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Studying Genomic Processes at the Single-Molecule Level

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Abstract
To understand genomic processes such as transcription, translation, or splicing, we need to be able to study their spatial and temporal organization at the molecular level. Single-molecule approaches provide this opportunity, allowing one to monitor molecular conformations, interactions, or diffusion quantitatively and in real time, both using purified systems and in the context of the living cell. This review introduces the types of application of single-molecule approaches that can enhance our understanding of genome function.

Online 'at a glance' summary

- We present key examples of the use of single-molecule approaches to study transcription, translation, splicing and replication.
- We highlight the particular advantages of using single-molecule approaches for the study of genome processing.
- We provide an overview of the force-manipulation and fluorescence techniques used in single-molecule studies.
- We highlight how single-molecule studies of transcription have provided novel insights into initiation, elongation, and termination.
- We discuss how in the field of translation, single-molecule methods have been used to dissect aspects of initiation, elongation, termination, and protein folding.
- Novel single-molecule fluorescence assays have enabled studies of splicing and nuclear export in unprecedented detail.
- We review how single-molecule techniques provided surprising insights on the replisome's stoichiometry and dynamics.
- We conclude with an overview of challenges and future directions in the application of single-molecule approaches to genomic processes.

Introduction

Cells rely on the correct readout, maintenance, repair, and replication of genomic information, processes which involve a stunning variety of carefully coordinated and regulated molecular actors. Although many of the key players were identified decades ago, quantitative and mechanistic insight into their (inter)actions remains a challenge to this day that has drawn in scientists from disciplines that neighbor molecular biology and biochemistry such as biophysics, bioinformatics, and nanoscience.

To obtain quantitative insight into the behaviour of genomic processes, probing at the single-molecule level has proven to be very successful. For example, single-molecule methods can detect transient intermediates or rare events that are masked when ensemble techniques that average the behavior of a large number of molecules are used. Single-molecule measurements require techniques capable of probing biological material with nanometer-scale spatial and millisecond temporal resolution. Furthermore, techniques must be compatible with aqueous environments, as genomic processing takes place in the context of the living cell. Single-molecule methods now exist that reach this capability in a range of environments from simple saline solutions used for *in vitro* studies to the densely crowded environment of the living cell.

Using single-molecule approaches, a wealth of quantitative information on the activity of proteins involved in genome processing has been obtained in recent years. For example, experiments have allowed us to distinguish the different stepping motions utilized by helicases, witness the progress of RNA polymerase basepair by basepair, and follow the activity of the replisome in live cells in real time, to name but a few. Complementing excellent studies using bulk approaches that for reasons of space we cannot detail in this Review, the results of single-molecule studies have provided us with excellent mechanistic insight into motor proteins' functions and mechanisms and even found a major commercial application in single-molecule DNA sequencing approaches (recently reviewed in (1)). Yet the field is by no means mature. Single-molecule studies are increasingly going "beyond single molecules": experiments are beginning to address multi-component systems and their mutual interactions, e.g. they study more than the interaction of a single protein with DNA. This development has gone hand-in-hand with the ability of techniques to detect different molecular components simultaneously and to perform many single molecule experiments in parallel, enabling high-throughput data collection while still resolving the fundamental behavior of single molecules. Indeed, the drive towards more complex systems and the development of new techniques can together provide the answers to an increasing array of questions pertaining to genome processing.

In this review, we provide an introduction to single-molecule approaches to study genomic processes and highlight examples of insights obtained using these methods. We first focus on transcription and translation, then highlight very recent progress in studying RNA export and splicing, and conclude by illustrating the dynamics of protein-protein interactions in replication. Other areas of genome processing that have benefited from the single-molecule approach include DNA repair and recombination have been reviewed elsewhere (2,3). As it is possible to monitor the motion of a single biological molecule both *in vitro* as well as inside of a living cell, we compare and contrast genome processing in these two different contexts. Throughout, we indicate how specific insights rely on the capabilities afforded by single-molecule methods.

Measuring at the single-molecule limit

While achieving the single-molecule limit is in and of itself relatively straightforward (e.g. by sufficient dilution of a molecular sample), the challenge has always been how to observe or manipulate single molecules. Signals are inevitably weak, and the measurement apparatus is always macroscopic. Techniques such as electron microscopy (4-6) and patch-clamp detection (7) were early advances along these lines. In this Review, we focus on the principal single-molecule techniques that are employed for the study of genome processing, namely force (Box 1) and fluorescence (Box 2) spectroscopy. The developments of atomic force microscopy, video-based tethered particle motion, and optical and magnetic traps have formed the key methodological advances in force spectroscopy. The optical and

magnetic traps have succeeded in combining the greatest flexibility in terms of molecular manipulation with the highest spatial and temporal resolution. The development of optical methods to visualize individual molecules (8,9), the introduction of genetically-encodable fluorophores such as GFP (10), and the introduction of fast detectors and sensitive cameras have been key to the widespread usage of fluorescence spectroscopy. Indeed, using this approach it has now become routine to monitor the motion of individual molecules of different types within living cells.

There are many possible reasons for choosing single-molecule methods to study genomic processes. Most obviously, they allow one to re-examine known biological processes in real time, directly as they occur. In many cases, the high spatial and temporal resolution afforded by single-molecule techniques have made it possible to gain unique insight into enzymatic dynamics. This has been particularly the case for studies of genome processing, where the ~3 Å basepair spacing sets a critical lengthscale. Importantly, single-molecule techniques provide inherent synchronization, in the sense that the starting point of enzymatic activity is always known. Hence, the signals of molecular activities are not affected by the averaging of asynchronous events. For example, studies of the repetitive cycles of elongation in transcription, translation, and replication benefit from this lack of averaging. Additionally, when studying biological systems of considerable compositional complexity (e.g. the ribosome and its associated factors), single-molecule techniques can permit the selection, and hence the study, of correctly assembled complexes only. Lastly, the sheer diversity of parameters that can be measured via single-molecule techniques, e.g. enzymatic stall forces (11), backwards motion of an enzyme along a DNA template (12), the presence of friction during enzyme motion (13,14), and many more, has also played an important role in their wide acceptance.

Transcription

Our understanding of the functioning of the key molecular motor that powers transcription, RNA polymerase (RNAP), has been greatly advanced by single-molecule methods. Following the lead of initial single-molecule work on *Escherichia coli* RNAP (15), studies have primarily focused on bacterial systems, but more recently eukaryotic RNAPII has also been examined.

Initiation. In bacteria, an important step in transcription initiation is the transition from the so-called closed promoter complex to the open promoter complex, a process in which the RNA polymerase holoenzyme locally melts the DNA upon binding. To shed light on the various substeps and their associated dynamics of this process for the case of transcriptional regulation by σ54 (16), an *in vitro* multi-color single-molecule TIRF-based assay (Box 2 , panel a) was employed. Such an approach is widely applicable and provides large datasets that report on the association and dissociation of multiple, differentially labeled molecules in a single experiment. Here, a critical advantage was provided by the low dissociation rates between σ54, *E. coli* RNAP, and DNA, which permitted long waiting periods prior to the arrival of a labeled component and hence the use of relatively low concentrations of labeled molecules to facilitate observation of individual molecules using TIRF microscopy (Box 2). In this way, the authors observed that the transition from an unbound *E. coli* RNAP to a closed promoter complex can be decomposed into two substeps, each characterized by its own lifetime, whereby the rate of the second substep is rate-limiting for the overall transition into the open promoter complex. They further determined that, in the presence of competing processes such as dissociation, on average thirty attempts are necessary for *E. coli* RNAP to reach the open promoter complex. Interestingly, these studies showed that the dynamics of this σ54–dependent initiation pathway differ significantly from those of the more common σ70–dependent pathway, whilst closely resembling eukaryotic pathways.

smFRET (17) (Box 2, panel c) and magnetic tweezers (18) (Box 1, panel a) have been employed to distinguish between three different models proposed for the transition of the *E. coli* RNAP holoenzyme from initiation into elongation: (1) transient excursion (in which *E. coli* RNAP would diffuse back and forth on the DNA between subsequent abortive initiations); (2) inchworming (in which *E. coli* RNAP would stretch further along on the DNA template with each successive ribonucleotide incorporation, followed by subsequent

release); and (3) scrunching (in which the DNA transcription bubble would increase in size with each successive ribonucleotide incorporation, followed by subsequent release). To probe these different possibilities, smFRET employed several dye-labeling strategies (Fig. 3a) (17). For example, labeling the trailing edge of *E. coli* RNAP and the upstream template DNA enabled the authors to monitor the relative distance between these molecules; as this distance did not change, they were able to discard the transient excursion model. Labeling the trailing edge of *E. coli* RNAP and the DNA downstream from the promoter revealed that their relative distance was unaltered during the transition into elongation, hence the inchworming model was similarly ruled out. Lastly, by labeling the leading edge of *E. coli* RNAP and the upstream DNA the authors showed that 7-9 basepairs are scrunched prior to elongation, to be released only upon σ^{70} factor dissociation. Similar conclusions were arrived at by Revyakin et al. following careful analysis of promoter unwinding on supercoiled DNA in magnetic tweezers (18). Initiation has also been studied for eukaryotic RNAPII (which is structurally related to *E. coli* RNAP) by Treutlein et al., who used a smFRET-based triangulation approach to observe the transition from the open complex conformation into elongation. By labeling RNAPII, the DNA template, and several transcription factors, they could observe a large conformational change of the initiation complex during the transition from initiation into elongation, facilitated by the intrinsic flexibility of eukaryotic transcription factor TFIIB (19).

