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Review

DNA replication machinery: Insights from *in vitro* single-molecule approaches

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ABSTRACT

The replisome is the multiprotein molecular machinery that replicates DNA. The replisome components work in precise coordination to unwind the double helix of the DNA and replicate the two strands simultaneously. The study of DNA replication using *in vitro* single-molecule approaches provides a novel quantitative understanding of the dynamics and mechanical principles that govern the operation of the replisome and its components. 'Classical' ensemble-averaging methods cannot obtain this information. Here we describe the main findings obtained with *in vitro* single-molecule methods on the performance of individual replisome components and reconstituted prokaryotic and eukaryotic replisomes. The emerging picture from these studies is that of stochastic, versatile and highly dynamic replisome machinery in which transient protein-protein and protein-DNA associations are responsible for robust DNA replication.

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Contents

1. Introduction

DNA replication is a fundamental process of life that has been a central focus of molecular biology. Only 5 years after the description of the double helical structure of the DNA in 1953 [1], the laboratory of Arthur Kornberg identified the first enzyme capable of synthesizing DNA, to which they referred as DNA polymerase (DNApol) [2,3]. More than 60 years later, we are still gathering evidence to fully understand the robustness and beautiful sophistication of DNA replication and its regulation. The fundamental principles of DNA replication are surprisingly similar from simple viral systems up to the more complex organisms. The elegant experiment of Meselson and Stahl, a few years after the discovery of DNA structure, demonstrated that DNA is replicated in a semi-conservative fashion in which the two original DNA strands sepa-

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rate and each one serves as a template for a new DNA strand [4], Fig. 1. The antiparallel nature of DNA strands and the 5′-3′ polarity of DNApol force one of the strands to be synthesized continuously (leading strand) while the other (lagging strand) is synthesized discontinuously in shorter segments (Okazaki fragments), which are later joined together [5]. Despite these differences, the synthesis of the two strands is coupled, and is carried out by the same replication apparatus, called the replisome. The replisome is constituted by a sophisticated molecular machinery in which DNApols work in coordination with a plethora of other molecular motors (proteins that couple chemical energy to a mechanical task) and specialized proteins to unravel, synthesize, edit and move in one direction along mega-base-pair long genomes (Fig. 1). For example, the replisome of the bacterium *Escherichia coli* (*E.coli*, Fig. 1) is formed by at least 14 different protein subunits that synthetize DNA at rate up to 1,000 nucleotides per second with an accuracy of 1 wrong nucleotide incorporated every ~ 10^7 nucleotide polymerized [6]. A copyist with comparable skills would copy Don Quixote's novel (~1,500 pages) in approximately 30 min without making a single typo. Over the last 60 years, biochemical, structural and genetic studies have been pivotal for identifying the components of the replication machineries in different organisms, and defining their functions and structures [5,7,8]. What is still missing is a detailed quantitative understanding of the dynamics and mechanical principles that underlie the operation of these molecular motors and their interactions with their partners at the replisome.

In the last two decades, the advent of *in vitro* single-molecule detection and manipulation methods has finally allowed researchers to begin to fill this gap (for review see [9–17]). These biophysical methods share the ability to follow the real-time trajectories of individual molecules with nanometer (<10 nm) and millisecond spatial–temporal resolutions [18–26]. In this way, rare or transient events of a reaction usually averaged out by ensemble techniques, such as pauses, backtrackings, and rate fluctuations become apparent, providing a dynamic picture of the reaction. Besides, *in vitro* single-molecule manipulation methods can be used to exert calibrated forces (0.1–100 piconewtons) on single biological molecules, and measure the forces that result from their operation. Direct access to these mechanical forces provides a unique opportunity to quantify the coupling of mechanical (motion) and chemical reactions that govern the operation of molecular motors [20]. Briefly, two main groups of techniques are being currently used to study DNA replication *in singulo*: fluorescence spectroscopy and force spectroscopy. Fluorescence-based single-molecule techniques allow the real-time observation of the trajectory of molecules labeled with single fluorophores, which are excited with a laser of the appropriate wavelength. There are two complementary fluorescence techniques that differ in their excitation and detection modalities, total internal reflection fluorescence (TIRF) and confocal [26]. When two different fluorophores are attached to the system of interest, single-molecule fluorescence resonance energy transfer (smFRET) can be measured between them [22,24]. The fluorophores can be attached to different molecules to study their association and relative movements or alternatively, to different sites of the same molecule, allowing the measurement of conformational changes. In force spectroscopy methods [21], the dynamics of the protein acting on DNA are obtained by attaching the protein DNA-complex under study between a surface and a micron-size bead that is subjected to an external field. The nature of this external field, which dictates some of the main pros and cons of each technique, can be magnetic (magnetic tweezers), photonic (optical tweezers) or hydrodynamic (tethered particle techniques). The basic principles of operation of some of these techniques are briefly explained in the legends of Figs. 2, 3, 4 and 6.

Here, we review the main highlights of recent *in vitro* single-molecule studies of some of the replisome's main components; replicative DNApols, helicases, and single-stranded DNA binding proteins (SSBs) as well as recent developments in single-molecule research on fully or partially reconstituted replisomes.

