amino-3,6-dioxaoctanoyl (ethylene glycol) units. The bis-PNA is synthesized by continuous solid-phase peptide synthesis and designed such that the Watson-Crick PNA strand is antiparallel to the purine DNA target and the Hoogsteen strand is parallel to the purine target (the N terminus of the PNA corresponds to the 5′-end of the DNA). Although standard thymine and cytosine bases are used in the Watson-Crick strand, cytosine (recognizing guanine) should be replaced by pseudoisocytosine (ψiC: J base) in the Hoogsteen strand, as this relieves most of the pH dependence of the DNA binding[30]: Cytosine (in contrast with pseudoisocytosine) must be sufficiently protonated at N3 for Hoogsteen recognition of guanine, and this requires a pH of <6. Finally, it is recommended to include three or four lysine (or ε-N,N-dimethyllysine, if postsynthetic, solution-phase N-conjugation is required) residues to accelerate binding kinetics of PNA to the double-stranded plasmid DNA[32].

Binding of PNA to dsDNA. The slow rate of formation of the bis-PNA–dsDNA triplex invasion complex is due to the slow rate of DNA helix opening. However, once formed, the triplex invasion complex is extremely stable. For 10-mer PNAs, the half-life is many days, even at physiological ionic strength conditions. If possible, binding should be performed at low ionic strength (<10 mM) in the absence of Mg^{2+} and other multivalent cations (including polyamines such as spermine or spermidine), as these stabilize the DNA helix, thus reducing DNA breathing and consequently slowing down PNA invasion kinetics. Subsequently, the medium can be changed for further manipulations (e.g., restriction enzyme cleavage) without substantial PNA-DNA complex dissociation. Furthermore, as negative supercoiling facilitates DNA helix unwinding and helix opening, the natural negative supercoiling of a plasmid can accelerate PNA invasion up to two orders of magnitude[20]. Thus, any studies conducted on plasmids relaxed by nicking should be free of residual supercoiled DNA. We have observed that isopycnic centrifugation in CsCl with ethidium bromide yields DNA that binds PNA more efficiently than the commercial cartridge or column purification systems. We suspect that some constituents of the initial plasmid lysate such as polyamines, which inhibit PNA invasion, are more efficiently removed by CsCl-ethidium bromide purification.

Tethering of the biological construct. One of the PNA handles is labeled with biotin and the other is labeled with digoxigenin at the N terminus. By surface functionalization of a cover glass with anti-digoxigenin and a sub-micrometer-sized bead with streptavidin, the circular DNA molecule with PNA handles can be tethered between the surface and a bead (**Fig. 2**). As a polystyrene bead of ~500 nm is too big to rotate through the circular DNA, the geometry of the circular DNA will remain constrained by the point of the tethers. Before starting experiments with the designed assay, a control TPM experiment should be conducted with three control plasmids to ensure efficient and correct binding of the PNA to the DNA: one control plasmid lacking the digoxigenin-PNA target, the second lacking the biotin-PNA target and the third with both targets intact. Our controls showed that tethers only formed correctly with the plasmid containing both PNA targets[16].

The tethered plasmid is subjected to TPM analysis[33]. This type of analysis monitors the *x* and *y* positions of a particle (in this case the polystyrene bead) tethered to an *x*-*y* plane and performing Brownian motion. TPM does not directly yield information on the overall length of the tether, but the overall length is reflected in the distribution of positions visited by the bead (the longer the tether, the larger the excursions of the bead). If the tether is linear, a calibration curve can be made that relates a certain lateral motion to an overall tether length[33]. For the naturally supercoiled plasmids, the spread in linking number and writhe give rise to a broad distribution of overall tether lengths of the plasmid (as further detailed in ANTICIPATED RESULTS). This is even without any clamping proteins present, and therefore it is not straightforward to make a calibration curve for the construct. Our linear control DNA had the same length as the shorter arc of the supercoiled plasmid, and for this linear tether we know the relation between its overall length and the observed Brownian motion[28] (as further detailed in the ANTICIPATED RESULTS section). The symmetry of the positions visited by the tethered particle gives information on whether the plasmid is catenated or whether multiple DNA tethers could be attached on one bead. Both such artifacts give rise to an anisotropic diffusion of the tethered bead. In our experiment, the TPM analysis gave information

on the kinetics of the plasmids and on the stability of CI-mediated DNA looping as a function of CI concentration.

Limitations

If one of the PNA handles is attached to a bead that is larger than a typical opening of the supercoiled DNA (as sketched in **Figure 2**), the plasmid geometry is locked and the supercoils are not able to 'slide' freely along the structure, as they probably do *in vivo*. This could be resolved by using a smaller marker, possibly a fluorophore instead of a bead. In conventional TPM studies using linear tethers, it is possible to establish a well-defined relation between the tether length and the magnitude of Brownian fluctuations of the bead and use this as a calibration curve[28]. For a linear assay, this calibration curve can give direct information on, e.g., whether a DNA molecule has been looped by a protein. In the assay described here, there is a large natural variation in the degree of supercoiling among the individual plasmids, and the distance between the bead and the surface will depend on the degree of supercoiling of the individual plasmid. Hence, a universal calibration curve cannot be established. A possible protein-mediated DNA looping is visible through a change in the bead's thermal fluctuations, as shown in Norregaard *et al.*[16], and not solely by the size of the fluctuations at any given instant. In addition, kinetic rates of protein-DNA interactions may be affected by the degree of supercoiling in the DNA molecule and on the exact location of supercoiled domains within the molecule. A final limitation regards the time scales resolvable by the methodology: at very short time scales (milliseconds) the motion of a tethered bead will be autocorrelated[16]; hence, it is advisable to use this methodology to investigate biological processes that appear on time scales larger than a millisecond.

MATERIALS

REAGENTS

▲ **CRITICAL** For the preparation of all growth media, do not tighten the cap of the bottle until it has cooled to room temperature (RT, 20–25 °C). If the cap is tightened while the contents are still hot, the cooling will create a vacuum. Any dust that falls on the shoulder of the bottle may be drawn in and may contaminate the medium on opening.

- M63 salts, 5× (see Reagent Setup)
- Acetic acid (glacial; Fluka, cat. no. 45731)
- Activated charcoal (powder; Merck)
- Agar (Becton, Dickinson, cat. no. 214010)
- α-Casein from bovine milk (Sigma-Aldrich, cat. no. C6780)
- α-Casein solutions (see Reagent Setup)
- Ammonium sulfate (ICN Biomedicals, cat. no. 808211)
- Ampicillin, sodium salt (Sigma-Aldrich, cat. no. A9518)
- Ampicillin solution (see Reagent Setup)
- Anti-digoxigenin (Roche, cat. no. 11 333 062 910)
- Anti-digoxigenin solution (see Reagent Setup)
- B1 stock solution (see Reagent Setup)
- Bromophenol blue (Serva, cat. no. 15375)
- Casamino acids (CAAs; Becton, Dickinson, cat. no. 228820)
- Cesium chloride (Cabot Specialty Fluids, 99.99%)
- Citric acid (Sigma-Aldrich, cat. no. C-1909)
- Decolorized CAA solution (see Reagent Setup)
- DIEA (*N*,*N*-diisopropylethylamine)
- Digoxigenin *N*-hydroxysuccinimide (NHS) ester (Sigma-Aldrich)
- Distilled H_2O
- DTT
- Dimethylformamide
- Dimethylsulfoxide (DMSO)
- DNA (see PROCEDURE)
- Ethanol (absolute)
- EDTA, disodium salt (Sigma-Aldrich, cat. no. E-1644)
- EDTA stock solution (see Reagent Setup)
- Ethidium bromide (Sigma-Aldrich, cat. no. E-7637)
- Ethidium bromide solution (see Reagent Setup)
- Ferrous chloride (Aldrich, cat. no. 22,029-9)
- Ferrous citrate solution (see Reagent Setup)
- Ficoll 400
- Glucose (Becton, Dickinson, cat. no. 215530)
- Glucose stock solution (see Reagent Setup)
- Growth medium (see Reagent Setup)
- Hydrochloric acid (Fluka, cat. no. 84419)
- Isopropanol (Fluka, cat. no. 34965)
- Isopropanol saturated with CsCl and Tris-EDTA (TE) (see Reagent Setup)
- K-Ac solution (see Reagent Setup)
- λ-Buffer (see Reagent Setup)
- CI (gift from D. Lewis and S. Adhya, Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, US National Institutes of Health)
- Lysozyme (Fluka, cat. no. 62971)
- Lysozyme buffer (see Reagent Setup)
- Magnesium sulfate ($MgSO_4$)
- $MgSO_4$ stock solution (see Reagent Setup)
- PBS buffer (see Reagent Setup)
- PEN buffer (see Reagent Setup)
- PIPES (Fluka, cat. no. 80636)
- Plasmid growth medium (see Reagent Setup)
- PNAs, PNA-1021 (biotin-labeled) and PNA-3593 (digoxigenin-labeled; see Reagent Setup and PROCEDURE)
- Potassium acetate
- Potassium chloride
- Potassium phosphate, dibasic, anhydrous (Sigma-Aldrich, cat. no. 60353)
- Potassium phosphate, monobasic, anhydrous (Fluka, cat. no. 60220)
- Sample buffer (see Reagent Setup)
- Sodium chloride (Sigma-Aldrich, cat. no. S7653)
- NaOH stock solution (see Reagent Setup)
- NaOH-SDS solution (see Reagent Setup)
- Restriction enzyme(s)
- SDS (BDH, cat. no. 436696N)
- SDS stock solution (see Reagent Setup)
- Sodium hydroxide (Riedel-deHaen, cat. no. 30620)
- Sodium phosphate, dibasic
- Sodium phosphate, monobasic
- Streptavidin-coated polystyrene beads (see Reagent Setup; Bangs Laboratories, cat. no. CP01N)
- TAE buffer for agarose gel electrophoresis (see Reagent Setup)
- Tris-EDTA (TE) buffer (see Reagent Setup)
- Trifluoroacetic acid; TFA, Sigma-Aldrich)
- Thiamine-HCl (Sigma-Aldrich, cat. no. T4635)
- Tris base (AppliChem, cat. no. A1086)
- Tris-HCl (Sigma-Aldrich, cat. no. T6666)
- Tris-HCl stock solutions (see Reagent Setup)
- Tryptone (Becton, Dickinson, cat. no. 211705)
- Uracil (Sigma-Aldrich, cat. no. U-0750)
- Yeast extract (Becton, Dickinson, cat. no. 212750)
- Yeast-tryptone (YT)-ampicillin plates (see Reagent Setup)

