Insights on the effect of macromolecular crowding on transcription and its regulation

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Abstract

Transcription of DNA into RNA is a fundamental cellular process upon which life depends. It is tightly regulated in several different ways, and among the most important mechanisms are protein-induced topological changes in DNA such as looping. *In vivo* neither transcription, nor protein induced looping dynamics exhibited by individual molecules are easily monitored. *In vitro* single-molecule approaches do offer that possibility, but assays are conducted in rarefied, saline buffer conditions which greatly differ from the crowded intracellular environment. In the following, we describe monitoring both transcription and *lac* repressor-mediated DNA looping of single DNA molecules in the presence of different concentrations of crowders to bridge the gap between *in vitro* and *in vivo* experimentation. We found that crowding shifts the preferred orientation of DNA strands in the looped complex. Crowding also attenuates the rate of transcript elongation and enhances readthrough at the terminator. Clearly, the activities of proteins involved in gene regulation are modified in surprising ways by crowding.

Introduction

The cell is a crowded and complex environment where various physical forces operate within the genome and modify its organization and function. For example, crowding gives rise to depletion forces that tend to favor aggregates and condensates (Asakura and Oosawa 1958, Marenduzzo,

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Finan et al. 2006, Toan, Marenduzzo et al. 2006, Renko 2010, Mardoum, Gorczyca et al. 2018, Collette, Dunlap et al. 2023). Variations in crowding may, therefore, induce liquid-liquid phase separation (André and Spruijt 2020, Kohata and Miyoshi 2020, Shakya, Park et al. 2020, Gao, Zhang et al. 2021, Collette, Dunlap et al. 2023) and confer a more gel- rather than fluid-like, or vice versa, nature to the genome (Odijk 1998, Pelletier, Halvorsen et al. 2012, Ramisetty and Dias 2015, Jorge, Nunes et al. 2016, Yang, Männik et al. 2020). Transitions from one phase to the other are expected to have significant regulatory implications for genomic transactions. It has been suggested that gel-like condensates may serve as factories or storage locations for DNA-processing enzymes, but also the genome may simply be more, or less, compacted, dynamic and accessible to external molecules and compounds depending on the phase in which it is.

Other forces, constantly at play on the genome, are mechanical in nature being due to the combination of tethering of DNA to membranes and the activities of processing enzymes acting as molecular machines that manipulate the double helix while consuming energy. These forces can pull and twist DNA, thereby changing its secondary structure and interaction with regulatory and remodeling proteins (Rouzina and Bloomfield 2001, Li, Wijeratne et al. 2015, Svidlov, Drobotenko et al. 2021, Collette, Dunlap et al. 2023). How entropic and mechanical forces, as well as their combined effect, change gene transcription is grossly understudied, despite the incredible insight that understanding the role of these forces would provide on transcription regulation.

Here, we report preliminary explorations of the effect of macromolecular crowding on: (i) LacI-mediated DNA looping topologies, (ii) Transcription rate and (iii) Transcription termination given the following motivations.

Protein-mediated DNA looping is a ubiquitous mechanism of regulation, implicated in DNA replication, repair, recombination and transcription. Since it brings distant segments of DNA close together, it is also an effective way to compact the genome (Amouyal 1991, Skoko, Yoo et al. 2006, Bouwman and de Laat 2015). Protein-mediated DNA loops may be antiparallel (AP), or parallel (P). In the AP configuration, the DNA going into the loop has opposite orientation from that exiting it and the DNA loop “extrudes”. In the P configuration, DNA has a solenoidal arrangement in space and the same orientation going in, or out, of the loop. When DNA wraps around proteins, such as in nucleosomes in eukaryotes or in the 186 CI protein of the 186 bacteriophage, it clearly adopts a P configuration dictated by interactions with the proteins. In the case of loops secured only at the ends by proteins, the physiological significance of the P *vs*. AP loop topologies is less clear, aside from the consideration that the solenoidal, P, is more compact than the extruded, AP, configuration.