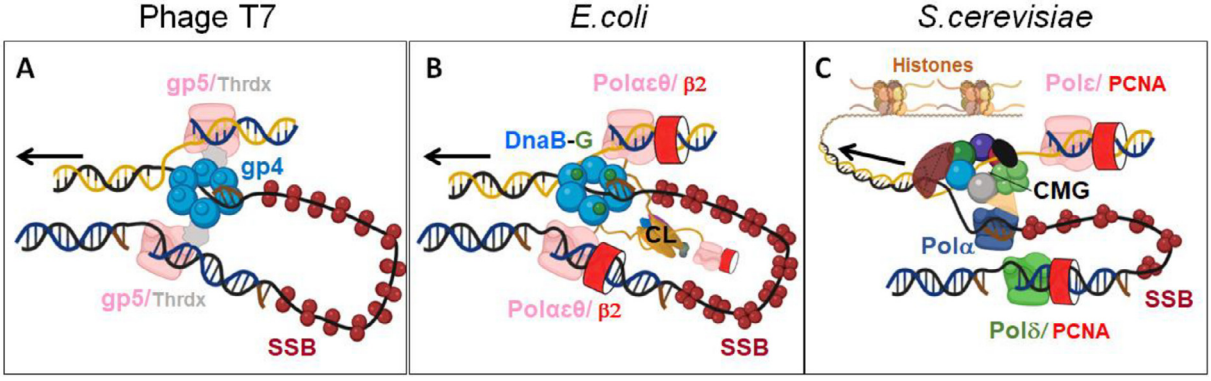

Fig. 1. Schematic representations of replisomes of increasing complexity. For all figures arrows show the direction of the replication fork and leading strand (top) is depicted yellow and lagging strand (bottom) black. A) The bacteriophage T7 replisome is formed by 4 proteins: DNApol (gp5) and its processivity factor thioredoxin (Thrdx), the helicase-primase (gp4) and SSBs (gp2.5). The helicase (gp4) translocates in 5′–3′ direction on the lagging strand and synthesizes primers (brown) for the discontinuous synthesis of the lagging strand. Two or more DNApols (gp5) interact with the C-terminal tail of the helicase and replicate the two DNA strands. DNApols can also exchange with external DNApols at forks. The SSB gp2.5 covers exposed ssDNA regions and interacts with the DNApols and the helicase, regulating their activities. B) The *Escherichia coli* (*E.coli*) replisome is composed of at least 14 different protein subunits. The DnaB helicase translocates in 5′–3′ direction on the lagging strand, promotes strand separation, and interacts transiently with one or more DnaG primases for RNA priming (brown). The DNApol III holoenzyme is responsible for DNA synthesis and is made up of three subassemblies: (i) the αεθ core polymerase complex that copy DNA, (ii) the β2 sliding clamp or processivity factor, and (iii) the seven-subunit clamp loader complex (CL) that loads β2 onto primer–template junctions and coordinates replication of the two strands. Up to three, readily exchangeable, core polymerase complexes bind to each fork. The coordinated synthesis of the two strands could be the outcome of the stochastic behavior of the DNApols at each strand. The SSB protein protects ssDNA and promotes helicase and DNApol activities. C) Up to 34 protein subunits built up the eukaryotic *S. cerevisiae* core replisome. The key components include: i) the 11-subunit heterohexameric CMG helicase that translocates on the leading strand in 3′–5′ direction, ii) three multi-subunit DNA polymerases: the leading-strand Pol ε, lagging-strand Pol δ, and Pol α-primase. Pol δ and Pol α are recycled to support the synthesis of multiple Okazaki fragments, iii) the replication factor C involved in attaching the processivity clamp (PCNA) to Pol δ, and iv) the RPA trimeric SSB protein. Numerous other proteins interact transiently with the eukaryotic replisome, some of which are known to be involved in checkpoint regulation or nucleosome handling, since in eukaryotes DNA is complexed to histones. (Adapted from [15]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Replicative DNA polymerases

Replicative DNA polymerases (DNApol) are the molecular motors responsible for synthesizing the new complementary strands of DNA. Helped by processivity factors, these enzymes use one strand of the DNA as a template and catalyze a processive stepwise addition of the corresponding complementary deoxynucleoside triphosphate (dNTP) on to the terminal 3′ end of the nascent DNA strand (primer).

The dNTP incorporation cycle involves large conformational changes of the DNApol subdomain referred to as the fingers, which pivots between 'open and close' positions in response to dNTP binding and hydrolysis reactions (Fig. 2) [27–29]. Structural and computational studies suggested that this conformational change could be coupled with translocation directly, pushing or pulling the DNApol to the next template position (power stroke models [30,31]). In contrast, *in vitro* single-molecule nanopore [32–36], and optical tweezers [37] studies argued for a Brownian ratchet mechanism. According to this model, upon nucleotide incorporation, the DNApol diffuses freely between pre- and post-translocated states, and binding of the correct incoming dNTP stabilizes the post-translocated state [38]. The actual mechanism of translocation of DNA and RNA polymerases along nucleic acids is still a subject under investigation [39].