Elongation. Single-molecule studies of elongation have revealed rich dynamics of RNAP nucleotide incorporation, in particular the presence of various kinds of pauses. Already the first *in vitro* single-molecule studies of RNAP (employing tethered particle motion, TPM) indicated the presence of heterogeneities in RNAP velocity (15). Since then, studies of elongation have primarily relied on the use of optical tweezers (Fig. 3c), benefiting from their high spatio-temporal resolution which in recent years has even achieved the limit of single basepairs within a 1 s bandwidth (Fig. 3d (20)). Such studies of transcription dynamics have highlighted how RNAP progression is interrupted by different kinds of pauses (21-26). For example, when Herbert et al. applied an assisting force to *E. coli* RNAP using optical tweezers (Fig. 3c, bottom panel, green arrow), they discovered that pauses occur at rates that are sequence- and force-dependent and have durations that are exponentially distributed and independent of the magnitude of the applied force (22,27). Such exponentially-distributed pauses could be modulated by the presence of co-transcriptional factors such as NusG (which decreases the pause density (28)) and NusA (which increases the pause density (27)). Conversely, the application of a hindering force on *E. coli* RNAP resulted in the observation of long pauses attributed to backtracking (23,29). Backtracking involves backwards diffusion of RNAP on its template while part of the mRNA is extruded, and is facilitated by nucleotide misincorporation (23). However, for *E. coli* RNAP studies disagree on the relative weight of backtracking pauses versus exponentially-distributed pauses (23,24,26). To obtain a unified model of *E. coli* RNAP elongation, it will be necessary to address the fact that the lifetime of the shortest pauses is comparable to the typical bandwith of optical tweezers (both ~1 second): either the experimental spatio-temporal resolution must be further increased, or more accurate analysis methods that can reliably distinguish pauses from elongation steps must be implemented. In both cases, increasing the size of datasets collected via single-molecule experiments will permit more stringent tests of different models (25,30,31).

Follow-up *in vitro* studies with optical tweezers have further investigated backtracking: for instance, transcription factors GreA and GreB, which cleave the protruding mRNA strand, were found to rescue of backtracked RNAP (23), illustrating a possible proofreading mechanism. Additionally, the role of backtracking in the dynamics of eukaryotic polymerases has been investigated: work by Galburt et al. on RNAPII showed that its pause dynamics are dominated by backtracking, to the extent that RNAPII cannot overcome forces beyond 8.5 pN (24). Interestingly, the addition of TFIIS (a eukaryotic transcription factor that acts similarly to GreA and GreB) rescued backtracked RNAPII and permitted it to overcome forces up to 16.5 pN. Lastly, it has been shown that RNAPII backtracking becomes more likely in the vicinity of a roadblock such as a nucleosome (32), and that overcoming such a barrier requires the aid of thermal fluctuations. It is expected that the application of these *in vitro* single-molecule techniques will continue to shed light on the mechanistic

details of RNAP progression in conjunction with the multiple factors that accompany transcription.

To see whether similarly rich dynamics of RNAP occur *in vivo,* Darzacq et al. observed the dynamics of RNAPII in mammalian cells by fluorescently labeling RNAPII and simultaneously monitoring an mRNA stem-loop specifically recognized by a GFP-labeled protein (33). Using this approach, they observed that the rate-limiting step in transcription is the transition between initiation and elongation (RNAPII transcribed the complete gene in only 1/90 of cases), and that transcription is interrupted by long-lived pauses likely related to backtracking. While these long-lived pauses are rare (only 4.2% of the polymerases are affected), they nonetheless have a significant impact, as their cumulative duration can total up to half the total elongation time. Curiously, the pause-free elongation speed observed *in vivo* (70 bp/s following the removal of aforementioned long-lived pauses) was significantly higher than the corresponding value observed by *in vitro* (~15 bp/s). Whether this can be attributed to a lack of assisting transcription factors in *in vitro* assays or to the reduced temporal resolution of the *in vivo* experiments remains to be determined. Nonetheless, the *in vitro* and *in vivo* approaches yield complementary insights relevant for our understanding of this complex enzyme.

Termination. The different pathways underlying the termination of transcription have been studied at the single-molecule level using optical tweezers (34). By applying force to the mRNA transcript generated by *E. coli* RNAP (Fig. 3c, top panel), Larson et al. investigated the efficiency of termination for various termination sequences that encoded mRNA hairpins. They discovered that the hairpins directly influence the efficiency of termination through their ability to shear the mRNA/DNA hybrid. The degree of shearing, and hence the efficiency of termination, could be enhanced by shortening the hybrid, whereas it could be decreased through the presence of additional secondary structure that interfered with proper folding of the hairpin.

Thus, we see that optical tweezers can successfully probe the activity of RNA polymerase by using subtle application of force to perturb its local energy landscape, while smFRET studies report on the kinetics of molecular conformational changes. Integration of the two approaches will determine whether pausing is predominantly a template-related process or rather a direct consequence of conformational changes in the enzyme. Future efforts to unravel the dynamics of co-transcriptional phenomena such as translation or splicing warrant further application of single-molecule techniques.

Translation

Single-molecule approaches have been used to study structural rearrangements within the prokaryotic ribosome. The structural rearrangements involve motions on a size-scale perfectly suited to smFRET measurement (35), adding dynamic information to the structural snapshots deduced via X-ray crystallography or cryo-EM. In addition, insights into the interaction of the prokaryotic ribosome with mRNA during elongation have been obtained using optical tweezers.

Initiation and elongation. To illustrate how smFRET has enhanced our understanding of the motion of ribosomal subunits, we consider the results from three separate studies. In the first (36), the two subunits of the bacterial ribosome (50S and 30S) were labeled with donor and acceptor dyes, respectively, and their interaction with initiation factor IF2 was studied. Using this approach, it was found that the association of the large subunit with the preinitiation complex could be accelerated by IF2 by a factor of four. The authors determined that IF2 subsequently hydrolyzes a single GTP to align the 30S subunit with the 50S subunit by rotating it with respect to the 50S. This rotation brings the ribosome into the elongation state. A related study (37) showed that this counterclockwise rotation did not require the presence of EF-G (elongation factor G, which supplies the necessary energy for ribosome translocation via GTP hydrolysis): the rotation itself is thermally driven, but it is stabilized in a pretranslocated (rotated, or unlocked) state by EF-G binding following peptide bond formation. Subsequent GTP hydrolysis by EF-G propels the ribosome into the locked state (non-rotated) to drive ribosome translocation. A third smFRET experiment then demonstrated multiple cycles of high/low FRET efficiency in a single trace (38), reflecting

the successive counter-clockwise rotations of the 30S subunit with respect to the 50S subunit required to incorporate multiple amino acids (Fig. 4a). These observations are in accordance with structural information from cryo-EM studies and with additional smFRET studies that utilized the fluctuations in the positions of labeled tRNA within the ribosome as their readout (39).