EQUIPMENT

- Zoom, 1.6× (fits the Leica DMI RB microscope)
- Collimated LED light source, 360 nm (fits the Leica DMI RB microscope)
- Air incubator (37 °C)
- Beakers
- Bransonic ultrasonic cleaner, model 2510 (Fisher Scientific)

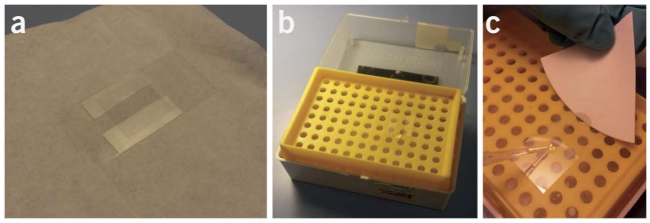

Figure 5 | Preparation of perfusion chamber. (**a**) Construction of chamber. (**b**) The finished perfusion chamber in the evaporation box. (**c**) Example of how to exchange the medium in the perfusion chamber.

- Centrifuge (Sigma-Aldrich 1-13)
- Clear and brown bottles
- Cover glass, 18 mm × 18 mm, no. 1 (Menzel-Gläser, VWR)
- Cover glass, 24 mm × 50 mm, no. 1.5 (Menzel-Gläser, VWR)
- Culture tubes
- Dialysis tubing
- Evaporation box (see PROCEDURE and **Fig. 5**)
- Filter paper (Whatman, Sigma-Aldrich, cat. no. 1001-0155)
- Flasks
- Funnels
- Graduated cylinders
- Graduated, low-binding pipette tips (Sorensen Biosciences, cat. no. 35090)
- High-numerical-aperture (NA, 1.45) 100× oil-immersion objective (Leica)
- High-speed refrigerated centrifuge with rotors and centrifuge tubes
- High-vacuum grease (Dow Corning)
- Inverted microscope (Leica, DMI RB)
- LabView particle tracking software (St. Andrews Tracker, National Instruments; http://www.ni.com/example/25948/en/)
- MATLAB program to perform principal component analysis (PCA)
- Microcentrifuge tubes, RNase/DNase free (Corning, cat. no. 3208/3207)
- Parafilm (VWR)
- ParaSequencer 1.6.3 program to record tethered beads with (Parameter)
- Petri dishes
- Piezo stage (Physik Instrumente, P-517.3CL)
- Plane block heater (Grant Boekel)
- Preparative ultracentrifuge with rotors and centrifuge tubes
- Progressive scan camera (Pike F-100B, Allied Vision Technology)
- Ring stand with clamps
- Standard oil-immersion liquid, $n = 1.518$ (Leica)
- Sterile plastic vials (Nunc and Falcon)
- Stir bars and magnetic stirrer
- Syringes and needles
- Temperature-controlled water bath
- Wooden applicator sticks

REAGENT SETUP

Bis-PNA synthesis The bis-PNA is synthesized by standard solid-phase chemistry[30] or obtained commercially (Panagene). It is important that a PNA containing only one reactive (primary or secondary) amine (e.g., the N terminus) be used. Dry PNA powder is stable for several years at 4 °C. Solutions >1 mg/ml) in water are stable for at least 1 year at −20 °C.

Tris-HCl stock solution, 1 M pH 8.0 (1 liter) Dissolve 42.4 g of Tris base and 102.4 g of Tris-HCl and adjust the volume to l liter; autoclave the solution. This solution is stable for years at RT.

Tris-HCl stock solution, 1 M pH 7.5 (1 liter) Dissolve 18.2 g of Tris base and 134 g of Tris-HCl and adjust the volume to l liter; autoclave the solution. This solution is stable for years at RT.

Tris-HCl stock solution, 1 M pH 7.4 (1 liter) Dissolve 14.2 g of Tris base and 138 g of Tris-HCl and adjust the volume to l liter; autoclave the solution. This solution is stable for years at RT.

EDTA stock solution, 0.5 M, pH 8.0 (1 liter) Suspend 186.12 g of disodium EDTA dihydrate in water. It will dissolve as the pH approaches 8. Adjust the pH with NaOH to 8.0 and the volume to l liter; autoclave the solution and store it in plastic for years at RT.

NaOH stock solution, 1 M Store the solution in plastic; this solution is stable for years if tightly sealed at RT.

SDS stock solution SDS is 10% (wt/vol). This solution is stable for months at RT.

TE buffer TE buffer is 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Autoclave the buffer and store it for years at RT.

2× PEN buffer To autoclaved, distilled water, add 0.5 M EDTA to a final concentration of 0.2 mM, 5 M NaCl to a final concentration of 20 mM and PIPES powder to a final concentration of 20 mM. Adjust the pH to 6.5 with NaOH and adjust the volume with autoclaved, distilled water. ▲ **CRITICAL** Do not autoclave the buffer. The buffer can be stored at −20 °C for at least 3 years.

λ Buffer Mix 10 mM Tris-HCl (pH 7.4), 200 mM KCl and 0.1 mM EDTA. Autoclave the buffer and allow it to cool. Remove any particles by filtering through a several-centimeter bed of fine Sephadex. Add DTT to a final concentration of 0.2 mM before use. The buffer can be stored at RT for at least 2 years.

0.1 M DTT Dissolve DTT to a 0.1 M concentration in autoclaved distilled water. ▲ **CRITICAL** Do not heat the solution, and never vortex it, as aeration will speed up oxidation. Divide the solution into small volumes to avoid thawing too many times. Store DTT at −20 °C for years.

PBS buffer Dissolve 1.42 g of Na_2HPO_4 in 10 ml of distilled H_2O to obtain a stock of 1 M Na_2HPO_4. Dissolve 1.19 g of NaH_2PO_4 in 10 ml of distilled H_2O to obtain a stock of 1 M NaH_2PO_4. Mix 5.77 ml of the 1 M Na_2HPO_4 stock with 4.23 ml of the 1 M NaH_2PO_4 stock, and add 90 ml of distilled H_2O. Withdraw 20 ml of this stock (pH 7) and mix it with 0.88 g of NaCl and 80 ml of distilled H_2O. Autoclave and filter the buffer through a several-centimeter bed of fine Sephadex. The buffer can be stored at RT for at least 2 years.

