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Parallelized DNA tethered bead measurements to scrutinize DNA mechanical structure

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Abstract

Tethering beads to DNA offers a panel of single molecule techniques for the refined analysis of the conformational dynamics of DNA and the elucidation of the mechanisms of enzyme activity. Recent developments include the massive parallelization of these techniques achieved by the fabrication of dedicated nanoarrays by soft nanolithography. We focus here on two of these techniques: the Tethered Particle motion and Magnetic Tweezers allowing analysis of the behavior of individual DNA molecules in the absence of force and under the application of a force and/or a torque, respectively. We introduce the experimental protocols for the parallelization and discuss the benefits already gained, and to come, for these single molecule investigations.

Highlights

- Single molecules techniques reach their full potential when parallelized
- Tethered Particle Motion and Magnetic Tweezers are candidates for parallelization
- The “free” motion of DNA tethered beads informs on DNA conformational changes
- Applying traction force or torque gives access to the mechanical action of enzymes
- Nanoarrays are affordable and easy to fabricate using microcontact printing

Keywords

Tethered Particle Motion; Magnetic Tweezers; high-throughput single-molecule techniques; microcontact printing; DNA conformational changes; dynamic activity of DNA enzymes.

1. Introduction

“Single molecule but multiple experiments” was the introduction of K. Kinosita’s keynote conference at the first Single Molecule international meeting in 1999 in Tours (France). In these early days of single molecules imaging, obtaining statistically significant measurements required multiple repeats of the observations, which was extremely time consuming. As quoted further by Kinosita, “having single molecule data to present was rewarding for lab heads but a nightmare to obtain for their students or collaborators”. While the visualization of fluorescently labeled individual single DNA molecules opened the route towards direct observation of DNA conformations [1], it was followed by the emergence of a variety of techniques that allow a more precise characterization of its mechanical structure, in the absence of labeling of the DNA filament itself. Those techniques are based on the observation of beads tethered to DNA molecules immobilized on glass slides by the opposite end. Molecules can be dsDNA, ssDNA, DNA hairpins, Holliday junctions; they have to be end modified, usually by biotin and digoxigenin moieties. Beads, and substrate, have to be functionalized with either neutravidin or anti-digoxigenin, accordingly. Optical videomicroscopy coupled to image analysis is used to detect and track the beads with a nanometer-scale precision. In the absence of an external force applied, it is then referred to Tethered Particle Motion (TPM), the bead is submitted to the sole effective force of repulsion due to a volume exclusion effect of only a few fN [2]. The bead is thus considered to freely explore a half-sphere around its anchoring point following the statistical fluctuations of the DNA molecule. TPM analysis consists of the measurement of the amplitude of motion of the bead. Initially determined as the size of the blurred image of the bead [3], it is now usually and more reliably measured from the bead’s trajectory as the standard deviation of the distribution of successive positions of the bead in a given time interval [4]. From the bead’s amplitude of motion, the DNA end-to-end distance can be extracted with a great precision, depending on the bead size and temporal resolution, by a careful treatment of the data explicated in section 3. This simple but powerful technique allows studying biological processes such as transcription or recombination involving a change in the DNA conformation. Notably, the TPM analysis of DNA looping provided major contributions to the full understanding of the regulation of gene expression by Lac repressor [5,6]. As illustrated in this chapter, fundamental questions can also be addressed regarding the physical properties of the DNA molecule such as the influence of temperature or of particular sequences.

Magnetic Tweezers (MT) experiments refer to the use of superparamagnetic beads to apply controlled traction force and torque to DNA molecules by tuning the position and rotation of external magnets. The force spectroscopy analysis relies on the “direct” measurement of the end-to-end distance given by the z position of the bead as a function of the applied force deduced from the x-y position fluctuations. MT give access to elastic properties of the DNA, and offer a mean to unveil the sensitivity of DNA enzymes activity to topology by applying torque [7,8]. MT, among other well established force spectroscopy methods such as Atomic Force Microscopy (AFM), Optical Tweezers and Biomembrane Force Probe (BFP), are more prone to efficient parallelization [9]. DNA tethered bead motion experiments under shear flow were also successfully parallelized but are less precise [10,11]. More recently, resonant acoustic waves proved to be applicable to hundreds of DNA tethered beads in parallel paving the way to a new generation of tool [12].

Recent developments in these tethered particle based techniques include parallelization, which is mandatory to reach their full potential. In this chapter, we first describe and discuss the respective advantages and limitations of the nanolithography techniques that have been used to produce single DNA tethered beads nanoarrays and to successfully develop high-throughput TPM (htTPM) and high-throughput MT (htMT). The following sections provide highlighting examples of the unique

advantages of the massive parallelization of TPM and MT measurements and discuss also for the latter the specific situations for which parallelization would not be critical. Finally, we give a glance at the future technological developments that could enhance the capabilities of htTPM and htMT.

2. Design of DNA tethered bead arrays using nanolithography

At that time of the premises of single molecule approaches, the studies focused at observing the molecules one by one and most frequently one at a time. Molecules were preferably isolated on glass slide homogeneously coated with an anchoring molecule. In order to keep the molecules at distance one from another the concentration of the solution incubated in presence of the slide was quite low. For single DNA experiments using DNA tethered beads, the two extremities of the DNA molecules were functionalized by a biotin and a digoxigenin, respectively. Neutravidin was adsorbed on the glass slide to immobilize the biotinylated end of the DNA and the bead coupled to an antidigoxigenin antibody was tethered through the free digoxigenin end of the DNA, or vice versa. These remain the most common combinations of linkers to date.

Microscopy images were recorded on videotapes that were further digitized. The standard image analysis tools were limited to the treatment of a single bead at a time. They allowed establishing the blurred image over a time interval [3,13] or the position image after image [4], i.e. the trajectory, of a tethered bead and to determine its z position by comparison with reference images [14].

Rapidly, as softwares for image analysis progressed, it became accessible and easy to analyze multiple beads simultaneously and therefore to optimize data collection. Videos were most generally treated after their recording which is satisfactory for a majority of experimental situations but teams with coding skills could develop real-time analysis tools [14,15]. Increasing the bead density on the video images was achieved by two different manners. First, the density of the beads on the microscope slides was raised by incubation with higher concentrations of DNA/bead complexes or DNA molecules prior addition of the beads. The need to avoid interferences between DNA molecules and between the beads themselves imposes a limit to the surface density of tethered bead density. Second, an additional gain can be expected from an enlargement of the field of view either by using larger camera detectors or smaller objective magnification. In both cases, the pixel size, which dictates the precision of the bead tracking, imposes a limit to the extension of the observed field. In the various DNA tethered bead approaches and whatever the method used for the determination of the x,y position of the bead, i.e. center of mass of the bead image, Gaussian fit of the intensity profile or cross-correlation with a reference image, the larger the number of pixels covered by the bead image is, the better is the precision on the bead's localization. Optimal image magnification to increase the resolution is however limited by the number of photons and background noise, and corresponds for most cases to a standard deviation of the point spread function about equal to the pixel size [16]. As a general rule of thumb, one needs to reach a compromise to have enough pixels for one bead for a good precision in localization, but not too many to keep enough photons per pixel for detection and keeping enough beads for parallelization.