Accuracy during DNA replication is a must [40]. One of the two main factors contributing to fidelity is the ability of DNApols to select the dNTP complementary to the template strand. smFRET studies using the Klenow fragment of DNApol I as a model system followed the conformational dynamics of the fingers subdomain under various conditions and revealed the existence of previously unrecognized intermediates states within the open and closed transitions, Fig. 2 [41–48]. These states may serve as kinetic checkpoints to discriminate against incorrect substrates during the dNTP incorporation cycle, conferring to the fingers conformational dynamics a novel role in replication fidelity. The second main factor contributing to fidelity is the capacity of DNApols to excise misincorporated incorporated nucleotides at the exonucleolytic active site (*Exo*). This site is separated by up to ~ 60 Å from the polymerization active site (*Pol*) and only binds single-stranded DNA [30], which imposes tight structural and kinetic requirements for efficient primer strand transfer. Single-molecule fluorescence [45,49–53], and force spectroscopy, Fig. 3A and 3B, [54–58] studies on several replicative DNApols revealed that the primer transfer between the distant *Pol* and *Exo* sites, far from a one-step reaction,

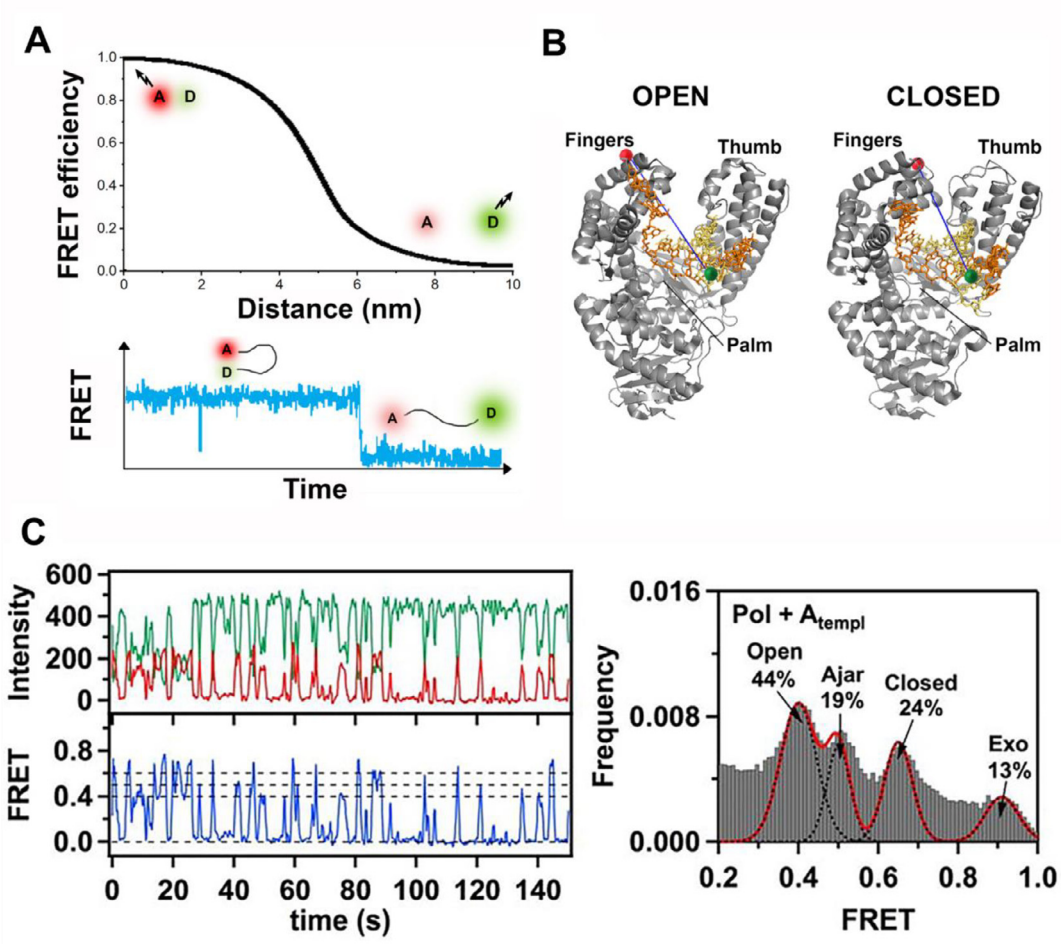

Fig. 2. Single-molecule Förster resonance energy transfer (smFRET). A) smFRET is based on the non-radiative energy transfer between nearby located donor (green) and acceptor (red) fluorophores, which results in a decrease in the donor (green) and a concomitant increase in the acceptor (red) fluorescence signals. Monitoring the degree of energy transfer reports on the distance and dynamics of intra- and inter- molecular interactions on the sub–10 nm scale. Bottom panel shows a characteristic trace of FRET efficiency depending on donor and acceptor proximity (adapted from [9]). B) Schematic illustration of labeling strategy used to probe the finger-closing conformational change in Pol I Klenow fragment. The donor fluorophore (green) is attached to the primer DNA and the acceptor fluorophore (red) to the tip of the fingers subdomain. As the fingers pivot between the open and closed positions the distance between the two fluctuates, which induce changes in FRET signal. C) Left panel. Characteristic fluorescence intensity time traces (green donor and red acceptor), and smFRET efficiency trajectories (blue) for a DNApol-DNA complex labeled as in B. FRET efficiency histograms (right panel) show 4 major populations that the authors assigned to the open, ajar (intermediate) and closed conformations of the fingers subdomain, and a population of DNA bound at the distant exonuclease site (B and C panels are adapted from [41]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