In addition, crowding changes the compaction state of polymers. In particular, crowding affects the condensation state and phase of DNA. It may also change the viscosity of the environment altering the diffusion of reagents (Heinen, Zanini et al. 2012, Skóra, Vaghefikia et al. 2020, Aporvari, Dang et al. 2022). Thus, crowding may also affect the rate of transcript elongation. Finally, viscoelastic effects may affect the probability of termination versus either recycling (Harden, Herlambang et al. 2020, Kang, Ha et al. 2020, Qian, Wang et al. 2024) or readthrough (Ray-Soni, Bellecourt et al. 2016, Vilborg and Steitz 2017, Xie, Libri et al. 2023, Caldas, Luz et al. 2024).

We tested these hypotheses using the tethered particle motion technique (Manzo and Finzi 2010, Manzo, Zurla et al. 2012, Kumar, Manzo et al. 2014, Kovari, Yan et al. 2018, Qian, Collette et al.

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2024) and magnetic tweezers (Yan, Ding et al. 2018, Yan, Leng et al. 2018, Kovari, Dunlap et al. 2019, Piccolo, Méndez et al. 2021). These are single molecule techniques particularly suitable to reveal conformational changes and the molecular details of dynamic processes such as RNA polymerase (RNAP) transcriptional elongation.

Materials and Methods

DNA template preparation for TPM experiments

The DNA fragments used in these experiments were produced by polymerase chain reaction (PCR) as previously described (Qian, Collette et al. 2024). In brief, primers (Eurofins Genomics, Louisville, KY) designed with"A plasmid Editor" (ApE) (Davis and Jorgensen 2022) and labeled with biotin or digoxigenin, were used alongside deoxynucleotide triphosphates (New England BioLabs, NEB) and Taq/Q5 DNA polymerase (NEB). The resulting DNA fragments were attached at the digoxigenin end to the microscope flow chamber, and the biotin end to a bead.

Microchamber preparation for TPM experiments

Microchambers were prepared as previously reported (Qian, Collette et al. 2024). In brief, the lower microscope slide (Fisherbrand, Thermo Fisher Scientific, Waltham, MA, USA) used to construct the microchamber supported a parafilm gasket that was cut to shape with a laser cutter (Universal Laser Systems, VLS 860, Middletown, CT, USA). The central observation section was connected via narrow channels to inlet and outlet reservoirs just beyond the edges of the top coverslip. Once the chamber was assembled, it was heated to approximately 85 °C on a hotplate and the top coverslip was pressed down with tweezers to seal the components together. The final volume was approximately 6 μL and provided a gradient of tether densities to optimize the utility of chambers. The sample preparation was conducted at room temperature (~20 °C) usingλ buffer (10mM Tris-HCl (pH 7.4), 200/100 mM KCl, 5% DMSO, 0.1 mM EDTA, 0.1 mg/mL α-casin, 0.2 mM DTT). Beads were polystyrene, streptavidin-coated and 0.32 μm in diameter (Spherotech, Lake Forest, IL, USA). Chambers were incubated with 10 μL anti-digoxigenin at a concentration of 5 μg/mL (Roche Life Science, Indianapolis, IN, USA) in PBS at room temperature for 1 hour. The anti-digoxigenin was then rinsed out with PBS and the chambers were then passivated with West-EZ Blocking Buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% α-casin (w/v)) (GenDEPOT, Katy, TX, USA) for 30 min. DNA tethers were then incubated in the chamber for 15 min to be anchored through a single digoxigenin to the anti-digoxigenin-coated coverslip. The other end of the DNA was anchored to a streptavidin-coated bead via a single biotin by adding 0.03 mg/mL beads solution in λ buffer for a 15 min incubation. Excess, untethered beads were then flushed out of the chamber with 120 μL of λ buffer. Beads were washed thrice in PBS and once in λ buffer before being resuspended in λ buffer prior to being introduced to the chamber. Lac repressor was added in a λ buffer solution containing different percentages (w/v) of crowder.

TPM Procedure and Analysis

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Microspheres tethered to a glass substrate by individual DNA molecules undergo Brownian motion. The bead diffuses throughout a hemisphere, the size of which is dictated by the tether length. The scatter of positions of the tethered microsphere in the XY plane is recorded by single particle tracking to identify the anchor point and the extent of excursions around it. The excursion amplitude,ρ, value can be converted to the effective tether length using an appropriate calibration curve. The technique is reviewed in (Beausang, Zurla et al. 2007, Beausang, Zurla et al. 2007, Beausang, Zurla et al. 2007, Kovari, Yan et al. 2018). All TPM measurements were performed as previously described (Yan, Leng et al. 2018, Xu, Yan et al. 2022, Qian, Collette et al. 2024).