Tethered Particle Motion experiments performed on homogeneously functionalized substrates [5,17–21] report a maximum of one hundred of valid beads observed simultaneously. This allows accelerating the data acquisition but remains insufficient to identify rare events, reporting on multiple co-existing populations and properly averaging the molecular details of the experiment[22].

In the case of magnetic tweezers experiments the beads need to be kept apart by a larger minimal distance than for TPM to neglect bead-bead interactions induced by the magnetic field. Accordingly,

Ribeck and Saleh [23] reported the manipulation and measurement of 34 beads in parallel, the positions of which they could track in real time. This score was surpassed by Crossen et al in 2014 [24] with more than 200 beads tracked simultaneously at about 60 Hz thanks to a 12 M pixel camera and a tracking software based on a parallel computing framework.

For a massive parallelization, i.e. a thousand of beads per field of view, the packing density had to be further increased. The best way to optimize the spacing between individual beads is to immobilize the DNA molecules on regular arrays of anchoring spots. Various nano or micro-lithography techniques can be implemented to realize such substrate patterning.

The first method reported in the literature is the deposition of gold which can be easily patterned at the nanometer scale by physical vapor deposition through a shadow mask [25]. Gold-thiol chemistry allowed the conjugation to the gold nanoposts (100 – 400 nm in diameter) of multiply thiolated DNA at one end, the other end being biotinylated and further attached to streptavidin beads. This procedure, followed by passivation steps, proved to be robust, as demonstrated by force spectroscopy measurement performed using optical-trapping microscopy. Although promising, these chemistry and patterning approaches have not spread widely. Apart from cost considerations, several limitations can be responsible for this. Masks can be obtained from technological facilities, reused multiple times and stored for long periods in the labs, but a direct access to a deposition machine is preferable since the anchoring of the thiolated-DNA on the gold nanoposts must be accomplished immediately after deposition. The occupancy rate is limited to hardly more than 10% of the posts in conditions that ensure a single molecule anchoring. Depending on their size, a minimum is necessary to optimize the occupancy of the array, the metallic deposits may not be compatible with the tracking of sub-micrometer sized tethered beads by optical microscopy. A variant of the method was designed to allow the application of a dielectrophoretic force to the tethered bead. Micrometer sized wells are patterned with a resin photoresist using photolithography on a continuous 130 nm gold coating that is consecutively chemically etched in the bottom of the wells before a new thin optically transparent gold layer is deposited and the DNA added, followed by a passivation treatment [26]. With successive deposition steps and chemical treatments, this solution hardly meets the quest for an efficient but as simple as possible solution.

The so-called soft lithography techniques bring responses to these issues [27]. Among those, microcontact printing, appropriate for the fabrication of microfluidic systems with applications in biotechnology, is extremely attractive because of its simplicity and affordability. Several microcontact printing techniques have been developed but here we will limit ourselves to the introduction of the variants involved in the sole two methods that allowed to really attain high-throughput (see fig 1A and B). Microcontact printing uses an elastomeric stamp, made of polydimethyl siloxane (PDMS) typically, and decorated by a pattern of anchoring molecules. In the patterning by subtraction method, a flat pre-cured PDMS is first inked with the anchoring molecule. After conformal contact with a silicon template patterned with a negative image of the desired features, the positive image of the features of anchoring molecules remain on the PDMS and are ultimately transferred on a glass slide by a second conformal contact step. Using this method for the parallelization of magnetic tweezers experiments, Vlaminck et al [28] fabricated regular hexagonal arrays of antidigoxigenin square patches of size down to 200 nm. With a field of view of 300 μm x 400 μm they could observe nearly 1000 sites of the array yielding statistical data for about 350 DNA molecules. Plénat, Tardin et al [29] proceeded by standard microcontact printing where the PDMS has a surface structured with relief features obtained by casting the elastomer against a silicon master with the complementary features. The surface of the PDMS stamp is inked with neutravidin and conformal contact with a substrate leads to the transfer of neutravidin features reproducing the design of the pattern of the

silicon master. Neutravidin patterns enable the anchoring of DNA molecules through their biotinylated end. The reverse procedure works also fine with the patterning of antidioxigenin to immobilize DNA through the digoxigenin moiety. In this procedure the silicon master can be reused extensively (more than 50 times) without having to proceed to a particular intermediate treatment or cleaning, which is a clear advantage compared to the subtraction method. Care must be taken, however, in this standard microcontact printing procedure, to the design of the PDMS stamp in order to avoid its collapse during the conformal contact with the glass slide. Optimal aspect ratios between the dimensions of the structures must be respected[30]. Fortunately, they are compatible with pre-requisites for TPM and MT experiments (see fig 1C). With arrays of 800 nm sized square features and 3 μm pitch, Plénat, Tardin et al cover about 2000 sites in a field of view of 150 μm x 125 μm which offer after the application of selection criteria between 500 and 1000 beads of 300 nm diameter tethered to 500 - 2500 bp DNA valid for TPM analysis (see fig 1D).

With a view to produce large series of arrays, the standard method seems to be more suitable in particular because the whole process starting from the inking of the stamp to print can be automated thanks to a dedicated machine [31].

When various geometries of arrays have to be tested, microcontact printing suffers from the need of an important initial investment in terms of time and budget for the mask fabrication and is not suitable as part of a trial-and-error approach. For such needs, a novel photolithography method called Light-induced molecular adsorption of proteins (LIMAP)[32] which allows for sub-micrometer-resolution printing of biomolecules should be considered. Based on this method, a commercial device to be docked on an optical microscope has been developed for custom micropatterning without mask or contact [33].