The direct observation of translocation by the ribosomal molecular motor during elongation has been a longstanding challenge. It was achieved by the Bustamante group using an optical tweezers assay that exploited the ribosome's ability to unwind mRNA hairpins (Fig. 4b) (40), and demonstrated that the elongation velocity is strongly influenced by the stability of the mRNA secondary structure. More recent investigations by the same group using hairpins with differing GC content showed that the ribosome employs two distinct mechanisms: interaction of ribosomal proteins S3 and S4 with the mRNA backbone opens weak secondary structures, whilst a lever mechanism involving a tRNA-mRNA interaction and GTP hydrolysis supplied by EF-G opens strong secondary structures, as previously suggested by cryo-EM studies (41). Detailed knowledge of the ribosome's unwinding mechanism is important in the context of ribosomal frame shifting, in which interactions between the ribosome and particular mRNA sequences shift the mRNA reading frame by a single nucleotide. Investigation of frame-shifting will thus provide a natural follow-up to these studies.

Recently it has become possible to observe ribosomal elongation via fluorescence detection, using zero-mode waveguides (ZMW) (Box 2 , panel a) to detect individual labeled tRNA or individual labeled amino acids above background at physiological concentration (42). Using this approach, Uemura et al. tethered the ribosome at the bottom of the ZMW via an mRNA template (43), and labeled three different tRNAs (lysine-tRNA, phenylalanine-tRNA, and N-formylmethionine-tRNA) with different colors. Since a ribosome must contain the $tRNA^{fMet}$ to elongate, detection of its associated color could identify tethered ribosomes in the ZMW. Subsequent incorporation of the other two labeled amino acids was then monitored by measuring the dwell times of tRNAs in the ribosome, for a total of twelve amino acid incorporation events. Interestingly, the authors observed that the ribosomal A and E sites almost never simultaneously contain a tRNA: most frequently, only the A and P sites contain tRNAs, as the exit of a tRNA from the ribosome occurs very rapidly once translocation has taken place (44). Future studies could use the ZMW approach together with labeled tRNA to observe more complex tRNA dynamics, e.g. during frame shifting or in the presence of high concentrations of acylated tRNAs.

Termination and protein folding. Translation termination is a multi-step process involving numerous factors whose dynamics have been studied using smFRET. Such experiments have provided detailed mechanistic insights into the association of release factors with the ribosome, and into the specific position of the large subunit with respect to the small subunit at every step of termination (45). A further aspect of translation, namely, the ribosome's influence on protein folding, has been the subject of recent single-molecule studies by Kaiser et al. (46). To study protein folding in the presence of the ribosome, they attached the ribosome to a bead held in a micropipette (Box 1, panel d). In the presence of an *in vitro* translation system, this ribosome synthesized T4 lysozyme, which was then coupled to an optically trapped bead via a DNA handle. By comparing the observed folding pathway to identical experiments performed in the absence of the ribosome, the ribosome's influence on the folding pathway was assessed. For example, only 28% of the ribosome-bound lysozyme refolded, whereas refolding always occurred in the absence of ribosome. The presence of the ribosome results in more compact folding, but at a rate that was more than hundred-fold reduced. The authors concluded that the ribosome slows down protein folding, acting as a chaperone to guide the proteins into properly folded structures.

Splicing and Export

In eukaryotes, many RNAs need to be exported from the nucleus, where they are transcribed, e.g. to the site of translation. Single-molecule studies have begun to reveal properties of transport through the nuclear pore complex in permeabilized cells (47-49) and of the individual nucleoporins *in vitro* (50,51). For example, a two-color super-registration microscopy approach was used to track mRNA transport *in vivo* (52) (Fig. 5a). Interesting

observations included the finding that that individual mRNA molecules often scan multiple pores before being exported and that not all NPCs are equally active in mRNA export. The authors were able to directly observe individual mRNAs being exported from the nucleus (Fig. 5a) and to propose a kinetic model for export that suggest that docking to and release from the NPC, rather than transport through the central channel, are the rate-limiting steps (52).

Several single-molecule studies of splicing, including of self-splicing introns, have used purified components and have revealed the dynamics of individual steps in splicing (53,54). Recently, Hoskins, *et al.*labeled individual spliceosomal subcomplexes in yeast whole cell extract with different organic dyes and using multi-color TIRF(55) (Box 2), they studied their assembly on fluorescently-labeled, surface-immobilized pre-mRNA constructs (Fig. 5b). The authors were able to deduce the order and kinetics of spliceosome assembly and found that the assembly steps are reversible and that the assembly rates of the different components are similar, such that no particular step is strongly rate-limiting. In the future, single-molecule methods have great potential to reveal further details of the various splicing pathways, including alternative splicing (56,57).

Nuclear export and splicing both involve very large (> MDa) and complex (nucleo)protein assemblies (the NPC and the spliceosome, respectively) that pose significant challenges to traditional high-resolution structure techniques and bulk biochemical assays. Overcoming these challenges and resolving the dynamics of these complex mechano-chemical machines will continue to require new experimental approaches at the single-molecule level.

Replication

The different enzymes that act in concert to carry out DNA synthesis are collectively known as the replisome (58-60). The architectural complexity of the replisome varies and encompasses four different proteins in bacteriophage T7 (61), eight proteins in bacteriophage T4 (61), twelve known in *E. coli* (62), and more still in eukaryotes (62). Our understanding of replisome dynamics has benefited from studies at the single-molecule level. *In vitro*, it has been possible to investigate the motor activity of individual polymerases (63) or individual helicases (64-66) using magnetic or optical tweezers (Box 1). In a recent study (67), a combination of fluorescence spectroscopy together with optical trapping was utilized to investigate the polymerization of single-strand binding protein (SSB), which plays a key role in lagging strand replication. More recently it has also become possible to study the activity of complete replisomes *in vitro* at the single-molecule level, relying on either direct reconstitution (for phage T4 (68), phage T7 (69), and *E. coli* (70) or the use of cell extracts (for eukaryotic systems) (71). These types of measurements can be compared to their *in vivo* analogues.

Replication dynamics: To illustrate how *in vitro* single-molecule assays can be used to probe the dynamics of replication, we briefly describe three recent studies that have focused on the interplay between helicase activity and primase activity. During replication, new primers need to be continuously synthesized to generate Okazaki fragments. The ssDNA template used for priming is formed by the helicase as it unwinds duplex DNA. However, the primase synthesizes RNA primers in the opposite direction to fork progression. Three possible ways to coordinate this process have been suggested: (1) pausing (leading to a cessation of unwinding); (2) DNA looping (resulting from continued synthesis by both helicase and primase whilst they remain associated); (3) disassembly (in which primase and helicase dissociate from one another). Manosas *et al.* (64) used a short DNA hairpin tethered in magnetic tweezers to investigate these models for the T4 primosome, in which the helicase and primase are thought to associate. For a given applied force, changes in the length of the DNA molecule provide information about changes in its conformation caused by the primosome complex. The authors concluded that the T4 primosome utilizes both the disassembly and DNA looping mechanisms, with a preference for the latter in the context of a full replisome (Fig. 6a). Two other studies, which investigated priming dynamics for the T7 replisome, used a flow-stretching assay (69) and smFRET (72), respectively. These studies revealed partially conflicting results: the former indicating that the primase slows down leading strand synthesis sufficiently to allow lagging-strand synthesis to keep up and the latter concluding that the leading strand T7 replisome did not pause (in contrast to the

observations by Lee *et al.*), that the leading strand synthesis is slower than the lagging strand synthesis, and that priming loops are formed on the lagging strand.

Replisome Stoichiometry: Both *in vitro* and *in vivo* single-molecule studies have recently shed new light on replisome stoichiometry. In a recent study using purified proteins (73), it was proposed that the PolIII holoenzyme contains three PolIII cores, in contrast to earlier models that suggested there were only two (bound at the leading and lagging strands, respectively) (74). Relying on the ability to localize individual DNA-bound fluorescent proteins inside living cells (75-77) (Box 2), Reyes-Lamothe *et al.* fluorescently labeled ten different components of the replisome in separate *E. coli* strains. Analysis of the number of bleaching events (whereby each event is generated by a single labeled molecule) and their intensities allows one to determine the number of molecules present within a diffraction-limited spot (Fig. 6b), and this enabled the authors to conclude that the *E. coli* replisome contains three PolIII cores. More recent *in vivo* work has confirmed the observation of three PolIII cores, with the caveat that the binding of the third polymerase appeared to be transient (78). A potential function for a third polymerase was investigated by using a single-molecule flow stretching assay (Box 1, panel d) together with a bulk bead-based assay (79). The authors observed that a tri-polymerase (tri-pol) replisome is more efficient at lagging strand synthesis than its di-polymerase (di-pol) counterpart (ssDNA gaps were observed in the di-pol case), and that it also displays increased processivity. That is, the DNA fragments generated by the tri-pol were nearly twice as long as those from the di-pol. From these *in vivo* and *in vitro* data, the presence of a third polymerase seems plausible, but further investigations are necessary to exclude other models and to determine the exact role of such a third polymerase *in vivo* (80).