The XY position of each bead was recorded at 50 Hz interlaced with a custom Lab VIEW (National Instruments, Austin, TX, USA) program. Vibrational or mechanical drift in the position of each bead was eliminated by subtracting the average location of reference beads that were adhered to the substrate within the same field of view. The effective length of each tether was then calculated as $< \rho >_{8s} = \sqrt{(x - < x >_{8s})^2 + (y - < y >_{8s})^2}$, in which $< x >_{8s}$ and $< y >_{8s}$ are eight-second moving averages representing the coordinates of the anchor point of a bead. The anchor point is determined by finding these average x and y positions. Changes in the effective DNA tether length are represented as changes in the excursion length of the bead. Any beads with (x,y) position distributions with a ratio of the major to minor axes greater than 1.07 were discarded to exclude beads tethered by multiple DNA molecules. Excursion data from the time records of beads, in the same experimental conditions, which passed the “symmetry test” were retained for the analysis described below.

The excursion data was then fitted by a Bayesian step-filtering algorithm, which proved efficient and effective in processing step-like high frequency signals, in this case, real-time signals with multiple fixed excursion levels corresponding to different looped and unlooped states. In brief, this method proceeds global filtering that finds an optimal time series $\rho_{opt}(t)$ that eliminates the noise but retains the piecewise constant stepwise signals in the original time series $\rho_{ori}(t)$. For this goal, we search for $\rho_{opt}(t)$ that minimizes the following cost function:

$D(\rho_{opt}|\rho_{ori}) = \frac{1}{2}\sum\left(\rho_{opt}(t) - \rho_{ori}(t)\right)^2 + \lambda \sum|\rho_{opt}(t+1) - \rho_{opt}(t)|$, where λ is a parameter that determines the weights of the mean square error term and the total variation term. The details of minimizing the cost function has been described previously (Qian, Collette et al. 2021). After this process, the original excursion time series were smoothed as piecewise signals with alternating excursion levels. Finally, we assign the corresponding looped and unlooped states to each piece according to its excursion level and acquire the fraction of each state.

Proteins

LacI was provided by Kathleen Matthews (Rice University). HA-labeled *E. coli* RNA polymerase was provided by Karen Adelman (Harvard University).

DNA template preparation for MTs experiments

DNA tethers were prepared using plasmid pZV_NI_400 by performing PCR amplification with Q5 Hot start High-Fidelity master mix (New England Biolabs, Ipswich, MA). Single biotin-labeled forward primer, 5’–ATCGTTGGGAACCGGAG, and unlabeled reverse primer, 5’–AGCTTGTCTGTAAGCGGATG, were used to amplify 3 kb DNA fragments. The transcribed region contained the T7A1 promoter 741 bp from the lac operator, O1. The λt1 terminator was 612 bp downstream of O1 (Qian, Collette et al. 2024). The anchoring point of the DNA at the chamber surface was located 1021 bp from the transcription termination site (Figure 1).

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Microchamber preparation for MTs experiments

A laser-cut parafilm gasket was placed in the center of a clean, rectangular glass coverslip and covered with a square coverslip (Kovari, Yan et al. 2018, Qian, Collette et al. 2024). The microchamber was heated for 15-20 s at 70 °C on a hot plate to prevent leaks. Next, anti-HA antibody was introduced to achieve a final concentration of 8 µg/mL into the microchamber and incubated for 30 min at room temperature. The microchamber was passivated with 15 µL blocking buffer (PBS with 1% casein, GeneTex, Irvine, CA) and incubated for 20 min at room temperature. Transcription elongation complexes (TECs) were stalled at position +21. They were prepared in an eppendorf tube by mixing 3 nM DNA template, 30 nM HA-RNAP, 100 µM GpA dinucleotide (TriLink, San Diego, CA), 5 mM AUG in transcription buffer (20 mM Tris glutamate pH8, 50 mM potassium glutamate, 10 mM magnesium glutamate, 1 mM DTT, 0.2 mg/ml casein) and incubated for 10 min at 37 °C. TECs were then diluted to 250 pM DNA:RNAP, added into the microchamber and incubated for 10 min at room temperature. Next, 20 µL streptavidin-coated superparamagnetic beads (diluted 1:100 in Transcription Buffer; MyOneT1 Dyna beads, Life Technologies, Carlsbad, CA) were added into the microchamber and incubated for 5 min at room temperature. Finally, the microchamber was flushed with 20 µL transcription buffer to remove the excess streptavidin-coated superparamagnetic beads.