After patterning, the biochips are sealed to bare glass slides with holes via spacers defining the channels of a fluidic chamber. Before introducing the pre-formed DNA-bead complexes, a critical step is required to prevent non-specific interactions between not only DNA and the non printed areas which occupy a vast majority of the surface but also between beads and the whole surface of the substrate. In the context of patterning by microcontact printing, several procedures have been successfully applied. Two of them proceed by a treatment after assembling the fluidic chamber. The simplest one uses a global passivation of the chamber by the incubation with a solution of blocking agents [29]. The other one [28] is based on the formation of an inert lipid bilayer on the bare surface areas by incubation with a suspension of small unilamellar lipid vesicles. Full coverage of the bare surface of the glass slide with bilayer should be proof checked carefully. A third approach consists of the pegylation of the glass slide with a mixture of PEG/PEG-biotin before the print of neutravidin [34]. A notable drawback of the two last methods is the additional time of implementation.

3. TPM a zero force method to investigate global and local changes in DNA conformation

On average, the double-stranded DNA (dsDNA) assumes the structure of a B-form double helix; however, some distortions of this ideal right-handed double helix have been observed locally for some naked dsDNA [35] as well as for some specific sequences when bound to proteins such as the transcription factors [36]. These local distortions include bends, kinks, G-quadruplex, B-to-Z transitions but also changes of dsDNA rigidity. These local distortions of dsDNA appear important to detect and characterize as they are very likely to be part of an indirect recognition process of specific sequences by proteins based on the DNA shape [37,38], which can affect any biological processes occurring in the chromosome. To achieve this goal with high precision, the tools of structural biophysics are usually employed as well as single molecule approaches such as Fluorescence

Resonance Energy Transfer and AFM [39]. Nevertheless, these techniques are highly demanding in terms of technology and time, and, in addition, constrain the DNA sample to severe experimental conditions such as crystalline organization or immobilization onto a surface. As previously explained, TPM alleviates these conditions of observation and makes it possible to characterize a dsDNA molecule submitted to a few fN force only. Thus, the large number of measurements obtained on the highly parallelized TPM biochip enabled us to quantify both local defects present at the core of DNA molecules as well as weak changes occurring globally on the molecule. The challenging issue in these two cases arose from the fact that the induced modifications in the amplitude of motion of DNA tethered particle measured by TPM could be extremely small, down to a few nm. To ensure the robustness of our final characterization, we first took advantage of the high throughput capacity of our biochip coupled to the large field of view acquired with a Dark-Field microscope. In practice, we perform the measurements on three different fluidic chambers containing each at least 300 tethered particles with 2D trajectories that were considered to be valid on the basis of their asymmetry factor (see fig 2). Secondly, the DNA modifications under study affect the entire DNA population; thus the distribution of the amplitudes of motion defined as $R_{\parallel\text{exp}} = \sqrt{\langle \vec{R}_{\parallel\text{exp}} \rangle}$ with $\vec{R}_{\parallel\text{exp}}$ calculated as the 2D-vector positions of the bead relative to the anchoring point of the DNA, which corrects for experimental drift of the tracked particles measured over time, is expected to form a normal distribution characterized by the mean $\langle R_{\parallel\text{exp}} \rangle$ and the standard deviation σ . We filtered out the tracked particles exhibiting an individual mean amplitude of motion out of the interval $[\langle R_{\parallel\text{exp}} \rangle - 2.5 \sigma ; \langle R_{\parallel\text{exp}} \rangle + 2.5 \sigma]$, which would correspond to 1.2 % of a normal distribution (see fig 2). Then, we applied a second round of similar filtering on the relaxation time of tethered particle. These abnormal cases, corresponding typically to $\sim 20\%$ of the supposedly valid tethered particles, could be due to aberrant DNA lengths, despite the selection and purification of the molecules by gel electrophoresis, or to the locally imperfect passivation of the chamber surfaces. Finally, the mean amplitude of motion was calculated as the arithmetic average of the mean amplitude of motion of each individual tethered particles and the uncertainty on the measurements is evaluated by calculating the standard deviation of means of 1000 distributions of amplitude of motion obtained by a bootstrap procedure (R software). This procedure was applied to derive both the angle of local DNA bend as well as to determine the influence of temperature on the persistence length of DNA.

In previous works on the molecular mechanism of action of recombinases [40,41], we had noticed slight changes induced by the binding of the proteins to a single interaction site present in a 2 kbp DNA, which we attributed to local protein-induced bends. To check this hypothesis and demonstrate the capacity of htTPM for detecting and quantifying local bends in DNA molecules almost free in solution, we extended the strategy of Rivetti et al [42] used to evaluate bend angles by AFM. Our method is directly applicable for the characterization of any intrinsic or protein-induced bend. It requires DNA molecules of ~ 600 bp with the bent sequence site located at mid-length, as a trade-off between a high impact on the DNA end-to-end distance and a correct TPM sensitivity. We showed that, when particles with a radius $R_p = 150$ nm with DNA molecules of $L = 600$ bp, the end-to-end distance of a DNA molecule, with a bend of angle θ localized at the l position of the contour length, can be correctly extracted using the basic formula $\langle R_{\text{DNA}} \rangle = \sqrt{\left[\frac{3}{2} \langle R_{\parallel}^2 \rangle - R_p^2 \right]}$. In this case, the dependence of R_{DNA} as a function of the bend angle is correctly described by the kinked worm-like-chain model leading to the following analytical formula where $L_p \approx 150$ bp is the persistence length:

$$\langle R_{\text{DNA}}(\theta) \rangle = \sqrt{2 L_p^2 \left[\frac{L}{L_p} - 2 + e^{-\frac{l}{L_p}} + e^{-\frac{L-l}{L_p}} + \cos(\theta) \left(1 - e^{-\frac{l}{L_p}} - e^{-\frac{L-l}{L_p}} + e^{-\frac{L}{L_p}} \right) \right]}$$

All the details about the establishment of these formula are given in the publication written by Destainville et al. at the end of this book. In the case of large bending angles ($> 33^\circ$), only two DNA

fragments with and without the bend can be enough. Nevertheless, the characterization will be more precise if the measurements are performed on a series of molecules with in-phase assembly of the identical bend. To do so, we designed a family of plasmids (pTOCx) derived from pBR322 with a replaceable 88 bp core [43,44]. We incorporated in-phase or out-of-phase assemblies of the intrinsic bends CA6CGG and showed that the angle of this intrinsic DNA bend free in solution would be equal to $\theta = 15^\circ \pm 2^\circ$ in good agreement with the results obtained by classical tools [34]. In fact, describing our entire 575 bp long DNA molecule as a worm-like chain polymer with a single local bend is not fully satisfactory. The publication written by Destainville et al. details the additional parameters that can be added to refine the modeling of the DNA.