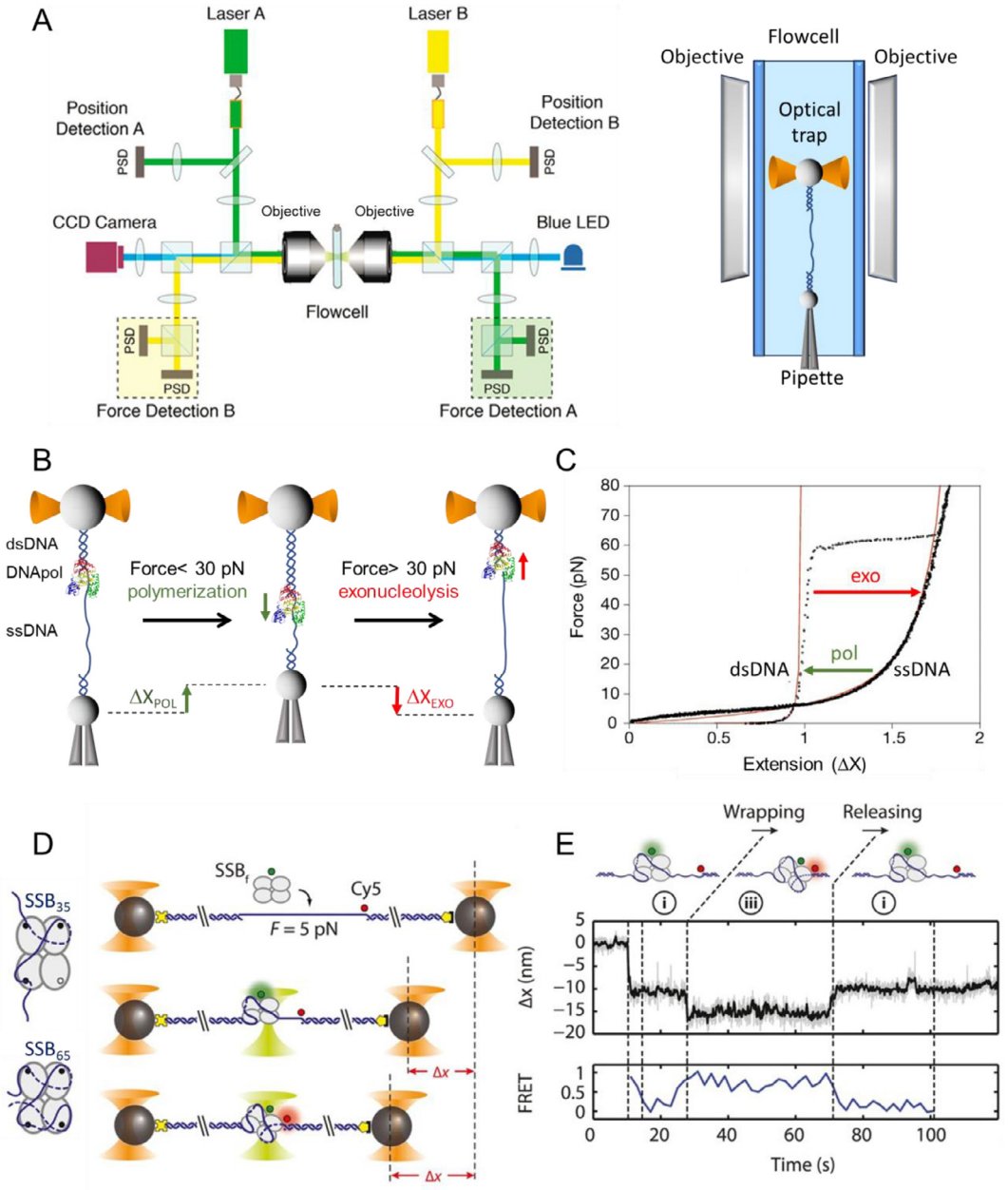

Fig. 3. Optical tweezers and 'Fleezers'. A): Diagram of a dual-beam optical tweezers setup. Two high numerical aperture objectives focus two counter-propagating 808 nm lasers, A (in green) and B (in yellow) inside a flow cell to form two optical traps. The position of each laser is controlled by piezo actuators. The two traps are superimposed in the same spatial position so that they function as one trap, effectively. To monitor the optical trap position beam-splitters divert a small percentage of the incoming light of each laser to position sensitive detectors (PSDs). The light leaving from each trap is sent to a different PSD to measure forces [100]. A CCD camera and a blue LED light (blue line) allow visualization of the interior of the flow cell (adapted from [101]). The panel on the right shows idealized lateral view of the flow cell showing a DNA molecule attached between two micron-sized polystyrene beads, one in the optical trap (orange cone) and the other on top of a micropipette. B) Experimental set-up to measure polymerization and exonucleolysis activities of individual DNApols with dual-beam optical tweezers [55]. A single DNA molecule containing a single-stranded gap is tethered to functionalized beads as in (A). At constant mechanical tension below 30 pN, the DNApol converts the single-stranded template (ssDNA) to double-stranded DNA (dsDNA). This activity is followed in real-time as a gradual shortening of the distance between the beads (Δx, green). Tension above 30–40 pN shifts the equilibrium towards the exonuclease activity, which is measured as a gradual increase in the distance between the beads (Δx, red). C) The force–extension curves of dsDNA and ssDNA can be described using polymer physics models (red lines) (reviewed in [102]). At constant force, the conversion from one polymer to the other by DNApol activities is captured as a change in extension D) Experimental set-up to measure the wrapping dynamics of *E.coli* SSB with a hybrid instrument that combines high-resolution optical tweezers with fluorescence detection (Fleezers, [103]). Polystyrene beads (grey) are held in separated optical traps (orange cones), tethered by a DNA molecule containing a short ssDNA region. The DNA is labeled with a FRET acceptor at the ss-dsDNA junction (red dot) and the SSB (tetramer) with the FRET donor (green dot). Fluorophores are excited by a ~ 500 nm laser (green cone). *E.coli* SSB binds to ssDNA and wraps either 35 or 65 nucleotides depending