Challenges and future directions

What are the most interesting challenges that remain in our understanding of genomic processes? And how will single-molecule techniques continue to contribute to them? Will certain single-molecule techniques become as commonplace as gel electrophoresis?

Challenges in molecular mechanisms: Many challenges remain in understanding genome processing at the molecular level. For example, while many molecular processes studied using single-molecule force spectroscopy have involved the motion of molecular motors along a linear template, the double-stranded helical nature of DNA also means that there are important roles for rotary motion. For example, the unwinding activity of replisomes or RNA polymerases generates torsional stress in the DNA template which may not always be dissipated sufficiently rapidly by topoisomerases (reviewed in (81)). By analogy to the way force-velocity relationships have shed light on the mechanochemistry of linear motion, the measurement of torque-angular velocity relations can be used to investigate the mechanochemistry of rotary motion for polymerases, helicases, and other genome-processing enzymes. Such investigations will be facilitated by the recent introduction of new measurement techniques that report on torque and twist such as magnetic torque tweezers(82-86).

A separate challenge at the molecular level is to understand how a molecular motor's conformational dynamics are linked to any physical displacement it executes along a DNA or RNA track. Single-molecule fluorescence techniques such as FRET (Box 2) are ideally positioned to report on the nanometer-scale conformational changes that proteins undergo. In order to correlate these (internal) changes with physical displacement along the motor's track, a feasible approach is the integration of fluorescence spectroscopy with force spectroscopy. Initial efforts along these lines have been published (87-89). In addition to monitoring an enzyme's conformational changes during translocation along a DNA track, such approaches could concurrently investigate the coordination of ATP hydrolysis.

Increasing complexity: An ongoing challenge is to apply *in vitro* single-molecule techniques to increasingly complex biological systems. Rather than investigating a single molecular motor in isolation, such a motor can be studied as part of a reconstituted protein complex, as in the case of DNA replication, or one could study the interplay between different types of molecular motors, as in the coordination between transcription and translation. The influence of more complex substrates (such as chromatin) and their influence on genomic

processing is another interesting challenge. A possible approach is to examine protein activity in cell extract (55,71,90), whereby the proteins of interest come with the appropriate post-translational modifications and native binding partners. However, technical challenges such as unwanted fluorescence background or unwanted adsorption will need to be overcome. An alternative approach to studying protein assemblies consists of purifying molecular complexes via immunoprecipitation and selectively adsorbing them on surfaces for *in vitro* analysis (91,92). This could shed insight into molecular complexes such as the RNA induced silencing complex (RISC complex), telomere complexes, etc.

Single-molecule techniques could also be utilized to investigate genome processing over a much wider range of sequences than typically considered, expanding to include full genomic coverage. At present, this is the case for single-molecule DNA sequencing, in which genome sequences are determined via single-molecule fluorescence measurements of a large number of genome segments in parallel. Potentially, one could interrogate different parts of the genome at the single-molecule level for their mechanical properties, transcription efficiency, transcription dynamics, etc. via single-molecule force spectroscopy or single-molecule fluorescence, or a combination thereof. These investigations and those of more complex biological systems discussed in the previous paragraph benefit from increased parallelization of single-molecule readouts. In certain approaches such as TIRF, parallel readout is already the standard approach, but in others such as magnetic tweezers it has become more widely available only recently (93).

Studies of living cells: Given that genomic processing in its full complexity occurs in the context of the living cell, the ultimate goal is to observe and manipulate molecular processes *in vivo* at high spatial and temporal resolution. To illustrate some of the possibilities, Fig. 7 depicts a range of genomic interactions that take place in and around the nucleus of a eukaryotic cell together with ways in which they may be probed via single-molecule methods. The DNA itself has an overall architecture that depends on the state of the cell cycle; a rapid and detailed snapshot of the entire architecture would be of great interest, for example, to understand its influence on transcriptional patterns. Already, fluorescence spectroscopy has been used as an aid to mapping the three-dimensional architecture of the *C. crescentus* genome, through its spatial calibration of chromosome capture data (94). Superresolution microscopy has made it possible to resolve the architecture of the bacterial genome in fixed cells to better than 100 nm (95), and there will no doubt be further improvements. It could also be of interest to monitor local stress and strain in DNA intracellularly; a FRET sensor integrated into the DNA could potentially fulfill such a role. Of similar importance is the monitoring of RNA and proteins *in vivo*. RNA is typically visualized via fluorescence in-situ hybridization (FISH), which can be performed at the single-molecule level (96), or via detection of RNA-bound proteins (97), but a recent development of interest is the development of genetically-encodable RNA aptamers that can directly bind fluorescent dyes (98). Building upon the ability to image protein dynamics intracellularly at the single-molecule level, multi-color imaging of different proteins could potentially reveal the dynamics of network interactions. For example, recent work has made it possible to image DNA-binding proteins in the bacterial nucleoid at high resolution (99,100). A similar approach could help to elucidate chromatin structure in live cells, for example by labeling histone proteins. However, for i*n vivo* imaging in particular an ongoing challenge is to improve the ease and specificity of introducing fluorescent labels, as well as enhancing their photon yield (101).

Another area for development is monitoring and applying forces or torques within the cell (Fig. 7). To date, force measurements on living cells have typically focused on the forces exerted by structures that execute large-scale motion such as the filopodia involved in cell migration (102), allowing measurement to be performed via AFM (103) or via the deflection of microfabricated pillars (104-106). Intracellular force measurements are more challenging (107,108) but can be achieved via the introduction of (sub)micron-sized particles (109,110) or careful design of calibrated fluorescent probes (111-113). More mature versions of these approaches can in the future be applied to genome processing events. Finally, it will be very interesting to monitor the extent to which genome processing is sensitive to external mechanical perturbations (114), which could be addressed via a combination of mechanical

manipulation via single-molecule force spectroscopy and intranuclear readout via fluorescence imaging.

Ultimately, our understanding of the genome and its processing will rely an input from both *in vitro* and *in vivo* techniques and from many fields: biology, bioinformatics, chemistry, physics, engineering, nanoscience, and nanotechnology, to name a few. Merging such knowledge into an understanding of cellular function will occupy us for decades to come.

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Highlighted references:

Friedman et al. (2012):
Studies using an *in vitro* TIRFM assay of the association and dissociation of the bacterial RNAP σ54 factor during the transition from initiation to elongation.

Kapanidis et al. (2006):
The first *in vitro* experimental complete study showing the scrunching mechanism of RNAP initiation by smFRET.

Abbondanzieri et al. (2005):
This study related the first force spectroscopy study of RNAP at the base pair resolution, using optical tweezers.

Aitken et al. (2010):
Use of smFRET to follow the intersubunit conformation of the ribosome during translation in real time.

Wen et al. (2008):
First *in vitro* observation of translating ribosomes using single-molecule force spectroscopy.

Uemura et al. (2010):
Demonstation of the utility of ZMW to study *in vitro* translation in the presence of a high concentration of labeled tRNAs.

Grunwald and Singer (2010):
Demonstration of tracking mRNA export through the nuclear pore in live cells using a super-registration approach.

Hoskins, A.A., *et al.*(2011):
Comprehensive study of spliceosome assembly using cell extract and multi-color TIRF microscopy.

Manosas, M., et al. (2009):
Thorough investigation of the T4 primose activity on the single-molecule level.

Reyes-Lamothe, R., et al. (2010):
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Glossary

EF-G: elongation factor G: A factor that provides the bacterial ribosome with the necessary energy (derived from GTP hydrolysis) required to translocate along the mRNA.

Mechanochemistry: Description of how the chemical reactions driving biological processes – e.g. ATP hydrolysis in a molecular motor- are coupled to mechanical motion –e.g. translocation along a nucleic acid template.

Primosome:A protein complex, consisting out of a helicase and primase, that is responsible for the synthesis of RNA primers during DNA replication.

Rayleigh criterion: The Rayleigh criterion quantifies the minimum resolvable distance between two objects that fluoresce at the same wavelength. This distance equals roughly half the wavelength of light.

Replisome: A multi-protein complex that carries out DNA replication.

Ribosomal A, P and E sites: respectively the aminoacyl, peptidyl and exit sites of the ribosome, the three different binding sites of tRNAs.