The same method extracts the persistence length, L_{pb} , of a local insert of contour length b on the basis of a derivation of the worm-like chain model that leads to the following equation:

$$\langle R_{DNA}(L_{pb}) \rangle = 2 L_p^2 \left[\frac{L-b}{L_p} + e^{-\frac{1}{L_p}} + e^{-\frac{L-b-1}{L_p}} - 2 + e^{-\frac{b}{L_{pb}}} \left(1 - e^{-\frac{1}{L_p}} \right) \left(1 - e^{-\frac{L-b-1}{L_p}} \right) \right. \\ \left. + 2 L_p L_{pb} \left(1 - e^{-\frac{b}{L_{pb}}} \right) \left(2 - e^{-\frac{1}{L_p}} - e^{-\frac{L-b-1}{L_p}} \right) \right] + 2 L_{pb}^2 \left(\frac{b}{L_{pb}} - 1 + e^{-\frac{b}{L_{pb}}} \right)$$

This method and more generally TPM measurements are thus sensitive to a local loss in DNA rigidity. We thus strived for detecting the stable DNA bubbles that were suspected to appear in growing number with the increase in temperature, but in vain although a single 3 bp long permanent DNA bubble was expected to reduce the amplitude of motion by a significant value of 3 nm. This study was only possible because we could track simultaneously a large number of DNA tethered particles, as temperature tends to destabilize the weak interactions linking DNA with particles and the coverslip, in our conditions. Reaching high precision requires not only high throughput capacity but also the appropriate correction to suppress the video averaging effect from the measured amplitude of motion [45]. This phenomenon usually biases the measured value of amplitude of motion as it depends on $T_{\text{exposure}}/\tau_{\text{relaxation}}$ with the duration of the camera exposure $T_{\text{exposure}} \approx 40$ ms and $\tau_{\text{relaxation}}$ ranging from 1 to 40 ms typically, theoretical development are given in the publication written by Destainville et al. at the end of this book [46]. It all particularly leads to dramatic biases when the relaxation time of the tethered particles changes as it is the case when the buffer viscosity is altered by temperature variation and when exact physical parameters such as the persistence length are sought. The precision brought by the large number of TPM measurements and the video-averaging correction is essential to give access to unbiased experimental amplitude of motion, which can be compared to simulated values allowing the extraction of the DNA persistence length in particular. Thanks to this instrumental and computational arsenal, we could measure precisely the influence of the ion concentrations on the persistence length and determine the appropriate physical model describing it [47,48]. Obviously, all this procedure will help in getting accurate data but requires that the biochemical conditions to study the nucleoproteins complexes are under strict control [48].

Our htTPM acquisition and analysis is also a valuable tool to dissect the dynamic molecular mechanism induced by the presence of proteins, in particular enzymes, as it permits to test many conditions in a reasonable time. In these types of samples, the recording is performed on a single field of observation and starts before the injection of the proteins in order to select the DNA molecules with an initial amplitude of motion comprised in the distribution of the naked DNA as in the first filtering step described above. We explored the mechanisms of looping and recombination between two *dif* sites induced by the XerC and XerD recombinases, which ensures the resolution of chromosome dimers in *E.coli*, and is implicated in the integration of mobile genetic elements in other prokaryotic chromosomes. An impairment of this looping step was thought to be responsible for the

surprising persistence of the pathogenic island in the *Neisseria Gonorrhoea* genome. Within a few months only, we could analyze series of thousands of tethered DNA molecules and observe that the assembly of a DNA synapse by the recombinase XerCD was still possible between *dif* sites located at the borders of the pathogenic island [22].

4. Stretching and twisting DNA using MT to unveil replication

Mechanical constraints are now accepted as a useful tool to probe biomolecular structure, interactions or activity. If this was obvious in the case of molecular motors involved in the movement in living organisms, it was less natural in the case of nucleic acid or protein structure. Nevertheless new knowledge could be obtained on the folding mechanisms of proteins, RNA, or DNA structure under torsional or elongation constraints for example [49].

Different techniques have been used in different contexts: AFM, Optical Tweezers, BFP, Acoustic Force Spectroscopy (AFS) and MT. They all have different characteristics (force/torque sensitivity, bandwidth, force range) but at the simplest level they can be divided in two categories: they are constant force or constant extension apparatus. In the first case, the force is imposed and the biomolecule extension adapts to the constraint, this is the case of MT or AFS if the force gradient is not too high. In the second configuration, the extension of the molecule is imposed and the force varies to keep the extension constant. This is typically the case of AFM or optical tweezers. One may switch between the two configurations by using a feedback loop, but this is at the cost of a reduction of the temporal bandwidth.

Another parameter in the choice of an experimental technique is the achievable parallelization. It may appear paradoxical to use intrinsic single molecule techniques and then require parallelization, but these are the only way to apply a controllable mechanical constraint with high detection sensitivity while throughput is important to get more statistics, more precision and, to detect rare events. This is important in the case of experiments using multicomponents where the requirement to have all individual components to be fully active at the same time is really restrictive.

Clearly AFM or BFP are not the best candidates when parallelization is required even if some efforts have been done in the case of AFM [52]. Optical tweezers may achieve multiple beads trapping [53–55]. As many as few hundreds of beads can be trapped, but in practice to be able to measure the force only few objects can be used. In that sense MT and AFS are much more parallelizable than the other techniques. The magnetic field gradient can be constant over more than few hundreds of microns on the sample as well as the acoustic wave that can impose the same radiation pressure on a large portion of the sample. If the particles used to manipulate the biomolecules are homogeneous this results in a relatively similar force (typically variation of less than 10 % on Myone™ beads on a field of view) on many beads[56,57].

An important parameter for the parallelization is thus the material of the beads. In the case of MT the beads used are superparamagnetic which implies magnetic interactions between beads in addition to, the always present, Van der Waals or electrostatic interactions. These magnetic interactions preclude a very high density as typically for beads of one micron diameter, a typical distance of 5 μm , seems to be a lower limit. This number might seem large enough (on a 250 μm x 250 μm field of view it allows studying 2500 beads in parallel) but in fact to fill all the spots on the surface treated to bind a bead, one has to incubate at a higher concentration than on a homogeneous surface. And this favors non-specific interactions between beads that play a larger role

than with non-magnetic beads. This is why until now in almost all the cases parallelization with magnetic tweezers was achieved mostly without arrays. Coverage is not as dense as it could be with an array system, but nevertheless hundreds of beads can be analyzed in parallel using a magnification allowing a few nanometer resolution [58]. In the case of enzymatic activity where monitoring the state of the biomolecule can be important to modify in real time the experimental parameters it is important to acquire and partially treat the data in real time. This adds an additional constraint. The data can be acquired in real time even for a large number of beads thanks to GPU despite a clear need of computational power as the computation is the same for all beads so it can be parallelized[24,59].