Procedure for magnetic tweezers experiments

Magnetic Tweezers were used to observe transcription and measure transcript elongation rates in real time by monitoring the change in bead height as RNAP moved along the DNA template (Figure 2). Non-moving beads were selected as reference and moving beads for transcription assay (over 10 beads). Then, 50 µM NTPs (New England Biolabs, Ipswich, MA) were added into the microchamber without interrupting the recording which was stopped after 1 hr for following data analysis. For crowding measurements, NTPs were added in a solution of polyethylene glycol (PEG). For percentages of PEG above 5%, the addition had to be done step by step with NTPs present only in the highest PEG concentration.

Results

In vitro measurements are arguably the simplest and, sometimes, the only way to study molecular interactions that may be obscured *in vivo*. However, most in vitro measurements are made in saline solutions that are far from mimicking the native crowded environment of cells. The cell contains many molecular species, including membrane-bound and membrane-less organelles, macromolecules and small molecules. Therefore, crowding is likely to exert entropic forces on molecules and macromolecules and affect intra-, as well as inter-, molecular interactions, including those relevant to transcription and its regulation. To begin addressing the gap between in vivo and in vitro measurements by characterizing the effect of macromolecular crowding on cellular processes, (i) DNA looping by the lac repressor (LacI) protein and (ii) transcription elongation by *E. coli* RNA polymerase (RNAP) were studied in the presence of a variety of polymeric, macromolecular crowders.

The tethered particle motion (TPM) technique (Kovari, Yan et al. 2018, Qian, Collette et al. 2024) was used to monitor loop formation by the lac repressor protein (LacI) in a DNA template where two copies of the wild-type lac repressor binding site O1 were separated by ~400 base pairs (Figure 1). Dextran70 (Dx70, 70 kDa in molecular weight) at 2.5 and 5% weight fraction in solutions containing either 100 or 200 mM KCl, or Bovin Serum Albumin (BSA) at 5 and 10% fraction were used as crowders and introduced simultaneously to the lac repressor protein. PEG

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was not employed in these looping measurements because it caused sticking of the bead to the surface as previously reported, possibly due to collapse of the DNA tether (Lin, Wuite et al. 2020). When LacI was introduced in the microchamber in a solution containing the crowder, looping was observed as a reversible shortening of the DNA tether. Both the parallel and antiparallel DNA looped conformations were observed (Figure 1A in (Yan, Leng et al. 2018) and (Virnik, Lyubchenko et al. 2003, Semsey, Tolstorukov et al. 2004, Semsey, Virnik et al. 2005, Lia 2008, Olson, Grosner et al. 2013)) and the probability of each was calculated as described in Materials and Methods. Figure 3 shows that while the overall probability of looping was unchanged in different crowding conditions (unlooped percentages remained constant), anti-parallel looping decreased as parallel looping increased in crowded conditions.

The effect of macromolecular crowders on the rate of transcript elongation was probed using PEG of two molecular weights (2 and 8 kDa: PEG2000 and PEG8000) in the magnetic tweezers where a gentle tension was applied to the DNA (Smith, Finzi et al. 1992, Strick, Allemand et al. 1998, Neuman and Nagy 2008, Seol and Neuman 2011, Seol and Neuman 2013, Kovari, Dunlap et al. 2019). This approach allowed monitoring transcription by RNAP in real time with better signal-to-noise levels avoiding DNA compaction. The top left panel in Figure 4 shows that increasing the concentration and molecular weight of PEG slowed down the rate of transcript elongation with the exception of 15% PEG2000.

The transcription traces also revealed that macromolecular crowding altered the probability of termination versus “run through” (Figure 4 bottom), as well as that of transcription “recycling” (Harden, Herlambang et al. 2020, Kang, Ha et al. 2020, Qian, Wang et al. 2024) (Figure 4 top right and bottom).