α-Casein solutions (λ buffer and PBS buffer) α-Casein reduces nonspecific binding; it is used to reduce nonspecific interactions between the cover glass surface and the tether complex and prevent the beads from aggregating. Dissolve 2 mg of α-casein in a tube containing 1 ml of λ buffer and in another tube containing 1 ml of PBS buffer. Filter-sterilize both solutions and store them at 4 °C for up to 1 month.

Bead suspension Withdraw 50 µl of polystyrene bead suspension from the stock and dilute it in 950 µl of PBS buffer. Briefly vortex the solution and then centrifuge it at 1,000*g* for 15 min at RT. Remove the supernatant and resuspend the beads with 350 µl of PBS buffer. Store it at 4 °C for up to 6 months. Before use, dilute 5 µl of the bead suspension with 95 µl of 2 mg/ml α-casein solution in PBS buffer, mix it in short pulses five times on a vortex and place it in a sonicator bath for 10 min to separate aggregated beads. ▲ **CRITICAL** We have found that the beads aggregate less if kept in the PBS buffer instead of λ buffer, possibly because of the lower salt concentration.

Anti-digoxigenin solution Dissolve the anti-digoxigenin pellet from Sigma-Aldrich in 1 ml of PBS buffer to a concentration of 200 µg/ml anti-digoxigenin. Distribute 10-µl aliquots of the solution into centrifuge tubes. Store the aliquots at −20 °C for up to 2 years. Before use, thaw the solution, and centrifuge it briefly (100*g* for 10 s at RT) to collect the sample, and then dilute it into 90 µl of PBS buffer to generate a final concentration of 20 µg/ml anti-digoxigenin.

Ferrous citrate solution Dissolve $FeCl_2$ and citric acid in distilled H_2O to a concentration of 20 mM each. Any brown precipitate that fails to dissolve can be removed by filtration through Whatman no. 1 paper. Autoclave the solution in a brown bottle and store it at 4 °C to protect it from light. This solution is stable for years.

5× M63 salts (1 liter) Mix 15 g of KH_2PO_4 (anhydrous), 35 g of K_2HPO_4 (anhydrous) and 10 g of $(NH_4)_2SO_4$. Add 2.5 ml of 20 mM ferrous citrate solution. Adjust the volume to 1 liter with distilled H_2O and autoclave it for storage. Store the salts at RT. If the solution is autoclaved, it should be stable for months. See Pardee *et al.*[34] for details.

$MgSO_4$ solution, 1 M Dissolve $MgSO_4$ to a 1 M concentration in distilled water. Store it at RT. This solution is stable for years.

B1 stock solution, 1 mg/ml Dissolve thiamine-HCl (vitamin B1) to a concentration of 1 mg/ml in distilled water. Filter-sterilize it and store it at 4 °C. This solution is stable for months.

Glucose stock solution, 20% (wt/vol) Filter-sterilize the solution and store it at RT. This solution is stable for years.

Decolorized CAA solution Dissolve CAAs to 25% (wt/vol) with distilled H_2O. Add 1 g of activated charcoal powder per 20 g of CAAs. **! CAUTION** Activated charcoal can stain clothes. Stir it at RT for 30 min. Filter the solution through doubled, fluted Whatman no. 1 paper. Measure and record the volume of the filtrate and adjust the pH of the filtrate to 7.0 with NaOH or HCl as appropriate. Adjust the volume of the neutralized CAA filtrate to 20% (wt/vol) CAAs. Distribute the solution into bottles and autoclave them; store the bottles at RT. Unopened bottles remain stable for years. Open the bottles aseptically and store them at 4 °C after opening.

Ampicillin solution, 100 mg/ml Weigh sodium ampicillin and dissolve it with distilled water to a concentration of 100 mg/ml. Filter-sterilize and distribute it into sterile plastic vials. Store it at −20 °C. Unthawed solution is stable for at least 1 year. Thaw and mix the solution before adding it to the medium. This solution can be re-frozen.

YT-amp plates Prepare the medium in a 2-liter Erlenmeyer flask covered with an inverted beaker. **! CAUTION** If mixing after adding antibiotics introduces bubbles, try placing a magnetic stir bar in the flask before autoclaving. To 1 liter of H_2O, add 8 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 15 g of agar. If you have a light touch, you can reduce the concentration of agar. Autoclave the medium for 15–20 min. Mix it well after autoclaving and allow it to cool to 50–60 °C. Aseptically add antibiotic solution to the cooled agar, and mix it without introducing bubbles. Pour it into Petri dishes and allow the agar to solidify overnight. See Miller[35] for details.

YT broth To 1 liter of H_2O add 8 g of tryptone, 5 g of yeast extract and 5 g of NaCl. Mix to dissolve them. Distribute 100-ml aliquots in bottles and sterilize them by autoclaving. Store the aliquots at RT. This medium is stable for months. Aseptically add antibiotic solution before use. See Miller[35] for details.

Plasmid growth medium For 1 liter of medium, autoclave together 200 ml of 5 × M63 salts, 25 ml of 20% decolorized casamino acids, 1 g of uracil and 751 ml of distilled H_2O. Store the medium at RT. Unopened bottles are stable for months. Immediately before use, add aseptically, with mixing after each addition, 1 ml of 1 M $MgSO_4$, 1 ml of 1 mg/ml B1, 20 ml of 20% (wt/vol) glucose and appropriate antibiotic solution (here, 2 ml of 100 mg/ml ampicillin). Distribute it into growth flasks. If you are using Erlenmeyer flasks and aerating by shaker, do not exceed 10% of the flask volume. See Norgard[36] for details.

Lysozyme buffer (100 ml) Mix 1 g of glucose, 2 ml of 0.5 M EDTA (pH 8.0), and 2.5 ml of 1 M Tris-HCl (pH 8.0); add distilled, autoclaved water to 100 ml. Store the buffer at 4 °C for months. Add lysozyme to 2 mg/ml to the amount necessary immediately before use.

NaOH-SDS solution (10 ml) This solution must be prepared daily. To 7 ml of distilled, autoclaved water, add 2 ml of 1 M NaOH and 1 ml of 10% (wt/vol) SDS. Mix it and leave it at RT before use.

K-Ac solution, 5 M, pH 4.8 (500 ml) Dissolve 147 g of KCH_3COO in ~300 ml of H_2O, add glacial acetic acid to pH 4.8 and bring the volume to 500 ml with H_2O. Autoclave the solution and store it at RT. The solution is stable for years.

Ethidium bromide solution, 10 mg/ml Dissolve ethidium bromide with autoclaved, distilled water and store it in a brown bottle at 4 °C. It is stable for years under these conditions. **! CAUTION** Ethidium bromide is mutagenic. Avoid contact with skin. Wear gloves. Avoid breathing the powder when you are preparing the solution. Please note that ethidium bromide is now commercially available in an aqueous suspension; in our laboratory, we will not make it from powder again.

Isopropanol saturated with CsCl and TE Add, in order, CsCl, then isopropanol, and then slowly add TE by shaking after each addition of TE. Add sufficient CsCl and TE so that when the phases resolve you see two liquid phases and some white powder (CsCl) at the bottom of the bottle. The top liquid phase is the organic phase. The solution is stable for years at RT. The bottle can be refilled by adding only isopropanol and TE.

50× TAE (500 ml) Dissolve 121 g of Tris base, 28.5 ml of glacial acetic acid and 50 ml of 0.5 M EDTA (pH 8) in H_2O and adjust the volume to 0.5 liters. Store it at RT. This solution is stable for at least 1 year.

Sample buffer Autoclave 25% (wt/vol) Ficoll 400. After cooling, store the Ficoll solution at −20 °C; it stable for years at this temperature. To prepare the sample buffer, mix 4 ml of 25% (wt/vol) Ficoll 400 with 1 ml of 0.5 M EDTA and add bromophenol blue until the solution is medium blue. The solution is stable for months at 4 °C and for years at −20 °C.

EQUIPMENT SETUP

Microscope In this protocol, we image tethered beads using a high-speed progressive camera mounted on an inverted bright-field microscope with a 100× oil-immersion objective and a 1.6× zoom. For a region of interest of 62 × 62 pixels^2, the frame rate is 225 Hz. The recorded output is obtained with the program ParaSequencer and is an AVI file of the tethered bead, a text file containing the number of recorded frames, frames per second (f.p.s.) and the number of lost frames, which in general is zero. The pixel resolution was determined to be 45.97 nm per pixel by moving a sample with immobilized beads on a cover slide in discrete steps by a piezo stage and taking images of these positions (see PROCEDURE).