on the experimental conditions (as shown on the left diagram). ssDNA wrapping decreases the extension between the beads (Δx). E) Simultaneous measurement of tether extension (top) and FRET efficiency (bottom) enables determination of both the position of SSB along the tether and the amount of ssDNA wrapped (adapted from [104]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is a highly dynamic process that involves numerous conformational intermediate states along the proofreading pathway. These states may work as fidelity checkpoints essential to fine-tune the equilibrium between the *Pol* and *Exo* cycles required for robust but simultaneously faithful replication.

In addition, many DNApols present an intrinsic ability to unwind the DNA fork during replication. Ensemble measurements showed that this strand displacement activity is limited to a few nucleotides by the partition of the primer from the *Pol* to the *Exo* domains [59]. Magnetic and optical tweezers studies revealed that individual DNApols destabilize the fork's next base pair with an average energy of 1–2 k_BT per dNTP incorporated [60–62]. This energy is smaller than the average stability of the fork (~2.5 k_BT/ *bp*) explaining why a stably closed fork junction slows down the polymerization rate, induces frequent pauses (as observed in smFRET studies too [63]), and shifts eventually the equilibrium towards the *Exo* conformation. These processes prevent excessive strand displacement activity by the lagging DNApol, which, as shown by *in vitro* ensemble studies, is detrimental for primer removal during Okazaki fragment maturation [64,65]. During replication of the leading strand, engagement of the helicase (and presumably SSBs) with the displaced strand would help decrease the energy barrier for DNA unwinding, preventing the *Pol-Exo* partition. Under these conditions, both enzymes would coordinate their DNA unwinding properties to promote processive DNA replication [66,67].

3. Replicative DNA helicases

Replicative helicases form hexameric rings that utilize energy derived from binding and hydrolysis of nucleoside triphosphates (NTPs) to translocate along ssDNA and partially destabilize the fork junction to facilitate DNA unwinding [68,69], Fig. 4. Interestingly, eukaryotic (and archaeal) helicases form hetero-hexameric rings that encircle the leading strand in its central channel and translocate in the 3′−5′ direction. In contrast, their prokaryotic counterparts form homo-hexameric rings that encircle the lagging