By definition the yield of single molecule experiments is not high and when multicomponents and complex systems are studied *in vitro* the ratio of desired events over observed molecules falls down sometimes dramatically. Without parallelization, obtaining enough data would sometimes not be possible. We will illustrate the interest of micromanipulations on DNA or RNA tethered beads with different examples and determine when parallelization is important.

The essential components of DNA replication are the replicative DNA polymerases. Due to different mechanical responses under tension of single stranded DNA (ssDNA) compared to double stranded DNA (dsDNA), it is possible to monitor in real time the replication of DNA at different forces, excepted the one, typically 5pN, where ssDNA and dsDNA have the same extension. Above this force, when a polymerase replicates one base, as dsDNA is shorter than ssDNA, the bead used both for applying a force on the DNA and measure DNA extension allows the determination of the number of bases replicated. Speed can be obtained by measuring the local slope of the curve extension vs time (up to 200 bps⁻¹ for T7 DNA polymerase at ~ 6 pN [60]). Pauses can be detected as constant extension periods. From the elastic response of ssDNA and dsDNA, one can determine, by a linear relation, at a given force, the change in extension in nanometers when a polymerase does replicate 1 base of ssDNA to create 1 bp of dsDNA, with typically ~ 0.1nm/bp. The final speed is thus in bp/s. Models have been proposed to interpret the dependence of the speed as function of the force as the signature of the number of bases adjusted by the finger to accommodate the template dsDNA [60,61]. At forces around 30 pN the polymerase could be stopped by the force. Surprisingly at higher force (~ 40 pN) a processive exonuclease activity was detected, likely because DNA deformation induce the proofreading function of the DNA polymerase (see fig 3). This observation is not limited to T7 DNA polymerase as it was also observed with Φ 29 polymerase [62]. Observing a force-controlled enzymatic activity at 40pN requires to pull at high force for a relatively long time. Then the linkers to bead or surface (usually biotin/streptavidin or dig/antidig) have a large probability to break. Like in previous TPM examples without force, starting with a higher number of beads would increase the probability to observe an interesting event.

Not only DNA but also RNA can be manipulated. RNA replication of RNA dependent RNA polymerases could also be studied by Dulin et. al [63,64]. To obtain the general behavior of the enzyme (speed for example) parallelization is not absolutely necessary. Nevertheless parallelization is necessary to reveal rare events such as the kinetics of rare mutation incorporation. Analysis of many polymerization traces allowed the Dulin et. al to propose to attribute all the measured/determined characteristic times to specific polymerization events and in particular among the detected pauses, the longest and rarest ones to mutation incorporation events. Their occurrence is indeed related to the average mutation rates detected in bulk measurements. Data accumulation was possible thanks to parallelization but also because the traces could be obtained with the same experimental parameters on all the beads (same force).

More recently another experiment using force revealed an unexpected exonuclease activity for some DNA polymerases (T4 and T7) in what looks like a more natural configuration [65]. In a DNA hairpin configuration, one pulls on a ssDNA but its sequence is partially palindromic so that at low force (below 15 pN) it forms a hairpin (see fig 3). With a primer that may bind to the remaining ssDNA, a polymerase may start replication in the direction of the apex of the hairpin. If the polymerase has a strand displacement activity, it may replicate the DNA inside the hairpin. An additional positive element in this configuration is that for every base that opens at a force of about 10 pN the DNA increase in extension is about 1 nm which is large enough to almost detect every base replicated. This assay is then more sensitive than the previous one. Here again the replication rate is determined by the change of the extension with time. Interestingly the extension increases, as expected for a replication, but there are some pauses and, more surprisingly, events where the extension decreases, the signature of an exonuclease activity. The frequency of switching from one mode to the other depends strongly on the force. At forces above 8 pN the polymerase mainly performs strand displacement and DNA synthesis. At forces below 8 pN mainly exonuclease activity is observed. So the switch between polymerization and exonuclease is force controlled. Such a behavior can of course be observed easily only in a micromanipulation experiment. In this type of experiment, the force is not so high. With active and stable enzyme, the typical current parallelization (tens to hundreds of beads in magnetic tweezers) is sufficient to obtain enough results as the force is not very high (typically one can observe a good number of events from about ten molecules to about fifty). It is even not useful to have a high parallelization because it is important to monitor and have a different feedback on each bead independently in real time. In the case of the hairpin assay, when the replication goes further than apex, the replication is no more in a strand-displacement mode but in the primer extension mode. The behavior is then different. The intrinsic asynchronous activity on different molecules in single molecule experiment may thus lead to the observation of different behaviors despite the fact that the starting point is the same for all the molecules.

The same limitation in the parallelization is present in the case of topoisomerase studies. Topoisomerases are enzymes that modify DNA topology. They can transiently cut one, or two strands of DNA, modify DNA topology by relaxing or adding torsion on the DNA before religating the DNA [7]. Their action is a mechanical action. They directly play on torsion and, in addition, there is a coupling between force and torsion. Micromanipulation tools can thus provide important new information. In particular, for topoisomerases, tools that can impose a number of turns to the DNA like the magnetic tweezers or the one that can control the torque on DNA. In bulk experiments to generate DNA under torsion, one must purify it from an organism (it is usually under-twisted i.e. negatively supercoiled) or use topoisomerases or intercalators that modify the helical pitch. In single molecule micromanipulation techniques one simply has to start from relaxed DNA and use electric motors to rotate magnets [66], an electromagnet to rotate the magnetic field or use the polarisation of light [67] to rotate the bead that through an adequate linker, a multilinker in fact, induce torsion, torque, on the DNA. As the majority of the topoisomerase relax torsion (it is not the case of the gyrase that can create negative supercoiling, and reverse gyrase that can create positive supercoiling), once the reaction has been completed the experiment is finished when performed in bulk. By contrast, using micromanipulation tools the experimentalist can regenerate a supercoiled substrate on which the enzymes can react again. As a bonus, as the generation of one turn can produce a change in DNA extension of about 40nm [66] it can be easily detected. Single enzymatic cycle on a single DNA molecule [7,8,68] can be observed, as well as the processivity of the enzymes. It has been recently possible to detect the insertion of individual topoisomerases in a DNA in real time [69].

Experimentalists then have a simple, but elegant, tool to investigate the action of drugs acting on topoisomerases like antitumor agents [70,71].