Particle tracking algorithm There are several different algorithms for tracking particles, e.g., based on cross-correlation, sum-absolute difference, center of mass or direct Gaussian fits. Here, we used the cross-correlation algorithm, as that approach is suitable for the size of particles used in this work[37]. We used a LabView program based on a normalized cross-correlation algorithm. The program can be downloaded via this link: http://www.ni.com/example/25948/en/. Note that to run the program a licensed version of LabView is required.

PROCEDURE

Preparation of digoxigenin-labeled bis-PNA ● TIMING 20 h

1| Dissolve 1.5 mg of PNA (H-(eg1)$_3$-(diMeLys)$_3$-TJTJTTTJTT-(eg1)$_3$-TTCTTTCTCT-Gly-NH_2) in 400 µl of DMSO (eg1: 8-amino-3,6-dioxaoctanoyl; diMeLys: ε-N,N-dimethyllysine; J: pseudoisocytosine PNA unit).

2| Dissolve digoxigenin-NHS ester (0.5 mg) in 400 µl of DMSO and add it to the PNA solution.

3| Add 25 µl of DIEA in 50 µl of dimethylformamide and incubate the mixture for 16 h at 20 °C.

4| Purify the reaction mixture by reversed-phase HPLC (C18, 5-µm column, 150 × 3.9 mm, eluant: 0–50% acetonitrile in 0.5% (vol/vol) TFA, linear gradient over 30 min with a flow rate of 1 ml/min and UV detection at 260 nm; see HPLC data in **Supplementary Fig. 1a**).

5| Check the identity of the product by performing MALDI-TOF or ESI mass spectrometry on the fractions that appear to contain the product (see MALDI-TOF data in **Supplementary Fig. 1b**). Combine the fractions, lyophilize them and store them at 4 °C for up to several years.

6| Dissolve PNA in pure water for use (store aliquots (~1 mg/ml) at –20 °C; concentrations are determined spetrophotometrically at 260 nm).
! CAUTION The PNA solution is very acidic, as the PNA is isolated as TFA salt.

Preparation of biotin–bis-PNAs conjugates ● TIMING 20 h
7| Prepare biotin–bis-PNAs conjugates. These conjugates can be synthesized as previously reported[20,30], or they can be obtained commercially (Panagene). The conjugates may also be synthesized fully analogously to the digoxigenin conjugates (see Step 2) using biotin-NHS ester (Sigma-Aldrich); to do this, perform Steps 2–6 using 0.5 mg of biotin-NHS ester instead of digoxigenin-NHS ester in Step 2. Handling and stability are as described above (Steps 5–6).
■ **PAUSE POINT** Lyophilized material can be stored at 4 °C for several years.

Growth schedule for purification of supercoiled plasmid DNA ● TIMING 3 d (after all media are prepared)
8| All DNA preparations begin with a single, well-isolated colony of the transformant harboring the plasmid. Prepare YT agar plates supplemented with the appropriate antibiotic(s). We have used ampicillin as our example of an antibiotic in the descriptions below. Many of our colleagues use LB agar, and both YT and LB are probably comparable.

9| *Revive transformants*. Scrape a small amount of frozen cell suspension from the –80 °C glycerol with sterile wooden applicator stick and inoculate the YT-amp plate.
▲ **CRITICAL STEP** Do not let the glycerol thaw, but return it to the –80 °C box. Streak the inoculated plate to produce single colonies and incubate it at 37 °C.

10| *Inoculate the starter culture*. On day 2 in the morning, inoculate 5 ml of YT-ampicillin broth and incubate it at 37 °C with aeration, such as on a roller drum.

11| *Large-scale growth of transformants*. At the end of day 2, dilute the YT-ampicillin culture into 0.5 liters of plasmid growth medium and incubate it overnight at 37 °C with shaking.

12| *Plasmid extraction* (modified from Birnboim and Doly[38]). On day 3, transfer the saturated culture to centrifuge bottles.

13| Collect the cells in a refrigerated centrifuge at 4,000*g* for 5 min at 4 °C.

14| Decant the supernatant and resuspend the cells with a 1/20th culture volume of 20 mM Tris-HCl (pH 8.0) and 0.1 M NaCl.

15| Collect the resuspended cells in a refrigerated centrifuge at 4,000*g* for 5 min at 4 °C. Decant and discard the supernatant.

16| Resuspend the washed cells with 4 ml of lysozyme solution (lysozyme buffer with 2 mg/ml lysozyme) per 100 ml of the original culture volume.

17| Incubate the cells with lysozyme for at least 30 min on ice.

18| Per 4 ml of lysozyme solution used, add 8 ml of NaOH-SDS, mix well and incubate the mixture for 5–10 min on ice. Examine the suspension and continue to the next step when the opacity starts to decline.

19| Per 4 ml of lysozyme solution used, add 6 ml of K-Ac, mix well and incubate the mixture for 45 min on ice.

20| Clarify the solution by centrifugation in a refrigerated centrifuge at 7,000*g* for 15 min at 4 °C.

21| Carefully decant the supernatant into a 50-ml conical tube. Note the volume.

22| Distribute the supernatant into the centrifuge tube noting the volume, and add 0.6 volumes of isopropanol (if it is convenient to increase volume of supernatant, e.g., when you are processing more than one sample and you want them all to be the same volume/weight so they can all be centrifuged in a single centrifuge run, use 30 mM Tris-HCl (pH 8.0), 5 mM EDTA and 0.1 M NaCl as diluent). Mix well.

23| Incubate the isopropanol suspension at –20 °C overnight.
■ **PAUSE POINT** The suspension is stable at –20 °C for at least 1 week.

Plasmid recovery ● TIMING ~0.5 d
24| Collect the precipitate in a refrigerated centrifuge at 6,000*g* for 10 min at 4 °C.

25| Decant and discard the supernatant.

26| Resuspend the pellet by vortexing with cold 70% (vol/vol) ethanol.

27| Collect the precipitate in a refrigerated centrifuge at 6,000*g* for 10 min at 4 °C.

28| Decant and discard the supernatant.

29| Centrifuge the pellet briefly (1,000*g* for 30 s at RT) to sediment the residual liquid.

30| Aspirate and discard the residual liquid.

31| Allow the recovered precipitate to dry in air.

32| Re-dissolve the dried pellet with a small volume (about one-half of the centrifuge tube capacity) of 20 mM Tris-HCl (pH 8.0) and 5 mM EDTA.

Plasmid purification ● TIMING ~2 h followed by overnight centrifugation

33| Dissolve 1.05 g of CsCl per ml of plasmid solution and add it to the plasmid solution[39].

34| Transfer the solution to an ultracentrifuge tube and fill it to 95% of the tube volume with Tris-EDTA-CsCl solution.

35| Gently fill the tube with 10 mg/ml ethidium bromide. If you are using quick-seal tubes, this step is easy with a 1-ml syringe with hypodermic needle.
! CAUTION Wear gloves for all manipulations involving the ethidium bromide solution.
▲ CRITICAL STEP DNA–ethidium bromide complexes are light-sensitive. Do not allow the ethidium bromide solution to mix with the DNA solution.

36| Seal the centrifuge tube. Verify that the tube is sealed by squeezing it.
! CAUTION Protect yourself with a gloved hand in case the tube leaks.

37| Mix the ethidium bromide and plasmid solutions thoroughly immediately before placing them in the centrifuge rotor.

38| Centrifuge the solutions using a Beckman Vti65 rotor for 20 h at 42,000 r.p.m. at 15 °C.

Recovery of supercoiled DNA ● TIMING ~2 h followed by overnight centrifugation

39| Remove the tubes one at a time and clamp them in a ring stand over a beaker to collect the waste.

40| Visualize the plasmid band with 366-nm mineral light in a darkened room (a 395-nm lamp from Xenopus Electronix can also be used). The lower of the two central bands will be the plasmid band. The diffuse fluorescent material at the bottom of the centrifuge tube is RNA.
! CAUTION Protect your eyes from UV light.

41| Puncture the tube near the top with a hypodermic needle to provide a vent hole.

42| Insert a fresh hypodermic needle on a syringe above the band with needle pointing downward.
▲ CRITICAL STEP It is prudent to puncture tubes above the desired band so that if the tube leaks you will not lose your sample.

43| Carefully withdraw the band and transfer it to a fresh ultracentrifuge tube wrapped in aluminum foil to protect the DNA-ethidium bromide complexes from excess exposure to light.

44| Fill the tube with Tris-EDTA-CsCl–ethidium bromide solution, seal it and mix. Prepare the balance tube without ethidium bromide and seal it.

45| Repeat the centrifugation and band recovery once (Steps 38–42).