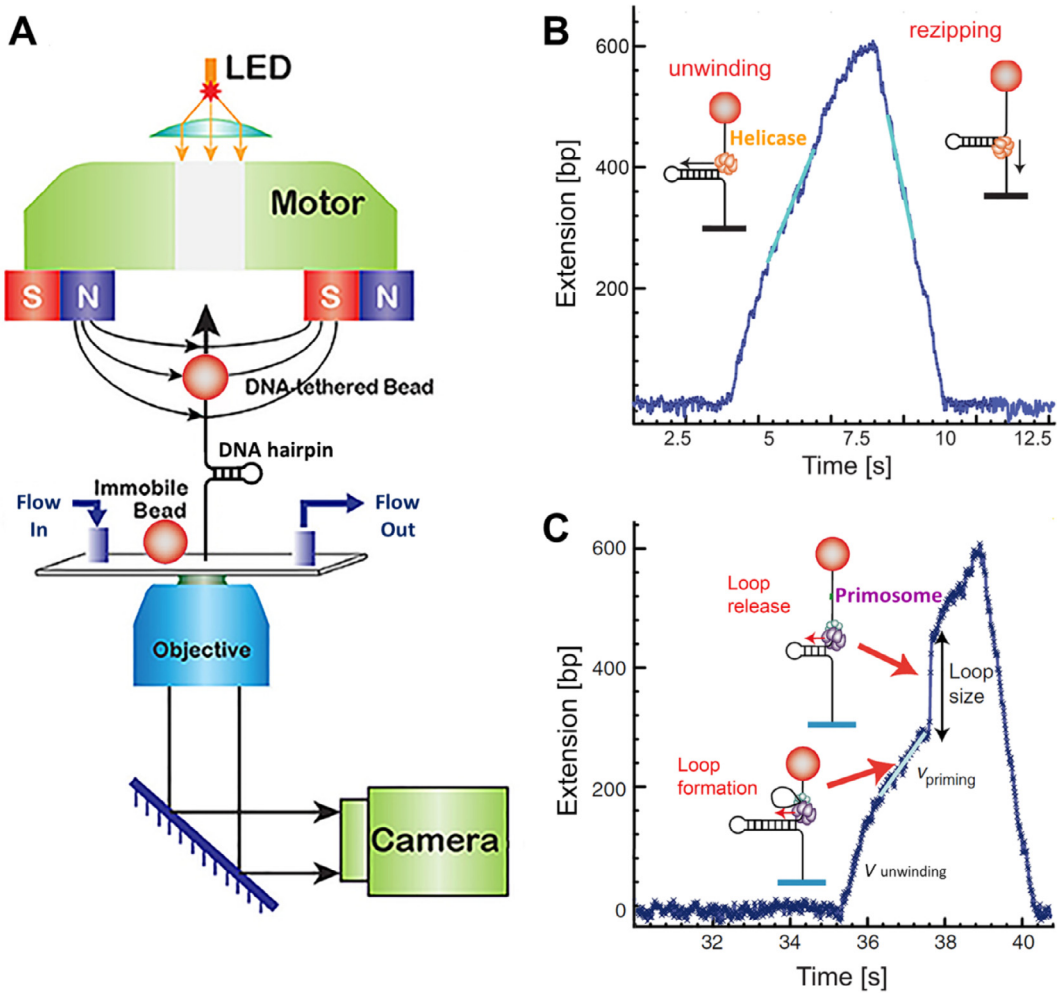

Fig. 4. Magnetic tweezers. A) Diagram of a magnetic tweezers setup. A paramagnetic bead is tethered to the surface of a flow cell via a functionalized DNA molecule. Beads stuck directly to the surface are used as a reference for drift correction. Permanent magnets produce magnetic field that pulls the bead in the direction of the field gradient (arrows). The orientation of magnetic field exerts horizontal and/ or vertical magnetic forces to stretch force or twist the DNA molecule. A CCD camera is used to follow in real-time the motion of the tethered bead. The changes in DNA extension are recorded in real time by computer-assisted analysis of the bead image (adapted from [133]). B) Representative DNA unwinding trace of a single T4 helicase using magnetic tweezers. A DNA hairpin is tethered between the paramagnetic bead and the flow cell surface. At constant tension, the DNA unwinding activity of the helicase opens the hairpin, which results in an increase of the DNA molecule extension. Upon full unwinding, hairpin rezipping rate is limited by helicase translocation rate on ssDNA (adapted from [134]). C) Detection of the T4 primosome helicase and priming activities on DNA hairpins using magnetic tweezers. Experimental run showing: *i*) initial DNA unwinding rate by the T4 helicase ($V_{unwinding}$), *ii*) apparent decrease in the unwinding rate due to priming loop formation ($V_{priming}$), and *iii*) sudden extension increase due to loop release upon primer synthesis by the primase (Loop size). After hairpin unwinding, rezipping is limited by helicase translocation on ssDNA (adapted from [135]).

strand and translocate in the 5′−3′ direction [70]. In both cases, unwinding of the fork is promoted by steric exclusion of the non-circled strand from the central channel [68,70–72]. In addition to DNA unwinding, hexameric helicases play a fundamental role as one of the central organizing centers of replisomes.

In vitro single-molecule studies have contributed significantly to decipher the operation of these molecular motors [73]. Together with ensemble studies, *in singulo* research showed that eukaryotic and archaeal helicases load onto duplex replication origin DNA as double-hexamers in a sequential manner [74–77]. Then, a set of 'firing factors' are required to convert each double hexamer into two active helicases competent for DNA unwinding and replisome progression [78–83]. Although the translocation mechanism of eukaryotic-type helicases is still under debate, magnetic tweezers studies suggested that the eukaryotic CMG translocate and unwinds DNA via an random walk biased by ATP binding/hydrolysis with a high propensity to pause in the absence of accessory factors [84]. For prokaryotic helicases, single-molecule studies (together with biochemical and structural measurements) supported a sequential hand-over-hand translocation mechanism with an overall kinetic step size of 1 bp/NTP, which may depend on the sequence context [85–88].

Overall, single-molecule studies revealed that the real-time kinetics of replicative helicases is frequently interrupted by pauses and slipping events, and strand separation is the rate-limiting step of their mechano-chemical cycle [87,89–94]. The poor unwinding 'activeness' of replicative helicases would avoid replisome uncoupling upon DNApol stalling [95], and suggest that their activity would be strongly regulated within the replisome to achieve rapid and processive replication. In fact, single-molecule and bulk studies have shown that slippage and pause events decrease and DNA unwinding rates increase when the helicase works at the fork in coordination with DNApol, primases [66,67,86,96–99] and/or SSB proteins (see below).