In these experiments one needs to work on a relatively large number of molecules because a non-negligible part of them are not sensitive to torsion due to a nick or improper attachment to the solid surfaces. In addition, as the topoisomerase activity events are not synchronized, when a relaxation event occurs on one molecule the experimentalist may regenerate new supercoils whereas other molecules are not yet relaxed. One has then to deal with a heterogeneous, in terms of supercoiling, population of molecules which is not easy to work with. In this particular case, parallelization may lead to difficulties in managing the experiment, but it will never be worse than without it as it is easier to reduce the number of molecules of interest than increasing the number of experiments.

In the case of DNA, another mechanical means to apply a force, which allows parallelization, has been successfully exploited. It uses a flow to stretch the molecules. The drag generated on the tethered beads by the flow dominates drag on DNA and drives the force acting on the DNA. DNA extension [10,72] is directly due to the drag on the bead. These techniques are not as versatile and precise regarding the force applied or the possibility to apply a torque like magnetic tweezers. In addition, it relies on a flow, so more biological material must be used to keep material concentration constant when DNA protein interactions are studied. These approaches are now easy to implement as stable pumps are available.

Many DNA interacting proteins have a mechanical action, even if they do not move, they often bend or twist DNA. Their action can thus be measured through DNA mechanical response. During replication, helicases separate the DNA strands. Some of them have been studied by single molecule micromanipulations [73] and likewise all components of the replication machinery can be studied separately but the goal is to try to get insight on the full replication machinery. Of course, the simplest systems are studied first. Nevertheless, this is a step towards more complexity. T7 phage has a simple replication machinery. It involves a helicase-primase, polymerases, and Single Strand Binding proteins (SSB). In order to monitor at the same time the synthesis of the leading and lagging strand, A. Van Oijen's group attached two beads at the extremities of a designed DNA molecule (see fig 4)[74]. One bead is attached at the extremity of the leading strand and the other at the one of the lagging strand. Leading and lagging strand can be distinguished thanks to different distances from the attachment point. A flow is used during the experiment in order to apply a drag on the beads and so a force on the two strands. The DNA molecule is designed so that when only replication of the leading strand synthesis is active the corresponding DNA extension increases. When the Okazaki fragment on the lagging strand is synthesized, the corresponding bead moves in the direction opposite to the flow. The leading strand attached bead has a net movement resulting from both synthesis. The movement of the two beads reveals the complex coordination of the replisome activity of both strands. This experiment shed light upon the plasticity of the replisome.

In this configuration, one may have access to a lot of information on a complex system but the cost is the very low rate of success of the construct. Duderstadt et al. had to observe tens of thousands beads in parallel in order to get data on the leading and lagging strand synthesis on a number of molecules varying between 53 and 535 [74,75]. With such a low yield one may see that, without parallelization and an efficient data analysis to extract the useful information, such observations would be impossible.

Note finally, that flow or electric field can also be applied without an attached bead [76–79] which may result in a less homogeneous stretching along the molecule as the force may depend on the position along DNA or distance from the surface [79].

5. Concluding remarks

Undoubtedly, the developments performed for parallelizing the TPM and MT techniques were worth the efforts. The advantages of high-throughput single molecule experiments are vast: an improved precision on the measurements due to the large number of simultaneous observations, a fast acquisition rate together with a sensitivity to a population heterogeneity or the occurrence of rare events, a robust method thanks to the rationalization of the microfluidic chamber preparation and the development of efficient protocols to limit non-specific interactions.

We foresee two major directions for future progress. First, true multiplexing, i.e. the simultaneous observation of a variety or similar molecules in various conditions, will drastically expand the data throughput and should be feasible by a smart coupling with microfluidics [80,81]. Second, as it has been already achieved for conventional MT experiments, the combination with fluorescence single molecule microscopy will provide additional information such as local conformational changes by FRET measurements [82–85].

Ten years after the first report of a single DNA tethered bead experiment on array, we must recognize that still only a very limited number of research groups have developed such methods. This is probably due to the belief that the technology is difficult to implement and training is long. The present article intended to provide an argument convincing the reader that actually the protocols are now quite robust and the necessary investment limited. We hope TPM and MT on arrays will become soon a routine tool of the biophysics and molecular biology laboratories.

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Figure Captions

Figure 1: Parallelization of TPM and MT experiments on nanoarrays.

- A. Fabrication of the arrays by microcontact printing. 1. Standard mode: 1a) an elastomeric stamp is molded on a silicon wafer with the inverse features, 1b) inking of the stamp by incubation of the stamp with the anchoring molecule solution followed by a drying step, 1c) the inked face of the stamp is put in contact with a clean and eventually pre-treated microscope glass slide, 1d) upon removal of the stamp, the anchoring molecules transferred from the stamp remain on the substrate. 2. Subtraction mode: 2a) a silicon template with a negative image of the desired pattern is applied to an inked planar elastomeric stamp, 2b) transfer of the anchoring molecules occur in the contact zones and the anchor patterns remain on the elastomer upon lifting the silicon, 2c) the elastomer is applied on a clean and eventually pre-treated microscope glass slide, 2d) the pattern of anchoring molecules is transferred to the substrate.
- B. Regular arrays of anchoring spots can be printed on mm^2 sized areas by the above methods chosen depending on the spot size and pitch desired.
- C. A minimum distance between nearest neighbors is required to avoid interferences between DNA and beads for TPM experiments and attraction between the magnetic beads for MT experiments.
- D. Preformed DNA-bead complexes self-assemble on the nanoarrays (dark-field microscopy imaging with a X51 magnification (see text))

Figure 2: Treatment of the data before analysis. After correction of the positions of the tethered particles by the drift of their anchoring point, those with symmetrical trajectories are selected if their symmetry factor $S < 1.35$. S is calculated as $S = \sqrt{(\lambda_{max}/\lambda_{min})}$ where λ_{max} and λ_{min} are the eigenvalues of the covariance matrix of the 2D corrected positions. Then the particles out of the normal distribution of the amplitude of motion $R_{//exp}$ and the relaxation time τ are discarded, and the video-averaging effect is corrected on the amplitude of motion. Finally, the mean amplitude of motion of the tethered particles are calculated and the uncertainty is obtained by bootstrap. All these steps permitted us to observe the decrease induce by the presence of increased number of bends assembled in phase (green circle) located at the center of a ~ 600 bp DNA molecule with a reduced uncertainty that is smaller than the marker size. This decrease disappears when the 4 intrinsic bends are assembled out of phase (black circle).