Removal of ethidium bromide ● TIMING 2–3 h followed by overnight incubation

46| Transfer the solution containing the supercoiled plasmid to isopropanol saturated with TE and CsCl, mix it well and let the phases separate. The top phase in the isopropanol/TE/CsCl bottle is the isopropanol phase.

47| Remove and discard the organic phase.

48| Repeat the extraction with isopropanol saturated with TE and CsCl until the organic phase shows no pink color, and extract once more.

49| Dialyze the aqueous phase against 100 volumes of 20 mM Tris-HCl (pH 8), 2 mM EDTA, and 0.1 M NaCl for at least 1 h.

50| Withdraw the dialysate, measure its volume and mix it with two volumes of ethanol. Incubate it at –20 °C overnight.

51| Recover the plasmid DNA by centrifugation at 10,000*g* for 10 min at 4 °C.

52| Discard the supernatant.
▲ **CRITICAL STEP** Be careful not to discard the pellet; it may be small.

53| Gently rinse the tube with –20 °C ethanol and let it dry in air. Dissolve ~200 µl of DNA per liter of original culture with TE.

Characterization of CsCl-purified plasmids ● TIMING 1 d
54| Determine the concentration of the purified plasmid. This can be done by measuring absorbance at 260 nm.

55| *Gel electrophoresis (optional)*. Analyze the samples prepared as described in the in-text table below:

Uncut preparation	To determine the supercoiled fraction (versus relaxed or catenated)
Complete digestion with a restriction endonuclease that cuts the plasmid once	To identify the mobility of the plasmid as linear DNA
Partial digestion with a restriction endonuclease that cuts the plasmid once	To determine if the plasmid is a monomer or multimer

! CAUTION Protect eyes from UV light.
▲ **CRITICAL STEP** To quantify relative amounts of the different forms, it is crucial that the gel be stained after electrophoresis. To be sure that the gel is uniformly stained, look at it from the side while it is on the UV transilluminator. If the bands are uniform across the thickness of the gel, the gel is uniformly stained.

Preparation of a chloroquine gel ● TIMING ~4 h of manipulation, plus overnight electrophoresis
56| (Optional) Determine the degree of supercoiling in the plasmid preparation by a chloroquine gel (shown in **Fig. 4a**).
! CAUTION Chemicals purchased today are often much purer than those purchased in 1987. Accordingly, start with concentrations as described by Esposito and Sinden[40], but you may have to repeat experiments with a lower concentration of chloroquine. This step is optional, as it is not possible to correlate the bead excursions to the degrees of supercoiling. However, when analyzing the data, it is important to be aware that there will be a spread of overall lengths of the tethered DNA molecules originating from the natural spread in degrees of supercoiling.

57| To determine the diversity of supercoiling in the plasmid preparation, electrophorese serial dilutions of the plasmid on a 1% (wt/vol) agarose gel in 1× TAE buffer supplemented with 2 µg/ml chloroquine-diphosphate. It is important to use low voltage, so the electrophoresis takes ~20 h. After electrophoresis, the gel is stained with 0.5 µg/ml ethidium bromide in 0.25× TAE. The stained gel is then photographed on a UV transilluminator.

Preparation of end-labeled linear DNA ● TIMING 1 d
58| To assess the DNA-PNA coupling conditions in the perfusion chamber, prepare linear DNA with one end labeled with biotin and the other labeled with digoxigenin. End-labeled linear DNA can be prepared by PCR amplification using the protocols provided by the thermophilic DNA polymerase manufacturer.
▲ **CRITICAL STEP** The only changes we made from the standard protocol were to use one primer bearing a 5′ biotin and the other primer bearing a 5′ digoxigenin. Although it is perhaps unnecessary, we removed the unincorporated primers by desalting over Sepharose 2B. The addition of a small amount of bromophenol blue to the sample lets you know when the smallest material has eluted. Fractions containing the desired fragment can be identified by agarose gel electrophoresis and staining with ethidium bromide.

Initial identification of PNA-DNA coupling conditions by gel electrophoresis ● TIMING ~2 h on the first day, plus most of the second day

▲ **CRITICAL** Gel electrophoresis allows you to determine the initial concentrations and buffer conditions for PNA-DNA binding. In these experiments, please keep in mind that you are looking for a band that goes missing, not for a single band that appears[41].

59| In a 10-µl reaction, add purified plasmid DNA to 10 nM. Vary the concentration of PNA. For an initial range use 0.1–1 µM PNA. Include a no-PNA control. In addition, prepare samples with the plasmid cut with a restriction enzyme that cuts adjacent to the PNA target, and do not add PNA. The digested plasmid will aid in identifying the target band. We examined two solution conditions: TEN (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and 10 mM NaCl) and PEN (10 mM sodium PIPES, pH 6.5, 0.1 mM EDTA and 10 mM NaCl).

60| After mixing the samples, incubate them overnight in a 37 °C air incubator.

61| After incubation, dilute the samples to 30 µl with the high pH restriction enzyme reaction buffer for the diagnostic restriction enzyme.

▲ **CRITICAL** Choose a diagnostic restriction enzyme that cuts the plasmid several times, functions at 37 °C and leaves the diagnostic band well resolved from the other bands.

62| Heat the diluted samples to 65 °C and incubate them for 10 min. Transfer the samples to ice.

63| Add the diagnostic restriction enzyme. Incubate the restriction enzyme reactions at 37 °C.

64| After digestion is calculated to be complete, add 10 µl of sample buffer.

! CAUTION Do not add additional tracking dyes to experimental samples, as xylene cyanol can prevent visualization of ethidium bromide–stained bands that co-electrophorese with xylene cyanol. Electrophorese samples through a polyacrylamide gel in TBE. After electrophoresis, stain the gel with 0.5 µg/ml ethidium bromide. A 1.5-mm thick gel will require ~45 min with agitation for staining to be complete. After staining, visualize the stained DNA with a UV transilluminator and photograph the gel. An example can be seen in **Figure 4b**.

Preparation of PNA and plasmid DNA coupling ● TIMING 15 h

65| Pipette 1 µl of 10 µM PNA-biotin into a low-binding tube, and mix it with 9 µl of PEN buffer. Pipette 1 µl of 10 µM PNA-digoxigenin into another low-binding tube and mix it with 9 µl of PEN buffer. Pipette 1 µl of 200 nM DNA into a third low-binding tube and mix it with 3 µl of PEN buffer. Withdraw 3 µl of each of the three prepared solutions and mix them in a low-binding tube. Incubate the mixture in an air incubator at 37 °C for 14 h.

▲ **CRITICAL STEP** Use low-binding pipette tips and tubes to prevent nonspecific adhesion of the PNA and DNA molecules to the surface of the tubes during preparation.

? TROUBLESHOOTING

■ **PAUSE POINT** After incubation, the solution can be stored at 4 °C for up to 3 d.

66| After incubation, pipette 2 µl of the PNA-DNA solution into a new low-binding tube and dilute it in 48 µl of PEN buffer.

■ **PAUSE POINT** The solution can be stored at 4 °C for up to 3 d.

67| Pipette 4 µl of the diluted PNA-DNA solution into a low-binding tube and mix it with 96 µl of λ buffer. Place this tube in a heat bath at 65 °C for 10 min.

▲ **CRITICAL STEP** The heat bath incubation is important to remove nonspecific binding between PNA and DNA, and it cannot be excluded from the protocol.

■ **PAUSE POINT** The solution can be stored at 4 °C for up to 3 d.

Construction of perfusion chamber ● TIMING 15 min

68| Place two strips of Parafilm on a clean 24 × 50 mm cover slide with a spacing of ~5–7 mm. On top of the Parafilm, place a clean 18 × 18 mm cover slide as a lid (**Fig. 5a**).

69| Heat a plane block heater to ~80 °C, and place the prepared chamber on it to melt the Parafilm. Gently press the two slides together. Remove the chamber from the heat stage, cut off excess Parafilm if necessary and place the chamber in a closed box with a little water in the bottom to prevent evaporation of the open perfusion chamber during sample preparation (**Fig. 5b**).

▲ **CRITICAL STEP** The sample will dry out during preparation if there is not water in the closed box.

Preparation of the PNA-DNA tethered sample ● TIMING 2.5 h

70| Add 20 µl of the 20 µg/ml anti-digoxigenin solution to the perfusion chamber. Incubate it for 30 min at RT. After incubation, wash the chamber with 90 µl of λ buffer.