4. Single-stranded DNA binding proteins (SSBs)

SSB proteins are essential for the replisome's proper operation and play pivotal roles during genome maintenance (for review [105,106]). During DNA replication, SSBs bind to the lagging strand with high affinity in a sequence-independent manner and constitute the nucleo-protein complex upon which other components of the replisome work. Many SSB contain several Oligosaccharide Binding domains (OB-folds), allowing them to bind a variable number of nucleotides *in vitro* (review in [107]). These different binding modes may be used selectively in different DNA maintenance processes [108].

Single-molecule studies have revealed new information about the equilibrium constants and energetics of the binding of several prokaryotic and eukaryotic SSBs to individual ssDNA molecules [109–120]. One of the most extensively studied SSB proteins at the single-molecule level is the homo-tetrameric SSB of *E. coli* (EcoSSB). Depending on the ionic conditions and SSB density on ssDNA, EcoSSB wraps *in vitro* ~ 17, 35 or (56)65 nucleotides/ tetramer [121]. smFRET and force spectroscopy measurements uncovered a highly dynamic binding of EcoSSB to ssDNA, in which the major binding modes can interchange reversibly in discrete steps [104,122] and, individual EcoSSB tetramers can diffuse along ssDNA by a reptation mechanism [123,124] while in different binding modes [104], Fig. 3D and 3E. These results explained how EcoSSB could be redistributed along ssDNA by genome maintenance proteins and remain tightly bound to ssDNA. Diffusion along ssDNA has also been reported at the single-molecule level for other SSB proteins [109,120,125]. Also, single-molecule fluorescence and force spectroscopy studies showed that on long ssDNA segments, EcoSSB can interact with distant intramolecular sites [126] and reposition itself via long-range intersegment transfer [127]. Single-molecule imaging of labeled EcoSSBs showed that intersegment transfer also occurs during DNA replication *in vitro and vivo* and SSB recycling for multiple Okazaki fragments would depend on the concentration of competing SSBs in solution [128]. Concentration dependent exchange was also reported at the single-molecule level for the eukaryotic RPA SSB protein [110].

In vitro single-molecule studies also revealed that at the replication fork SSBs stimulate the average rates and processivity of the lagging and leading strand DNApols as well as those of the replicative helicases, by establishing functional and/or physical interactions with these molecular motors [80,129–131]. Simultaneously, the gradual release of the lagging strand during DNA replication has been shown to select the binding mode of the human mitochondrial SSB [132], highlighting the reciprocal interactions between the replisome components at the fork.

5. Replication machineries: Replisomes

The composition of the replisome varies among different organisms extensively. However, the structure and physical–chemical properties of the DNA impose basic operating principles to replisomes, Fig. 1. Next, we will summarize the main findings of single-molecule studies on the operation of model prokaryotic and eukaryotic replisomes.

As the first step for DNA replication, the replisome components assemble at the replication origin. smFRET studies revealed the ATP-dependent assembly pathway of the T4 replisome. Interestingly, while the T4 DNApol could use multiple pathways to load on the leading strand [136], the primosome (helicase and primase) assembles into the lagging strand in a single and orderly fashion [137,138]. Upon helicase loading, 1–3 primase molecules bind to the helicase hexamer, which in turn, stabilizes the complex on the DNA fork and stimulates helicase activity [138–140]. Simultaneously, helicase loading turns on the activity of the leading strand DNApol holoenzyme [141]. Overall, these results showed a finely-tuned orchestration between replisome components to ensure a proper replisome assembly on to the DNA.

The antiparallel nature of the lagging and leading DNA strands forces a precise series of highly coordinated events within the replisome to ensure the synchronized synthesis of the two strands. On the one hand, leading and lagging strand DNApols move in opposite directions (Fig. 1). In prokaryotes, ensemble studies showed that this problem is solved by forming of a 'trombone loop' in the lagging strand to reorient the lagging-strand DNApol to advance in parallel with its leading-strand counterpart, Fig. 1 [142,143]. Single-molecule fluorescence and flow stretching assays with the reconstituted T7 replisome followed the dynamics of 'trombone loop' formation and revealed that two events ensure the timely release of loops: the primer synthesis and the actual completion of the Okazaki fragment [144]. On the other hand, the primosome faces a similar directionality problem; the primase (usually associated with the helicase) makes primers opposite to helicase movement (Fig. 1). Single-molecule studies showed that T7 and T4 replisome components overcame this problem by the transient formation of a 'priming loop' between the helicase and the primase [135,145], Fig. 4C and Fig. 5. This mechanism keeps the primer in physical proximity to the replication complex and ensures hand-off to the lagging-strand polymerase without transiently blocking the replisome advance [145]. The T4 study also showed that one of the primase subunits can dissociate from the primosome complex to remain with the newly synthesized primer [135].