RNA aptamers: RNA molecules that specifically bind to a target molecule.

RNA polymerase holoenzyme: The initiation complex composed of the RNAP core enzyme and the sigma initiation factor.

σ54: One of the initiation factors that can bind to *E. coli* RNAP during initiation to allow it to recognize a specific promotor sequence.

σ70: The most common and most widely-studied initiation factor that can bind to *E. coli* RNAP during initiation to allow it to recognize a specific promotor sequence.

Tethered particle motion: A single-molecule technique that employs tethered beads to study biological molecules in the absence of any externally applied force. Changes in the bead's position can report on changes in tether length and hence on enzyme activity.

Box 1. Single-molecule force-manipulation techniques. A range of techniques can be used to apply forces (and in some cases torques) to individual molecules or molecular assemblies (115-117). These single-molecule manipulation techniques rely on specifically attaching the extremities of the molecule(s) of interest between a surface and a force-transducer, e.g. to a magnetic or dielectric bead, or an atomic force microscope (AFM) cantilever (117). Specific attachment is accomplished via biotin-streptavidin linkage, thiol bonds, antibodies, etc.

In magnetic tweezers (figure panel a), permanent or electrical magnets are used to manipulate surface-tethered magnetic beads by controlling the external fields. The force on the magnetic beads depends on the gradient of the magnetic field (118). In addition to forces, magnetic tweezers can also routinely apply torques, by rotating the magnetic fields (82,119). In flow-stretch experiments (panel b), liquid flow exerts drag forces on tethered beads (120). The force can be controlled by adjusting the flow rate. In AFM forces are applied to individual molecules tethered between a functionalized AFM cantilever and a surface (117,121) (panel c). Optical tweezers hold a dielectric particle or bead in the focus of an intense laser beam (117,122,123) (panels d-f). Common optical tweezers geometries have one end of a nucleic acid tether attached to a bead held in an optical trap, while the other end is attached to either the surface (panel f), to another optically trapped bead (panel e, a so-called dumbbell or dual bead assay), or to a bead held in a micro-pipette (panel d). In flow-stretch and magnetic tweezers set ups the tether extension is typically monitored by CCD-camera based tracking of the bead positions. In optical tweezers or AFM, the laser signal is usually read out with a position sensitive device or quadrant photodiode (121,122). Flow-stretch and magnetic tweezers intrinsically operate in constant force mode, i.e. for a given setting of the flow speed or magnetic fields, the stretching force exerted on the tether is (very nearly) constant. In contrast, optical tweezers and AFM intrinsically control the extension, by controlling the position of the optical trap or cantilever. Constant extension techniques can be operated in (approximately) constant force mode by using feedback control or by exploiting trap nonlinearities (124). All four techniques can apply forces in the pico-Newton to tens of pico-Newton range (Fig. 1g) most relevant to biological processes at the molecular scale (e.g. the stall force for polymerases are in the range of 10 pN). AFM measurements are at the high force end on this scale, while magnetic tweezers are particular well-suited to accurately apply and measure very low forces (117).

Box 2 – Fluorescence microscopy. Fluorescence microscopy is a versatile, widely-used tool used to study biological processes both *in vivo* and *in vitro*. It relies on the fact that molecules can emit light after they have absorbed light at a shorter wavelength . The efficiency of this process depends on the type of molecule used. Many different kinds of fluorescent probes are available, each with its own specific set of characteristics (125-127). They can be divided into two broad classes, fluorescent proteins and organic dyes: the former are a common choice for live cell imaging (126) given their high specificity, whilst the latter have an increased brightness but suffer from a reduced specificity inside living cells.

In addition to the choice of the fluorophore, an important consideration is the excitation method employed. Panel a shows four frequently-employed excitation strategies: wide-field, confocal, total internal reflection fluorescence (TIRF), and zero-mode waveguides (ZMW). These methods differ from one another by the excitation volume, shown here in green. In wide-field microscopy, the sample is excited by a nearly collimated light beam. Out-of-focus fluorophores are also excited, increasing the background noise and rendering probe localization more difficult. Confocal microscopy reduces the excitation volume by making use of a focused laser beam and furthermore employs a spatial filter in front of the detector to eliminate any out-of-focus fluorescence light. Both TIRF and ZMW employ an evanescent waves (parallel optical waves with an exponentially decaying intensity that occur near a surface when incident light impinges at an angle greater than the critical angle for refraction) to reduce both the excitation and detection volumes, making it possible to only excite molecules that are within ~100 nm from the surface and as such significantly reducing the background fluorescence (128). In ZMW the excitation volume in the imaging plane is further restricted compared to TIRF by making use of nanofabricated

structures with typical dimensions of ~ 100 nm (inset). An important parameter in determining the most suitable technique is the required concentration of fluorescently-labeled components. For example, physiologically-relevant ligand conditions are preferred for studies of enzymatic activity. Higher concentrations of fluorescently-labeled ligand result in higher background fluorescence, with a consequence an increased need to reduce the excitation and detection volumes. The typical concentrations of fluorophore-labeled molecules (ranging from pM to uM) that can be investigated with these respective techniques are indicated on the left.

Fluorescence excitation of individual fluorophores gives rise to diffraction limited spots that can be detected with sensitive detectors, typically EMCCD cameras or photodiodes. The position of these fluorescent foci can be determined using single-molecule localization methods with an uncertainty in the nanometer range (dependent on the probe's signal-to noise ratio). Single-molecule localization involves fitting the spatially distributed intensity of the detected fluorescence spot to a mathematical function (typically a Gaussian) to determine its center (panel b) (129-132). The newly-developed super-resolution techniques (recently reviewed in (133,134)) have developed ways to successively localize neighboring molecules to build up an image with resolution beyond that permitted by the Rayleigh criterion.

The preferred microscopy method is highly dependent on the type of investigation. Low background techniques such as TIRF and confocal are commonly used to probe 3D conformational changes. This is can be done in combination with FRET (Förster Resonance Energy Transfer). FRET is based on the non-radiative energy transfer between so-called donor (green) and acceptor (red) fluorophores in close proximity (17) (panel c). Following excitation, the donor molecule can non-radiatively transfer its energy to a sufficiently close acceptor molecule, resulting in decrease in the (green) donor fluorescence signal and a concomitant increase in the (red) acceptor fluorescence signal. Monitoring the degree of energy transfer therefore reports on the distance and dynamics of intra- and intermolecular interactions on the sub-10 nm scale (135).

Figure Captions

Fig. 3. Studies of RNA polymerase at the single-molecule level. a-b) Schematic of an smFRET experiment by Kapanidis et al. (17) in which the polymerase and the DNA template strand are labeled by an acceptor and a donor fluorophore, respectively. **b)** Representation of the results from this experiment. At the start of the reaction, the FRET signal is weak (distribution in top histogram). Subsequent advancement by *E. coli* RNAP results in higher FRET efficiency (distribution in bottom histogram). Even small structural changes in the *E. coli* RNAP can be monitored in this way. **c)** Example of a force spectroscopy experiment that relies on a dumbbell assay in an optical trap to monitor transcription. The direction of *E. coli* RNAP motion is indicated by the arrows. Note that force can applied in different ways: to the synthesized mRNA (top panel); to the DNA template as a hindering force (bottom subpanel, red arrow); and to the DNA template as an assisting force (bottom panel, green arrow). Schematic adapted from (34). **d) T**he observation of transcription elongation by a single *E. coli* RNAP at basepair resolution. Raw data shown in red, smoothed data shown in black. The blue dashed lines are spaced by the distance between nucleotides. Data adapted from (20).