▲ **CRITICAL STEP** Fluid placed at one end of the channel will be drawn into the chamber by capillary forces and can be withdrawn at the other end of the channel by placing a filter paper to suck up the liquid (**Fig. 5c**). This method is used for all buffer exchanges during the sample preparation. Be careful not to remove more liquid than what is added, as a temporarily dried chamber will acquire air bubbles when new liquid is added and can damage the sample. Preferably always keep an excess of the liquid at the inlet and at the outlet of the flow channel.

71| Add 20 µl of the 2 mg/ml α-casein solution to the perfusion chamber. Incubate it for 30 min at RT. After incubation, wash the chamber with 90 µl of λ buffer.

72| Add 20 µl of the prepared PNA-DNA solution that has been heated in a water bath of 65 °C for 10 min (Steps 65–67) to the perfusion chamber. Incubate it for 60 min at RT. After incubation, wash the chamber with 90 µl of λ buffer.

73| Add 20 µl of the bead suspension containing 2 mg/ml α-casein in PBS buffer (Reagent Setup) to the perfusion chamber. Incubate it for 30 min at RT. After incubation, wash the chamber with 90 µl of λ buffer.

74| Seal the perfusion chamber with high-vacuum grease to prevent evaporation.

▲ **CRITICAL STEP** When you are conducting protein-mediated DNA conformational change studies, add the protein solution before sealing the chamber.

? TROUBLESHOOTING

Identifying noise in the experimental setup ● TIMING ~2 h

▲ **CRITICAL** Before conducting a TPM experiment, it is important to quantify the drift in the system to minimize the level of noise that otherwise can lead to bias in the data. This is especially important for experiments longer than 10 s, in which temperature, stage and microscope drift could be significant factors. In our experiments, we recorded long time series, up to 60 s, in which reduction of drift in the setup was important. Allan variance analysis has proven to be a reliable method to identify low-frequency noise[42,43]. Also, Allan variance is useful to determine the optimal measurement time of an experiment that is subject to low-frequency noise with a possible bias[42,43]. It should be noted, however, that the biological process of interest should also be considered while determining the measurement time. To quantitatively address and to possibly minimize the noise present in a TPM experiment assay, we suggest following Allan variance analysis before the experiments.

75| Sample a long time series of position data of an immobilized bead in the microscope with a sufficiently high acquisition frequency.

76| Import the time series to MATLAB.

77| Calculate the Allan variance, which is defined as

$$\sigma_x^2(\tau) = \frac{1}{2}\left\langle (x_{i+1} - x_i)^2 \right\rangle_\tau$$

of adjacent time series for a set of measurement times τ, where τ is defined as the number of elements in the interval divided by the sampling frequency. A free Allan variance analysis program can be downloaded from http://www.mathworks.com/matlabcentral/fileexchange/26659-allan-v3-0.

78| Determine the optimal measurement time τ where the Allan deviation is minimal, or quantify the drift for a given measurement time.

79| Quantify and possibly minimize noise sources in the setup using the results of the Allan variance calculation.

Imaging condition setup and pixel-to-nm calibration ● TIMING ~1 h

80| Dilute microspheres with diameters of 0.5–3 µm, 1:10,000, in filtered Milli-Q water.

81| Perfuse the diluted microspheres into a perfusion chamber and seal it with vacuum grease to prevent evaporation.

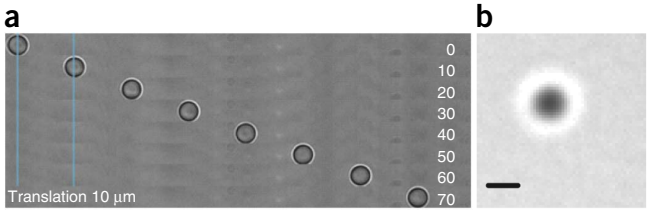

Figure 6 | Image analysis. (**a**) A 3-µm sphere immobilized to a coverslip is moved in 10-µm steps (shown as the distance between the blue vertical lines) by a piezo-stage with nanometer accuracy. The total movement in this figure is 70 µm. In our work, the conversion factor was 45.97 nm/pixel for maximum optical zooming. (**b**) A typical microscope image of the *x*,*y* projected plane of a 0.5-µm tethered bead. Scale bar, 500 nm.

82| Add a droplet of immersion oil on the objective and make sure that no air bubbles are present.

83| Adjust the microscope for bright-field imaging and optimize the illumination and contrast of the sample according to the standard procedure. If the microscope is equipped with differential interference contrast, this mode can be used to enhance the contrast of the beads.

84| Place the perfusion chamber filled with the sample on the microscope stage. Wait until a few microspheres are immobilized to the surface, typically 5–15 min.

85| Find an immobilized microsphere and zoom in using the optical zoom.
▲ **CRITICAL STEP** Digital zooming will not improve the resolution.

86| With a microsphere positioned at one side in the field of view, move the microscopic stage with discreet steps of a few µm (e.g., 5–10 µm) and at each step capture an image. Repeat this step for a few beads.

87| Import the images to an image-processing program (e.g., Gimp or Photoshop) or MATLAB to stitch the images together as shown in **Figure 6a**.

88| Determine the distance-to-pixel conversion factor by measuring the number of pixels between each discreet step (adjacent beads) and dividing that by the known step length (5–10 µm) obtained from the piezo-stage. Repeat Steps 86–88 also for small displacements, i.e., 100-nm steps.

Setup of the sample and image recording preparation ● **TIMING ~15 min**
89| Add a droplet of immersion oil on the objective; make sure that no air bubbles are present.

90| Place the sample perfusion chamber on the microscope stage.

91| Adjust the microscope for bright-field imaging, and optimize the illumination and contrast of the sample according to the standard procedure. If the microscope is equipped with differential interference contrast, this mode can be used to enhance the contrast of the beads.

92| Optically zoom on a bead that is tethered and that performs Brownian motion.
? TROUBLESHOOTING

93| Set an area of interest/region of interest (AOI/ROI) to minimize the imaging region in order to generate smaller data files and to achieve a faster acquisition rate. If possible, simultaneously image an immobilized object with the bead under investigation to correct for system drifts. In an ROI of 1,000 × 1,000 pixels (ref. 2), there are, on average, one or two tethered beads in a supercoiled sample.
▲ **CRITICAL STEP** Be aware that a small ROI gives a faster acquisition rate compared with a larger ROI, and hence it can capture faster kinetic rates of the DNA conformational changes. However, for investigations in which the time resolution is less important or the transitions occur on time scales on the order of a second, a larger ROI where multiple tethered beads can be imaged simultaneously is a better option as the method is fairly time-consuming.

Acquiring an image of a tethered bead ● **TIMING ~5 min**
94| Run ParaSequencer and connect to the camera.
▲ **CRITICAL STEP** Make sure that the full bandwidth (maximum package are set) of the camera is used for full-speed image acquisition. We show here the protocol using the frame capture program ParaSequencer 1.6.3 from Parameter Sweden. The same approach can, however, be used for other Institute of Electrical and Electronics Engineers (IEEE)-interface cameras.

95| At the settings dialog, set the number of packages to 7,576 and IEEE speed to 800 Mbps. This allows for 60 f.p.s. at 1,000 × 1,000 pixels^2 (ref. 2).

96| Unclick 'Auto Sequence Numbering', set the 'Video File Path' and name the file. Set a specific number of frames or the acquisition time determined by Allan variance analysis (Steps 75–79).

97| Set the AOI (width and height) and place the marked-up box on the bead under investigation. For higher f.p.s., set an AOI/ROI that minimizes the imaging region; 62 × 62 pixels (ref. 2) will cover a region of 2.85 × 2.85 μm^2 (sufficient for a 0.5-μm bead tethered by an ~7-kbp DNA plasmid performing Brownian fluctuations), and it will allow for 225-Hz recording and thus an exposure time <5 ms. A typical *x*-*y* image plane of a bead in an ROI of 62 × 62 pixels (ref. 2) is shown in **Figure 6b**. Press 'Set AOI' to reset the frame.

98| Press 'Live' to fine-tune the light/contrast and correct the position of the bead to the center of the ROI using the piezo-stage.

99| Press 'Start' and the frames will be recorded and stored in an AVI file format. In addition to the saved AVI file, a .txt file containing the number of recorded frames, the FPS for the sequence and the number of dropped frames is saved.

Particle tracking of a tethered bead ● TIMING ~15 min

100| Import the AVI file into the St. Andrews Tracker program that performs postprocessing of the recorded frames. Choose the .avi file tab and locate the file to be analyzed. Mark the 'SubPixel' accuracy check box. For high-contrast videos, 700 is sufficient for 'Minimum Match Score'. Keep the default settings of the other controllers. Set the ROI by checking the 'New template(s)' box and set the 'End' frame to the number of frames of your AVI file. Start the program.