Discussion

The cell is an environment crowded with small and macro molecules where many processes take place simultaneously and, often, in competition. This limits the reach of in vivo experimentation to understand the structure, function and physico-chemical properties of biomolecules in their native environment. On the other hand, the behavior of molecules and their biochemical interactions can be dissected with extreme precision in saline solutions in vitro. To bridge the informational gap between in vitro and in vivo measurements and enhance the relevance to the cellular scenario of the knowledge gathered from in vitro experiments, we monitored the dynamics of two processes, which are fundamental to the life of the cell: (i) protein-mediated DNA looping and (ii) DNA transcription, in crowded environments.

DNA looping by proteins which can bridge two, or multiple, sites is a broadly recognized, fundamental mechanism of regulation in almost all genomic transactions, such as DNA transcription, replication, repair, recombination, etc. A loop may form in either of two conformations, the parallel or the antiparallel, depending on the relative orientation of the DNA double helix going in and out of the loop closure point. The antiparallel conformation “consumes” more DNA than the parallel conformation and, perhaps, exposes the loop more to the external environment. On the other hand, a parallel loop has a solenoidal geometry which may favor more compact packing with long helical fibers (Bancaud, Silva et al. 2006). Our finding that crowding the solution with either of two different polymers, Dx70 and BSA at two different salt concentrations (for Dx70), favors one loop conformation versus the other (Figure 3) suggests that modulation between the two conformations may be attained with local variations in crowding fraction alone, independently of salt concentration, or even crowder chemo-physical properties.

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Although these results are preliminary and more crowders and ionic strengths should be explored, these results indicate that the amount of crowding of the local environment may have important regulatory consequences, controlling accessibility *vs*. compaction of DNA in the genome.

The effect of crowding on DNA transactions, such as transcription and its regulation could, in principle, be also important. Our measurements do not inform on possible effects on transcription initiation but do show changes in transcription rate and the possibility of effects on transcription recycling and the probability of run off transcription. Higher molecular weight PEG showed stronger effects than lower molecular weights, indicating that crowder size plays a role. A crowding-induced decrease in the rate of elongation may allow for check-point and repair mechanisms to take place. The effect of crowding by PEG on transcription recycling and termination remains to be further investigated with other macromolecular crowders.

In summary, the measurements reported here show that local crowding variations could have significant regulatory effects on several DNA transactions and that the field is primed for investigation.

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Author contributions

D.C. performed the TPM LacI-induced DNA looping measurements, J.Q. analyzed them and made figure 3, W.X. run the magnetic tweezers experiments in the presence of PEG, analyzed the data and made the panels in Figure 4. D.D. designed the plasmids and led the project with L.F. L.F. conceived and co-led the project. All participated in the writing of the manuscript.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure 1