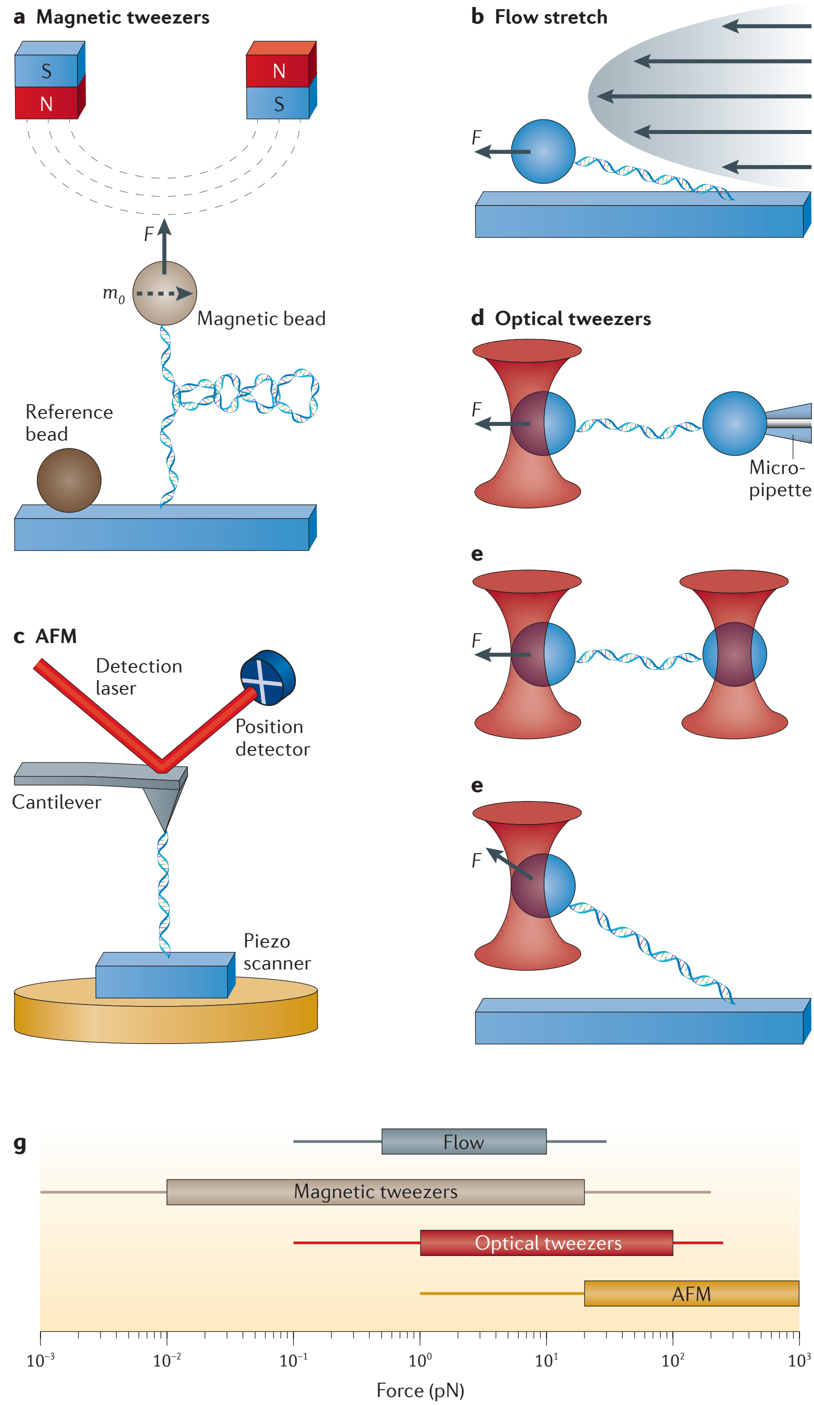
Fig. 4. Studies of ribosome and translation at the single-molecule level. a) Study of the ribosomal subunits' dynamics over multiple elongation cycles. Here, mechanical motions within the ribosome from two perspectives: a side view showing the tRNA translocation, and top view in which subunit rotation is visible. The two subunits are labeled with dyes for single-molecule FRET (small subunit labeled with donor, green circle; large subunit labeled with acceptor, red ciricle). At the start of the experiment, the labeled 30S subunit is bound to the tethered mRNA and labeled 50S subunit is flushed into the flow cell. The three yellow slots represent (from right to left) the ribosomal A, P and E sites. Inset: experimentally-observed smFRET signal. Initiation is clearly visible by the first appearance of a signal from the acceptor dye (red trace). At each step of the elongation cycle, the rotation of the large subunit with respect to the small subunit was monitored, represented

by the signal of the donor (green trace) and the acceptor (red trace). A high signal on the green (red) trace implies an increased (decreased) distance between the dyes. Images and data adapted from (38). **b)** Schematic of a force spectroscopy study of ribosome translation dynamics adapted from (40). Here, the ribosome unwinds and translate an mRNA hairpin held at its extremities by two beads, one of which is maintained in an optical trap.

Fig. 5. Observations of nuclear export and splicing at the single-molecule level. a) Following mRNA export from the nucleus by super-registration microscopy. Tracked positions of β-actin mRNA labeled by multiple copies of YFP fused to a MS2 protein tag (in green) overlaid on the position of the NPC scaffold protein POM121 labeled with tandem Tomato (in red). "N" denotes the nucleus, "C" the cytoplasm. Units are in pixel = 64 nm. Data are adapted from (52). **b)** Ordered and dynamic assembly of spliceosomes. Shown here is a single-molecule fluorescence trace of labeled spliceosome subcomplexes U1. The trace reveals multiple binding and dissociation events to pre-mRNA that was co-localized (not shown), suggesting reversible binding. The arrows indicate the duration of binding events that can be analyzed to determine the lifetimes. Similar traces were obtained for U2, U5, and the Prp19-complex NTC. Data are adapted from (55).

Fig. 6. Replisome architecture and dynamics. a) Looping of DNA during primer synthesis and unwinding. Here an experimental trace is shown from which the priming velocity and loop size can be inferred. A magnetic tweezer is used and the changes in the length of the DNA is measured. Images and data are adapted from ref (64). **b)** Stoichiometry of replisome components. **b1)** An overlay of bright-field (gray) and fluorescence images (yellow) of an *E. coli* strain with one of the replisome components fluorescently labeled. **b2)** Photobleaching traces of fluorescent proteins in living *E. coli* indicating either one (left) or two (right) replisomes. Data are adapted from ref (75).

Fig.7. Genome processing taking place inside a cell nucleus, and various potential ways to monitor it at the single-molecule level. A schematic view inside the nucleus, showing DNA in various stages of condensation being acted upon by molecular motors such as RNAP and the replisome and various potential ways to probe the genome and its processing via single-molecule techniques.



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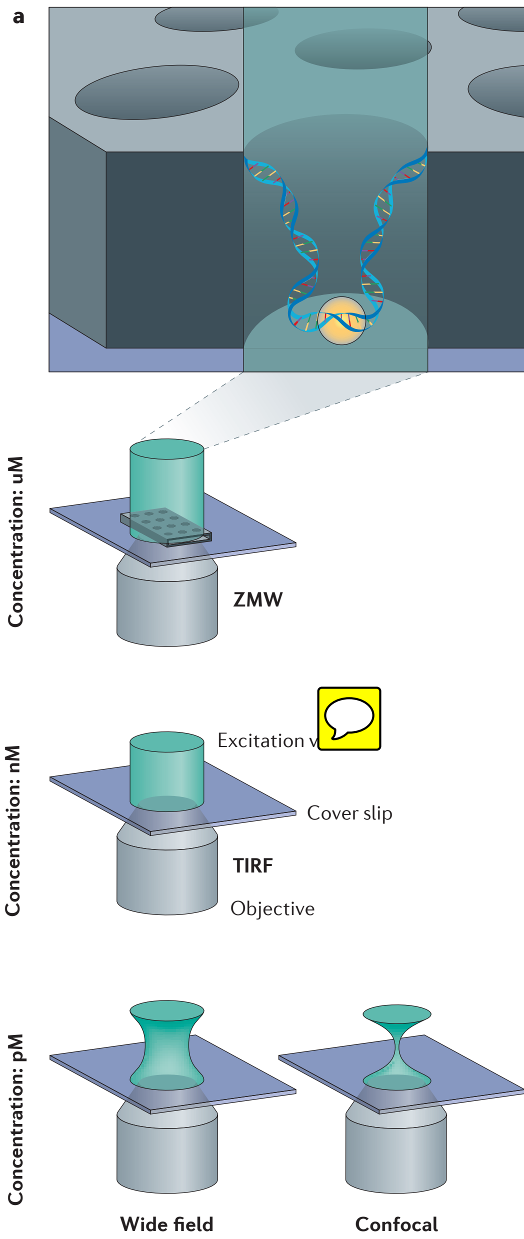