101| An additional window is opened. Zoom in on the bead and set an ROI around the rim of the bead by clicking and dragging with the mouse in the movie window. An ordered ASCII data file containing the *x* (first column) and *y* (second column) pixels of the bead's center position in each frame is created.

Time series analysis of a TMP data set ● TIMING ~5 min

102| Import the ASCII data file into MATLAB.

103| Subtract the mean of the *x*- and *y*-coordinates $(x(t)-\langle x\rangle)$, $(y(t)-\langle y\rangle)$ from the times series to have the bead positions distributed around the tether point.

104| Plot the *x* and *y* data in a scatterplot and histogram to visually inspect the position data. The data should be centered at position 0.

105| Calculate the time-dependent projected displacement vector ρ, i.e., $\rho^2(t) = (x(t)-\langle x\rangle)^2 + (y(t)-\langle y\rangle)^2$ to quantify the 2D projected displacement. Filter ρ with a sliding window (moving average) (we used a moving average of 20 ms). Plot ρ versus time and inspect the data set.

106| *x* and *y* are random directions, so we perform a principal component analysis (PCA) to determine the principal axes of the positions visited by the bead and interpret the data accordingly to the principal axes. The two principal axes denote the orthogonal axes along which the data have the largest and smallest spread. Use the data from Step 103 to find the principal axes by calculating the covariance matrix as

$$C = \begin{pmatrix} \sigma_{xx} & \sigma_{xy} \\ \sigma_{yx} & \sigma_{yy} \end{pmatrix}$$

where σ_{ij} represents the covariance of the in-plane coordinates (i,j), and where the covariance is defined as

$$\sigma_{ij} = \frac{1}{N}\sum_{k=1}^{N} i^k j^k - \frac{1}{N^2}\left(\sum_{k=1}^{N} i^k\right)\left(\sum_{k=1}^{N} j^k\right)$$

with *N* representing the number of frames and $k = 1,2,\ldots N$. An example of a PCA analysis on an isotropic and an anisotropic data set is shown in **Figure 7**.

107| Transform the *x* and *y* data (defined by the black axes in **Fig. 7a–c**) into the new coordinate system, defined by the principal axes *PA1* (red lines) and *PA2* (green lines), using the minimum and maximum eigenvalues of the eigenvectors from the covariance matrix. In this way, the data are now represented by the axes defining the largest and smallest spread in the data set.

108| Calculate the s.d. of the two transformed data sets along the principal axes std^2_{PA1}, std^2_{PA2}. If the data are not expressed in terms of *PA1* and *PA2*, the spread in an asymmetric data set would be underestimated (compare the black lines in **Fig. 7c** with the red and green lines in **Fig. 7d**).

109| The method of systematically deciding which data sets are qualified and which should be rejected without bias are based on two selection criteria suggested in literature[44–46]. These criteria relate to the size of the r.m.s.d. and the symmetry of the positions visited by the bead and are described in Steps 109–111. The r.m.s.d. gives information of the mean bead displacement and thereby the conformational state of the DNA. The more compacted the molecule, e.g., as a result of supercoiling or protein-mediated looping, the shorter the r.m.s.d. (see ANTICIPATED RESULTS section). The r.m.s.d. value can be found by taking the square root of the added squared s.d. values $\sqrt{std^2_{PA1} + std^2_{PA2}}$. Use this quantity to evaluate whether the bead is correctly tethered and to exclude, e.g., surface-stuck beads that have a low r.m.s.d., ~50 nm or less (**Fig. 8**). We set 60 nm as the lower limit of the r.m.s.d. that could be expected to originate from correctly formed plasmid tethers.

110| From the minimum and maximum eigenvalues, s_1 and s_2 of the covariance matrix, *C*, calculate how symmetric the data set is via the 'symmetry', *s*, defined as the ratio between the two eigenvalues, $s = s_1/s_2$ (where $s_1 \leq s_2$).

111| Set a criterion on the symmetry calculated in Step 110 to assess whether the bead is correctly tethered by a single DNA tether. As the calculations of the covariance matrix gives two values of unit length, the criterion ranges from 0 to 1. As we experience that the data sets originating from the plasmid tethers normally have a high symmetry, we set the symmetry criterion to $s > 0.8$ (compared with 0.5 in Tolic-Norrelykke[44]), thus permitting only data sets similar to **Figure 7a**,**b** in the final analysis (the length of the red and green lines are very close, symbolizing high symmetry). In contrast, a bead tethered by, e.g., multiple tethers or catenated plasmids typically performs an anisotropic motion, as shown in **Figure 7c**,**d**, and is excluded by this criterion (the lengths of the red and green lines are very different). After being subjected to the selection criteria in Steps 109–111, ~50% of the data sets remain from a sample containing a ~7.6-kbp supercoiled DNA molecule[16].

Identification of PNA-DNA coupling conditions in TPM chamber ● TIMING ~1 week (without plasmid preparation)

112| Confirm that the PNAs are binding at their specific targets by TPM analysis. Here we describe the method used in Norregaard *et al.*[16]. Prepare two plasmids lacking either the target for the biotin-labeled PNA or the target for digoxigenin-labeled PNA. Prepare a third plasmid with both targets intact.

113| Perform TPM analysis and subject the data sets to the r.m.s.d. and symmetry criteria. Compare the r.m.s.d. of plasmids lacking a PNA target with the r.m.s.d. of plasmids with both targets intact. Plasmids that are incorrectly tethered will have a very low r.m.s.d.. Hence, if the PNAs bind correctly, only the plasmid with both targets intact should remain after the r.m.s.d. criterion is applied.

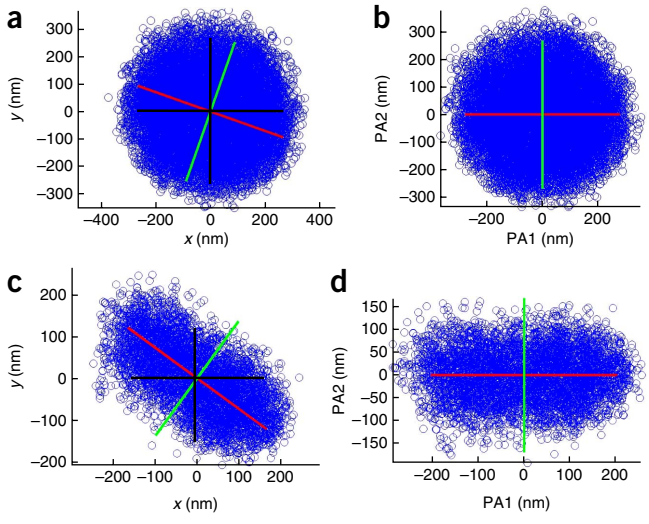

Figure 7 | Example of PCA. The data are rotated so that the maximum variance of the data is along the ordinate axis. (**a**,**b**) A scatter plot of a data set accepted for analysis with r.m.s.d. = 193 nm and $s = 0.95$. The red and green lines represent the principal axes. In **a**, the s.d. of the data set is found along the *x* and *y* (black lines) directions. In **b**, the data set has been transformed and the principal axes *PA1* (red line) and *PA2* (green line) are found. (**c**,**d**) show an example of an anisotropic data set with r.m.s.d. = 132 nm and $s = 0.47$ before (**c**) and after (**d**) the assignment of principal axes. If the s.d. values, which are compared to find *s*, are not calculated along the principal axes (red and green lines) both *s* and the r.m.s.d. of the data set would be underestimated. Because of the low symmetry ($s < 0.8$), the data set shown in **c**,**d** is discarded, and it probably originates from tethering of the bead by multiple tethers.