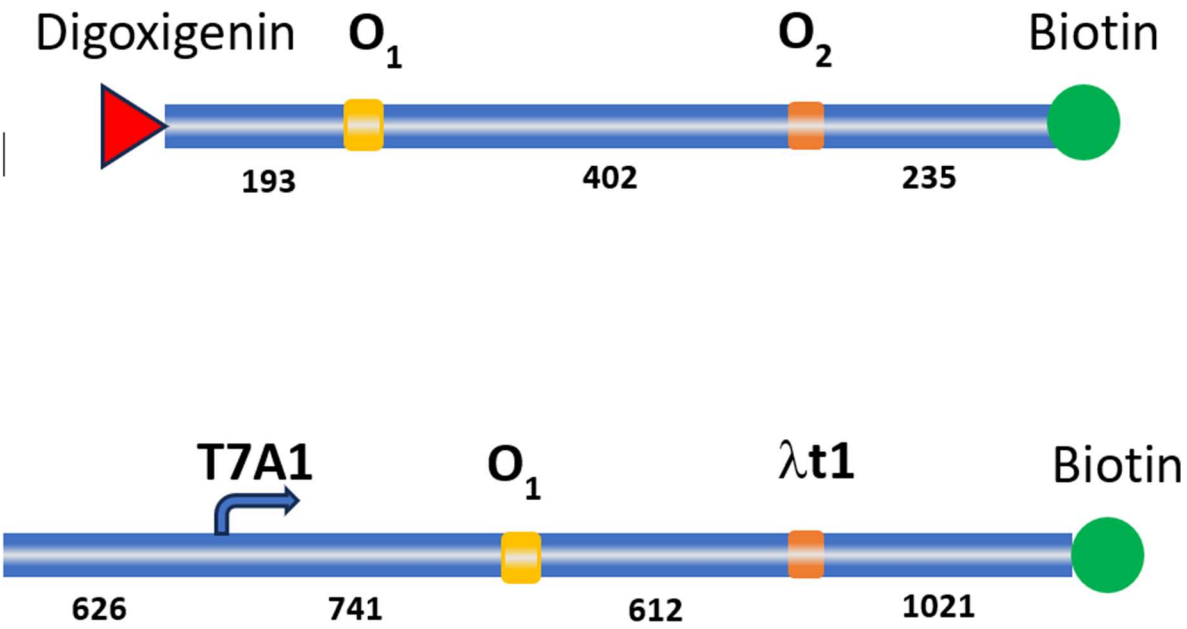

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Figure 2

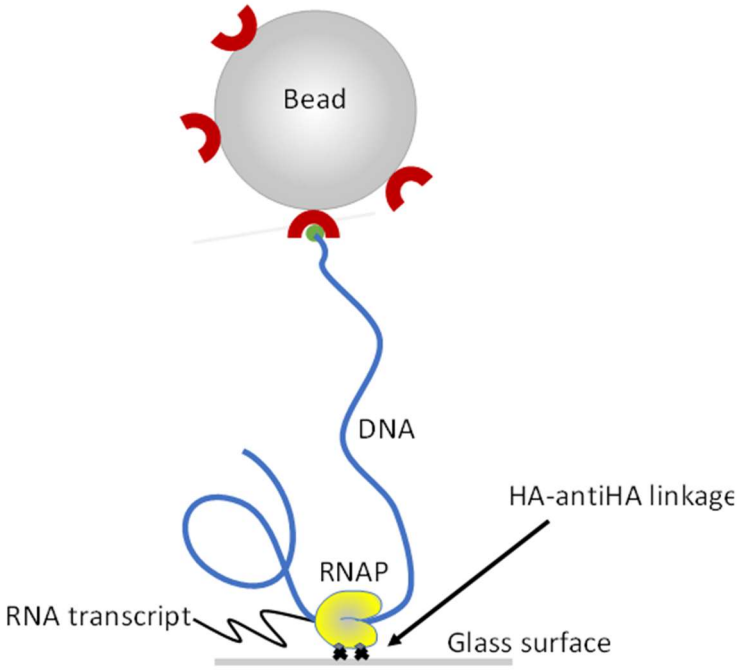