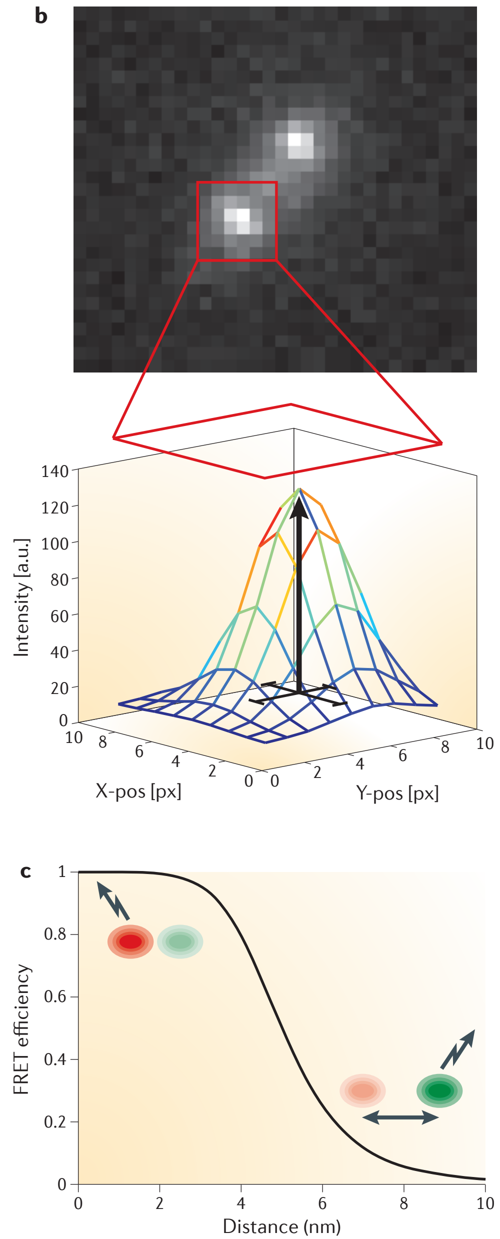

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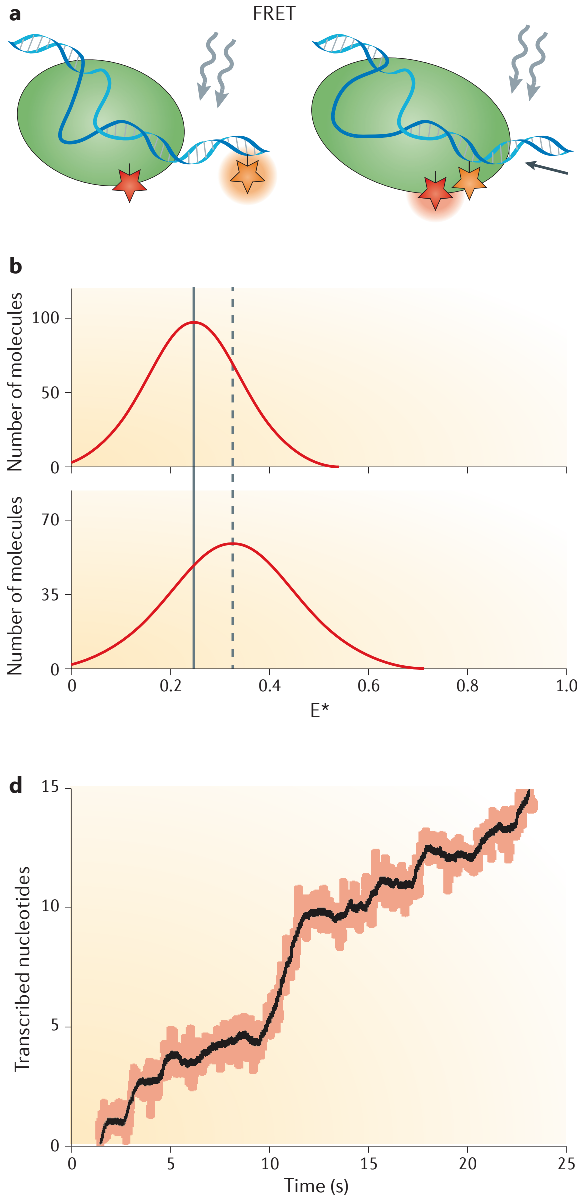

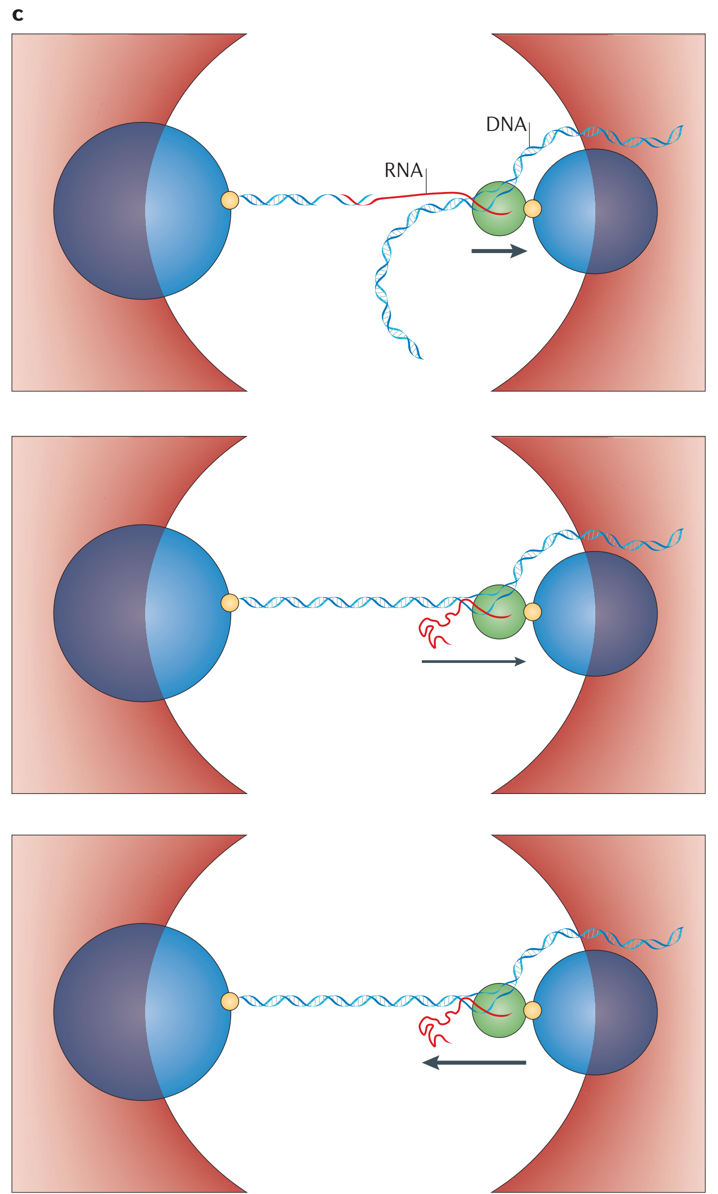

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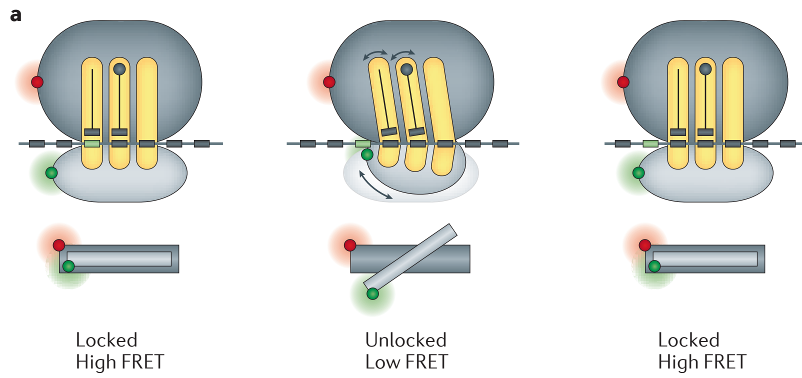