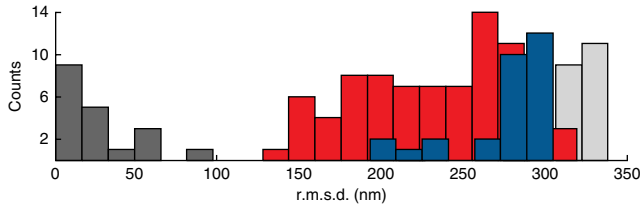

Figure 8 | r.m.s.d. of various plasmid constructs usable for troubleshooting the overall length of the tether. The red bars show the distribution of the r.m.s.d. taken over an entire experiment (not a moving time window as in **Fig. 9**) for a supercoiled plasmid. The r.m.s.d. distribution from a relaxed tether is shown by blue bars, the light gray full bars show the distribution from a linear control DNA and the dark gray bars show the r.m.s.d. from a stuck bead. The linear plasmid has the longest r.m.s.d. with a narrow distribution. The relaxed plasmid (the shortest arc of which has the length of the linear plasmid) has an r.m.s.d. that lies just below the linear plasmid. This is reasonable because it is less flexible than the linear DNA. The supercoiled plasmid has a large distribution of r.m.s.d., which originates from the different number of supercoils that are naturally present in the distribution. As expected, the stuck bead has the lowest r.m.s.d. distribution (with an average value of 36 nm).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
65	PNA invasion in relaxed plasmid DNA	PNA binding to the DNA requires opening of the DNA helix, which is less efficient in relaxed DNA compared to the supercoiled state	Identify optimal PNA concentration and/or PNA-DNA incubation time. In our study[16], we increased the concentration of the digoxigenin-labeled PNA to obtain tethers with relaxed plasmid DNA
74	Beads aggregate	Low stability of the beads either from the supplier or possibly because of low functionality of the α-casein	Prepare a new α-casein solution. If this does not help, contact the supplier of the beads to see if the stock solution has an appropriate quality. A cup horn sonicator appears to be more efficient at breaking up aggregates than normal ultrasound
92	No tether formation	Inactive PNA-DNA coupling	Produce a linear DNA molecule to identify the correct PNA-DNA coupling (Step 58). We made a linear DNA molecule with the same length (2.6 kbp) as the shorter of the two arcs in the circular DNA plasmid. If an experiment with PNA tethered circular DNA failed, we could use this linear DNA molecule as a control to examine the reagents involved in the PNA-DNA coupling. This linear DNA molecule could also be used to compare the excursions of the relaxed circular plasmid (see ANTICIPATED RESULTS)

● TIMING

Steps 1–6, preparation of digoxigenin-labeled bis-PNA: 20 h
Step 7, preparation of biotin–bis-PNAs conjugates: 20 h
Steps 8–23, growth schedule for purification of supercoiled plasmid DNA: 3 d in total (20 min on day 1, plus 20 min in the morning and 45 min at the end of day 2)
Steps 24–32, plasmid recovery: ~0.5 d
Steps 33–38, plasmid purification: ~2 h followed by overnight centrifugation
Steps 39–45, recovery of supercoiled DNA: ~2 h followed by overnight centrifugation
Steps 46–53, removal of ethidium bromide: 2–3 h followed by overnight incubation
Steps 54–55, characterization of CsCl-purified plasmids: 1 d
Steps 56–57, preparation of chloroquine gel: ~4 h of manipulation, plus overnight electrophoresis
Step 58, preparation of end-labeled linear DNA: 1 d
Steps 59–64, initial identification of PNA-DNA coupling conditions by gel electrophoresis: ~2 h on the first day, plus most of the second day
Steps 65–67, preparation of PNA and plasmid DNA coupling: 15 h
Steps 68–69, construction of perfusion chamber: 15 min
Steps 70–74, preparation of PNA-DNA tethered sample: 2.5 h
Steps 75–79, identifying noise in the experimental setup: ~2 h

Steps 80–88, imaging condition setup and pixel-to-nm calibration: ~1 h
Steps 89–93, setup of the sample and image recording preparation: ~15 min
Steps 94–99, acquiring an image of a tethered bead: ~5 min (In 2 h, ~50 time series of 60 s can be recorded when the sample search time is included.)
Steps 100–101, particle tracking of a tethered bead: ~15 min
Steps 102–111, time series analysis of a TPM data set: ~5 min
Steps 112–113, identification of PNA-DNA coupling conditions in the TPM chamber: ~1 week (without plasmid preparation)

ANTICIPATED RESULTS

This protocol for specific tethering of circular DNA to surfaces, beads or other markers via PNA handles allows the study of the dynamics of DNA. Circular DNA can either be in its natural supercoiled state or the supercoils can be enzymatically removed, thus leaving the plasmid relaxed. **Figure 9** shows typical data stemming from an experiment where a bead is attached to a naturally supercoiled plasmid DNA incorporating λ bacteriophage operator sites OL and OR (as illustrated in **Fig. 2**). The length of the projected displacement vector, $\sqrt{\langle \rho^2(t) \rangle 20\ \text{ms}}$, of the positions visited by the tethered bead and averaged over a 20-ms time window is shown as a function of time in **Figure 9a**, the corresponding histogram in **Figure 9b**. **Figure 9c** shows $\sqrt{\langle \rho^2(t) \rangle 20\ \text{ms}}$ of a similar supercoiled DNA tether but in the presence of 20 nM (~1/10 of the lysogenic concentration) of the CI. With CI present, DNA looping events are visible as abrupt decreases in $\sqrt{\langle \rho^2(t) \rangle 20\ \text{ms}}$ (for instance, from 700 s). The on and off times can be read off directly from plots such as **Figure 9c**. **Figure 9d** shows the corresponding histogram; the two peaks signifying two distinct distributions represent the looped and unlooped states, respectively. The ratio of the areas of the two distributions gives the probabilities of being in the looped versus unlooped state. In time series stemming from the plasmid alone (**Fig. 9a**), we did not observe changes of $\sqrt{\langle \rho^2(t) \rangle 20\ \text{ms}}$ that would result from a change in writhe. Hence, for a given naturally supercoiled plasmid, both the linking number and the writhe seem to remain constant.

As expected[20], it was more difficult to make the PNA handles attach to DNA if the DNA was enzymatically relaxed. To troubleshoot this problem and to obtain an idea of which r.m.s.d. distribution to expect from the enzymatically relaxed DNA plasmid, we constructed a linear DNA with exactly the same sequence as the arc of the plasmid containing the PNA attachment sites and the λ immunity region (purple part of **Fig. 3a**). The r.m.s.d. distributions from the supercoiled plasmid, the relaxed plasmid and the control linear DNA are shown in **Figure 8**. The linear DNA (light gray bars) has a very narrow distribution of r.m.s.d. The length of the linear control DNA was 2.6 kbp; hence, we know that a r.m.s.d. of ~340 nm corresponds to a tether length of ~870 nm (assuming that each bp is 1/3 nm). The natural distribution of writhe number in the supercoiled plasmids gives rise to a rather large spread of the r.m.s.d. (red bars in **Fig. 8**). This distribution has an r.m.s.d. from 150 nm up to 340 nm, and thus it signifies a large variety of overall tether length among the supercoiled plasmids. The relaxed plasmid gives rise to a narrower r.m.s.d. distribution (blue bars), which is centered at the upper end of the r.m.s.d. distribution from the supercoiled plasmid. This is reasonable because the relaxed plasmid should behave similarly to the fraction of supercoiled DNA with the lowest writhe number. In addition, the center of the r.m.s.d. distribution from the relaxed plasmid lies close to but slightly below the r.m.s.d. distribution from the linear tether. As an arc of the relaxed plasmid is only ~17 times the persistence length of dsDNA and hence not totally flexible, it is expected that the r.m.s.d. distribution of the relaxed plasmid is slightly lower than that of the linear tether. In addition, for a comparison, we show (dark gray bars on **Fig. 8**) the r.m.s.d. distribution for a stuck bead. These types of analyses are recommended if there is doubt about the correctness of the r.m.s.d. values obtained.

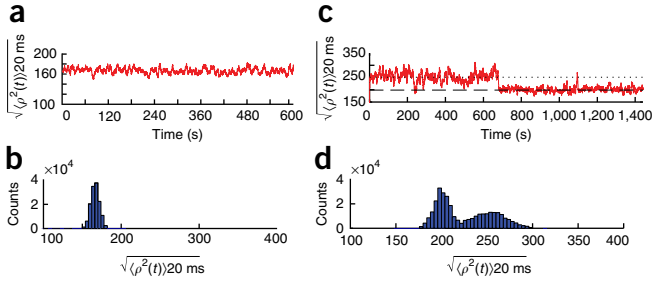

Figure 9 | Typical data resulting from TPM experiments with a supercoiled DNA plasmid. (**a**,**b**) The lateral displacement of a bead tethered to a supercoiled plasmid averaged over a time window of 20 ms as a function of time (**a**) and the corresponding histogram (**b**). (**c**) The lateral displacement of a bead tethered to a supercoiled plasmid, similar to that shown in **a** but in the presence of 20 nM CI, which occasionally clamps the DNA present in the sample (**c**); the corresponding histogram (**d**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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