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Figure 3

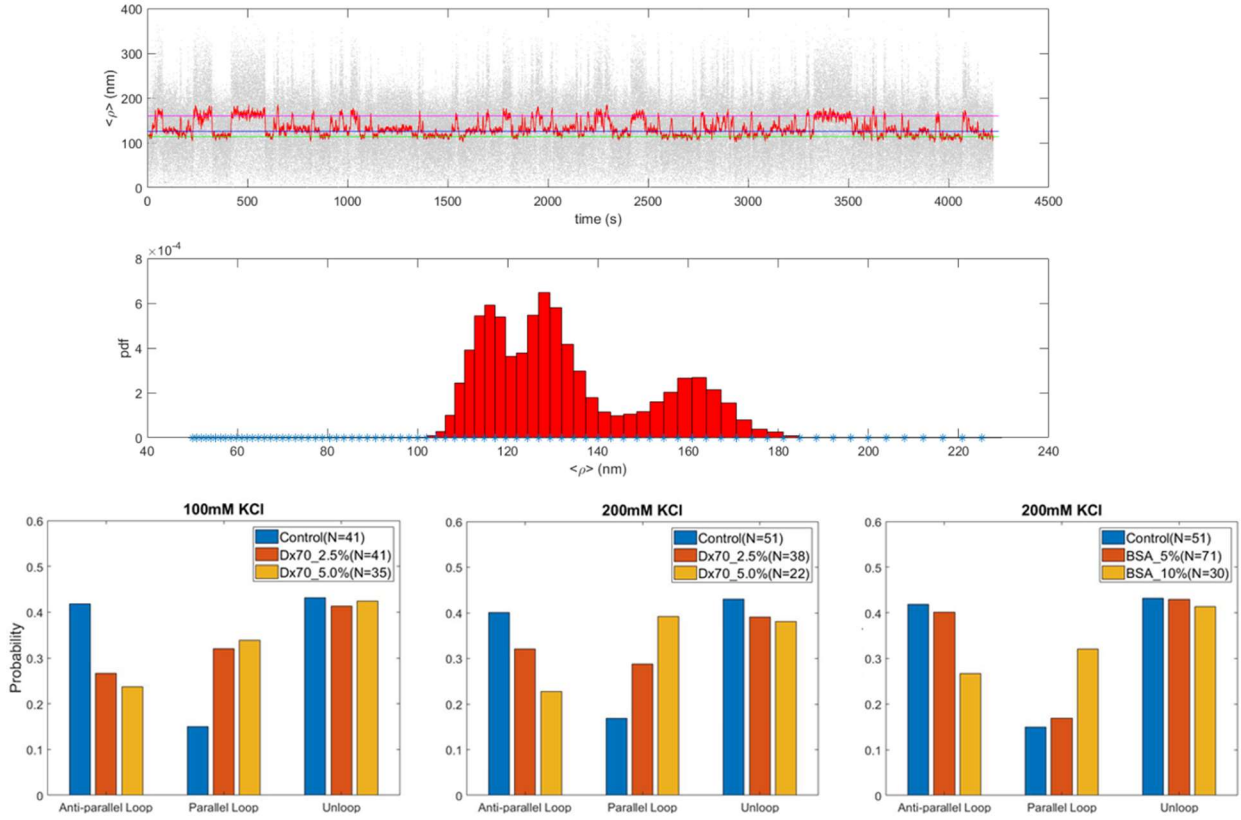

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Figure 4

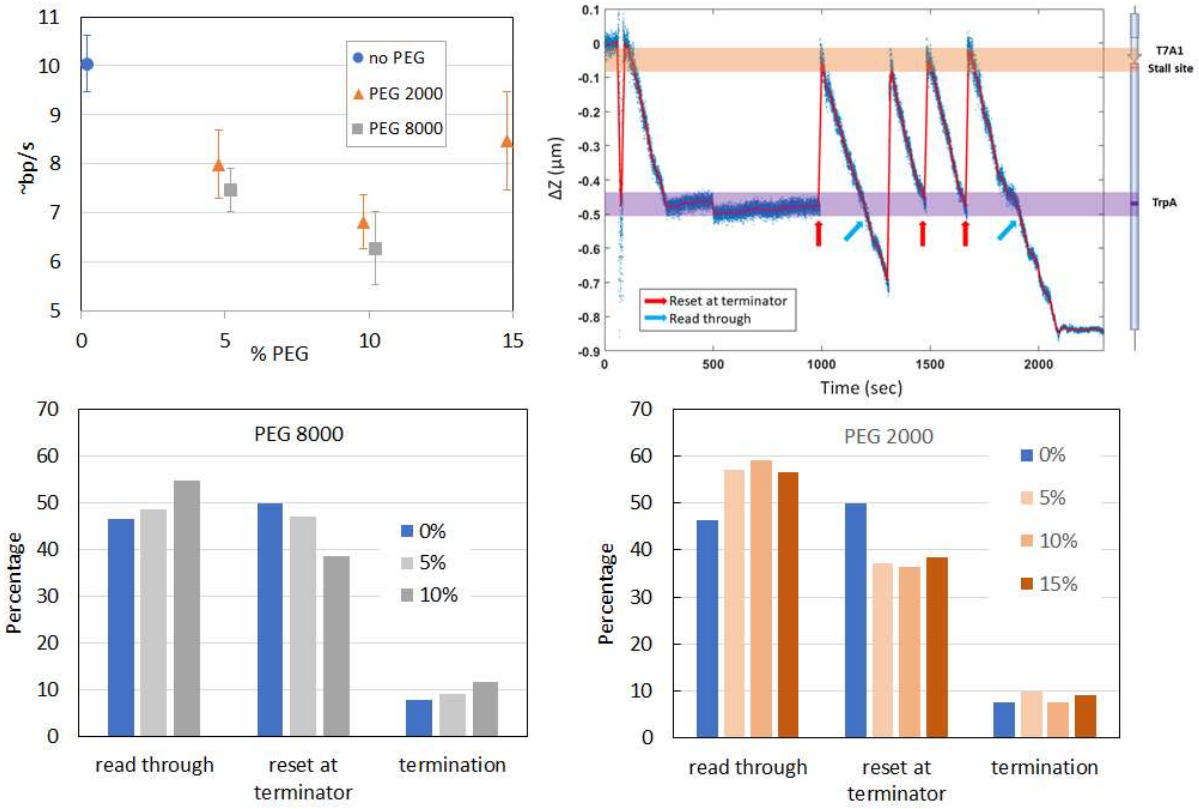