Figure 3: Enzymatic activity can be force controlled. Using optical tweezers A) Wuite et al observed that at high force T7 DNA polymerase can switch from primer extension activity, where DNA extension shortens, to exonuclease activity where bead to bead distance increase with time B). Adapted from [60]. Similarly on a DNA hairpin, the exonuclease activity can be switched on from a strand displacement activity on T4 and T7 DNA polymerases (C and D). Polymerase speed is positive when polymerizing and negative in the exonuclease mode G). Speed is measured thanks to the change in DNA extension. Around 8pN the speed is almost 0 as it is the critical force where the polymerases continuously switch from one mode to the other. Increasing the force increase the polymerizing time and as a consequence the average speed becomes positive. Adapted from [65].

Figure 4: Duderstadt et al designed a construct with 2 beads attached to study T7 replisome A). The success rate of the construct, where different outcomes may happen (C), is low. Parallelization is needed to observe enough events. A low magnification microscope with a camera with 29 Mpixels are used to ensure the observation of a very large field of view B). To detect interesting events one must detect in the image, among about 20000 beads the ones with 2 close beads aligned by the flow. Their movement in the flow, in the direction of the flow for leading strand synthesis or in the opposite direction for loop synthesis provides information on the kinetics and coordination of the

synthesis of the two strands. These observations lead to the conclusion that the replisome has plasticity in its operation modes. Adapted from [74].