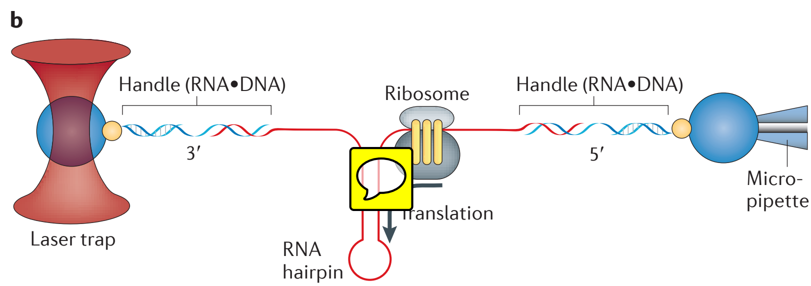

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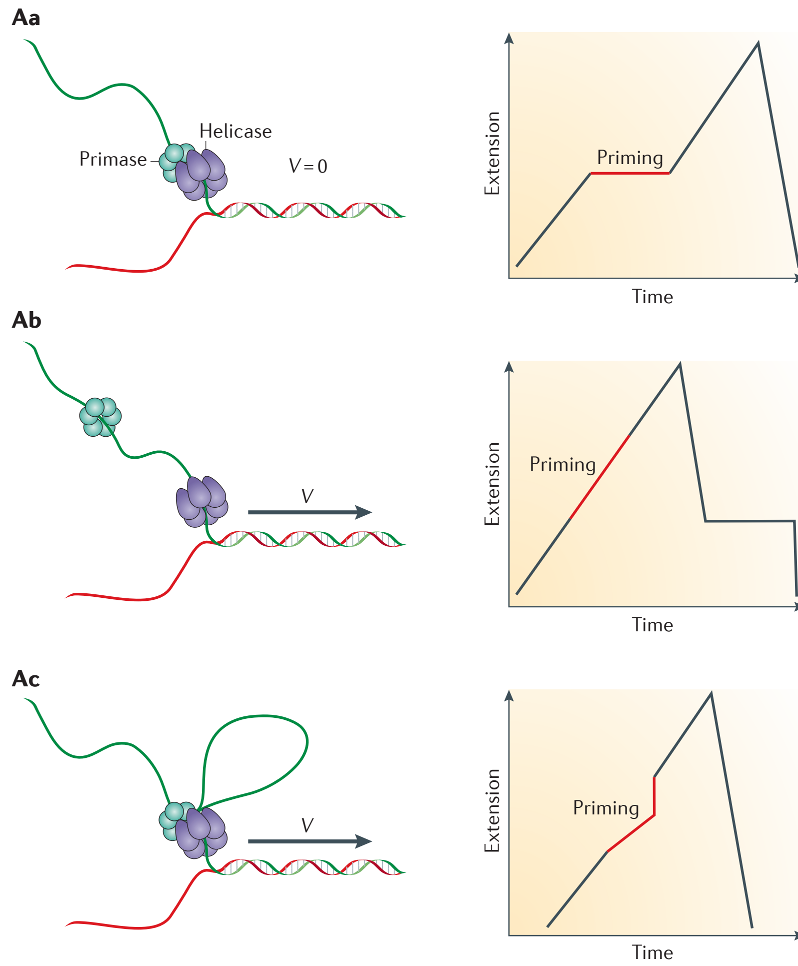

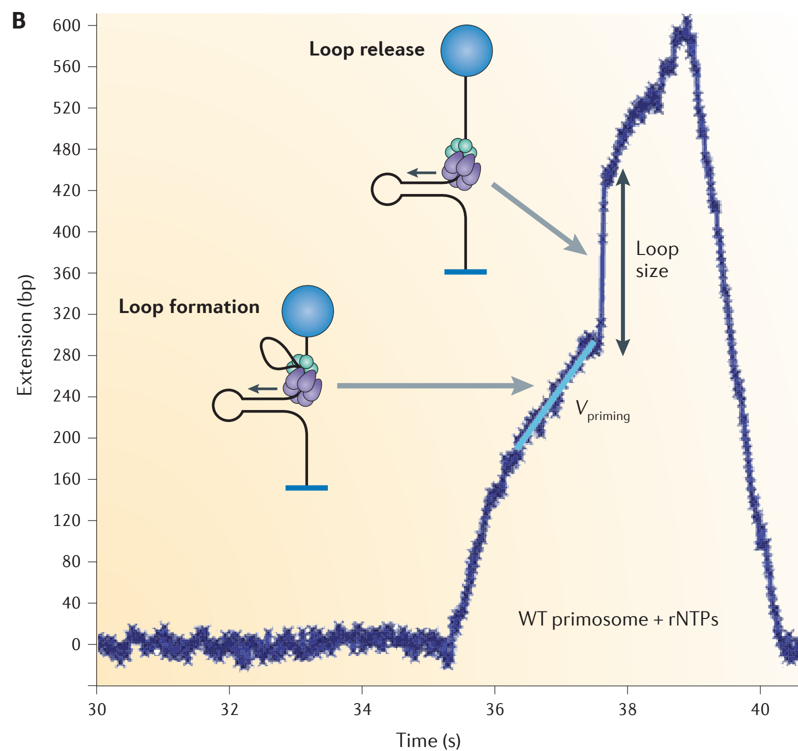

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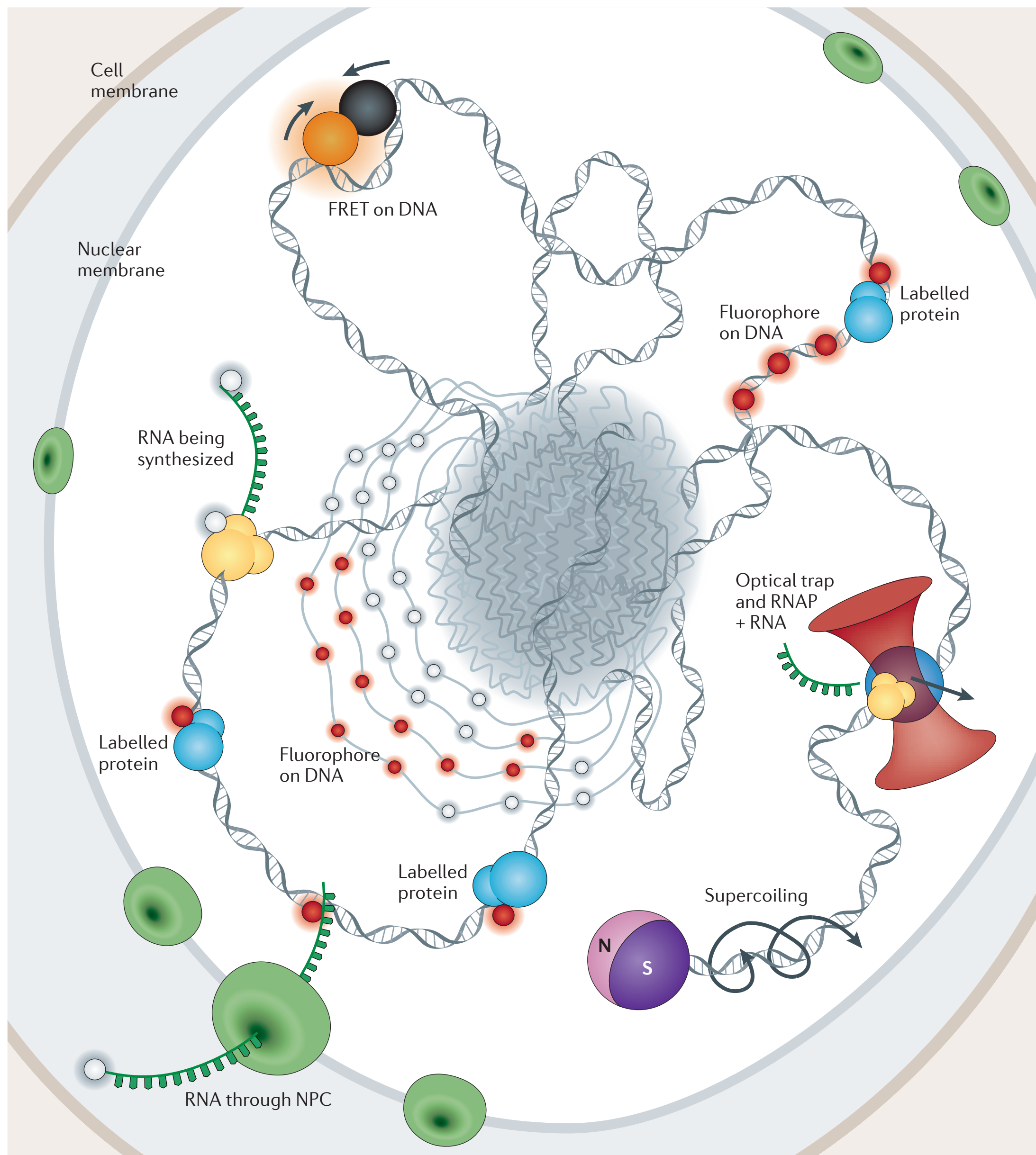

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