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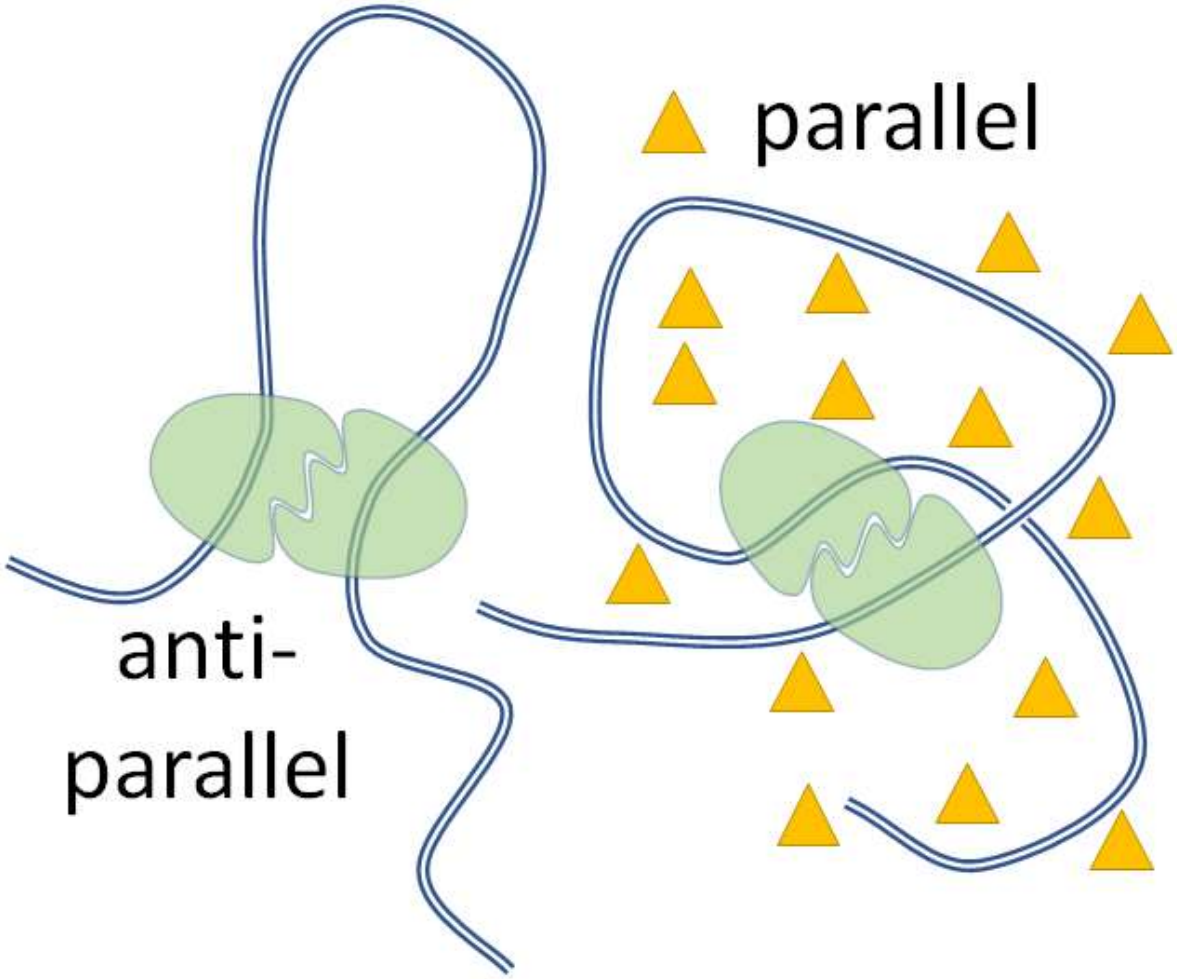
Figure 5



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