Figure 1

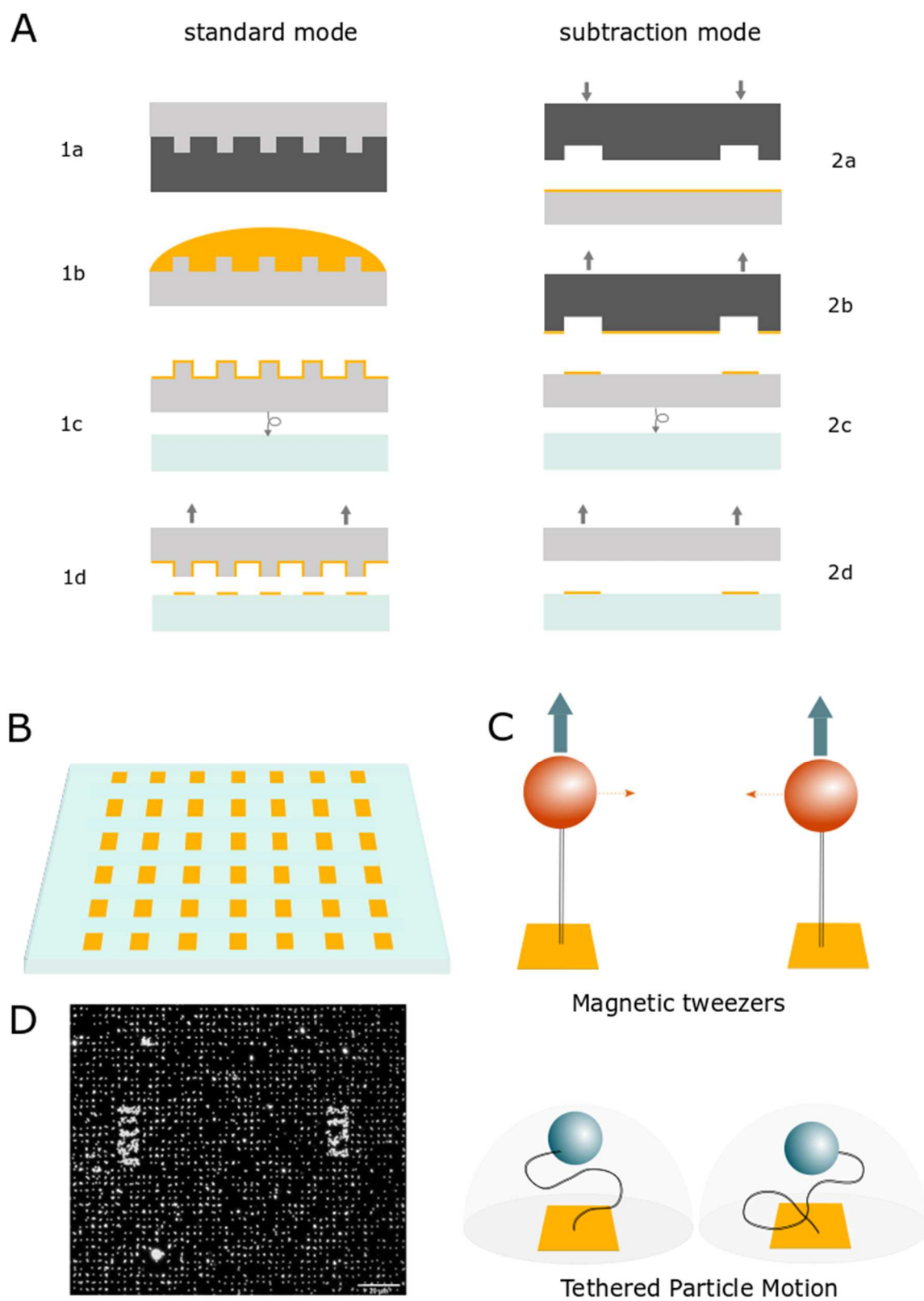


Figure 2

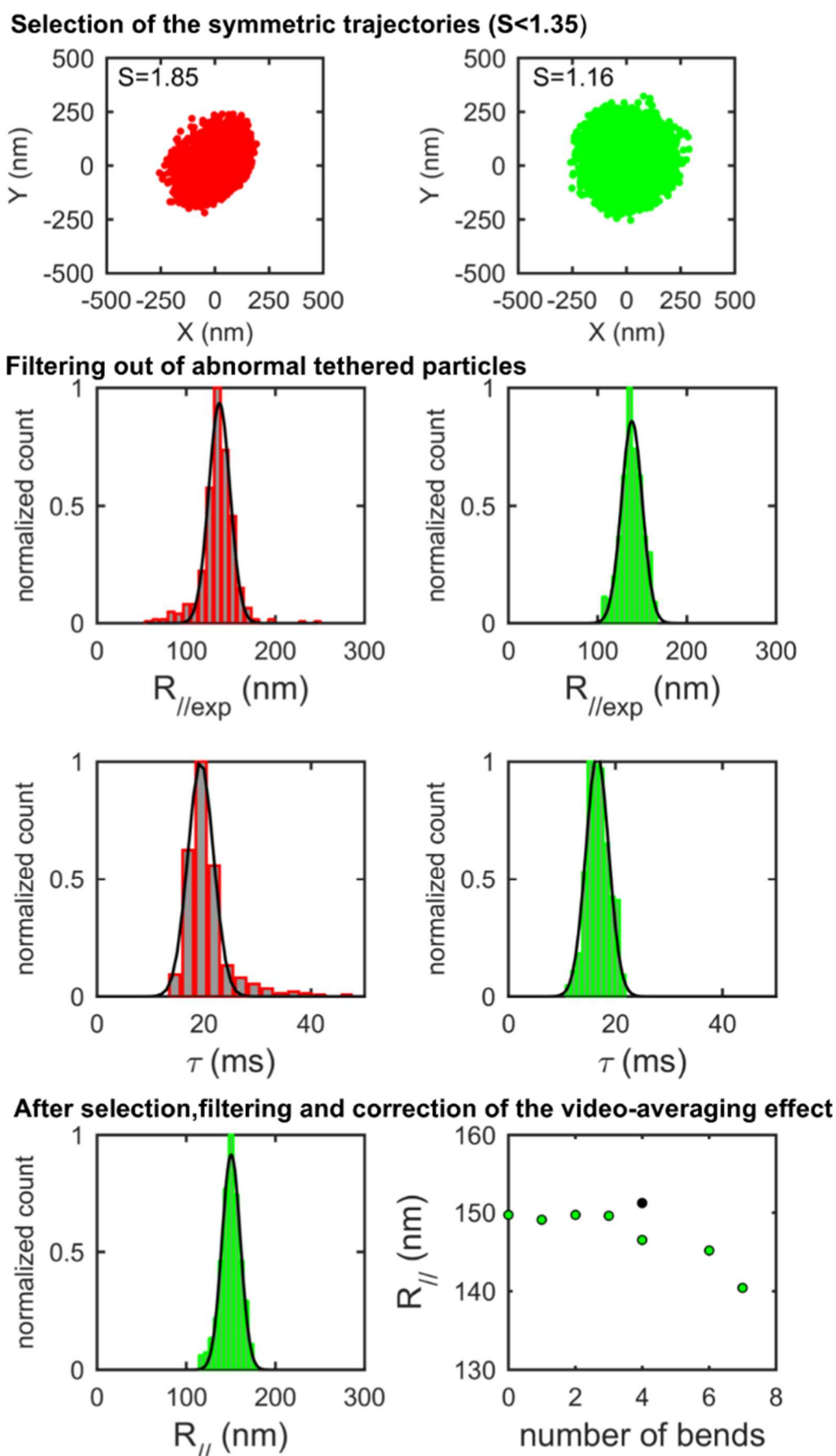


Figure 3

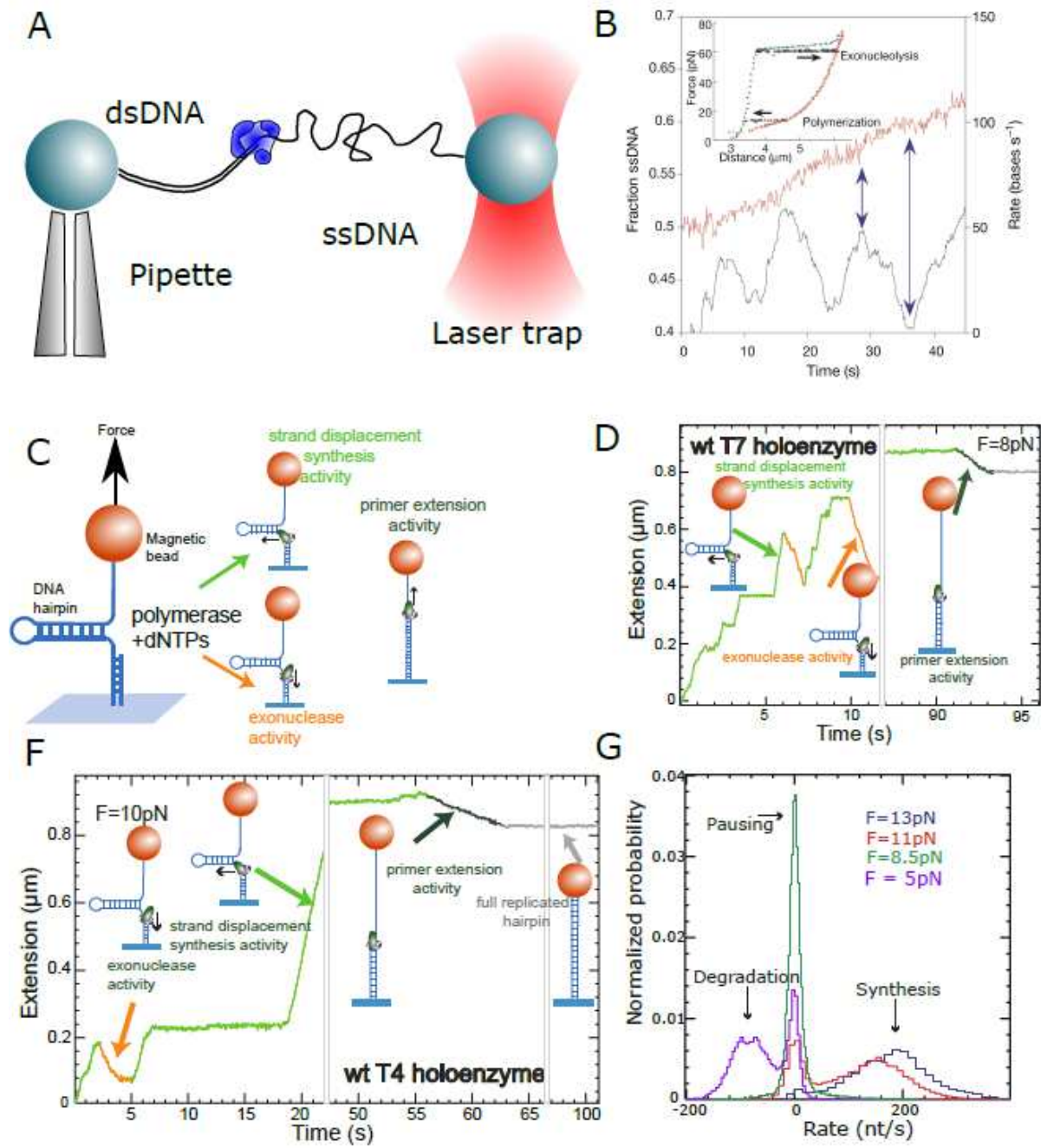


Figure 4