The discontinuous synthesis of the lagging strand also requires either replacement or recycling of the lagging DNApol to the next

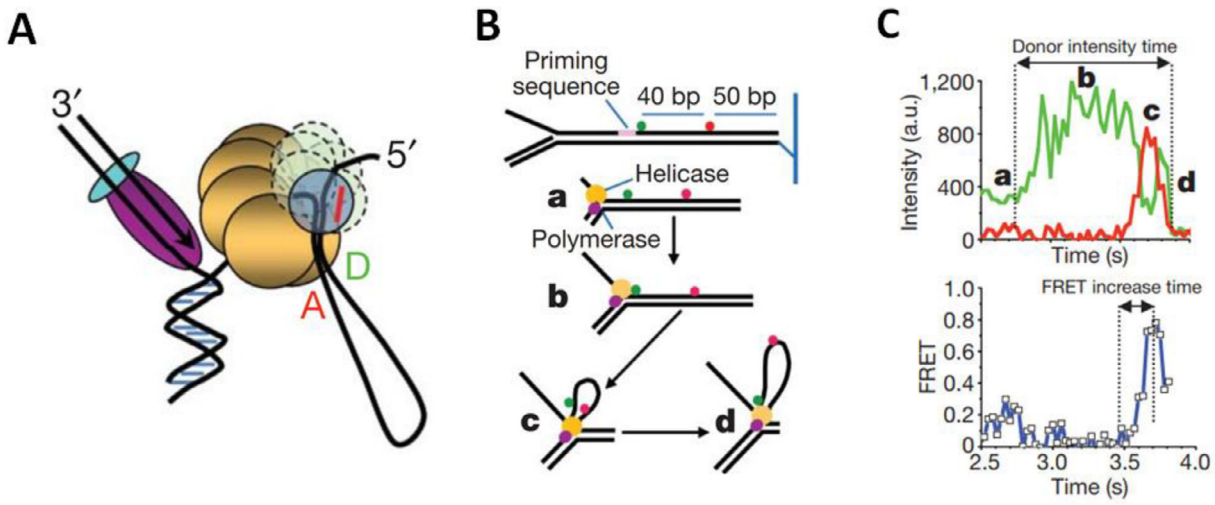

Fig. 5. smFRET detection of priming loop formation by the T7 replisome. A) Diagram showing a priming loop during the activity of a partially reconstituted T7 replisome. In T7, helicase and primase activities are carried out by the same polypeptide (gp4). During primer synthesis (red line), the excess DNA unwound by the helicase activity loops out allowing the primase-DNA interaction to stay intact as leading strand synthesis proceeds. Red A and green D, represent DNA bound acceptor and donor fluorophores, respectively, used to detect primosome activity. B) Schematic representation of fluorescently labelled DNA fork to investigate priming loop formation by smFRET. Red and green dots show the location of the acceptor and donor fluorophores, respectively, with respect to the priming sequence (pink). C) smFRET unwinding assays show: a) Before DNA unwinding the distance between the two fluorophores prevents FRET (bottom plot). b) As the T7 replisome unwinds the dsDNA, the donor shows an increase in intensity (green trace) due to protein-induced fluorescence enhancement. c) When the replisome reaches the priming sequence, the primase domain engages the lagging strand at this position causing the acceptor (red trace) to come close to the donor, as DNA unwinding continues. This event is detected as an increase in FRET. d) As the priming loop grows in size the donor and acceptor move apart, this was detected as a decrease in FRET. Adapted from [145]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Okazaki fragment. *In vitro* single-molecule fluorescence studies showed that the T7 replisome addresses this issue by associating several DNApols with the replisome [96,146], which are exchanged continuously at the lagging strand at a frequency similar to that of Okazaki fragment synthesis [147]. In addition, some of the lagging strand DNApols can be released from the replisome to complete Okazaki fragment synthesis behind and independent of the replication complex [148]. Similarly, ensemble and *in vivo* studies revealed that the *E.coli* replisome also contains more than two DNApols; Up to three DNApol (Pol III) cores could work in coordination and exchange at the fork while remaining attached at the replisome [149,150], Fig. 1. A tripolymerase replisome has been shown to present functional advantages such as increased processivity and increased efficiency in lagging-strand synthesis [151]. In addition, *in vitro and in vivo* single molecule fluorescence experiments on T7 [146,147] and E. coli [152,153] replication systems showed that DNApols associated with the replisome can also be exchanged with other DNApols in solution in a concentration dependent manner. DNApol exchange was also demonstrated in ensembles studies for the bacteriophage T4 [136,154]. In addition to DNApols, dynamic exchange has been reported also between different types of polymerases [155–157] and for other components of the replisomes [158]. These observations depict the replisome as highly dynamic molecular entity. The dynamic exchange of polymerases at the fork, by molecules already associated with the replisome or by proteins in solution, promotes the processivity of the replication complex and may allow the recruitment of factors necessary to correct lesions, overcome protein barriers in the DNA template, or replace a damaged polymerase without dismantling of the replisome structure [159].

Overall, the discontinuous synthesis of the lagging strand implies a series of 'slow' steps, which are not required for the continuous synthesis of the leading strand. Single-molecule studies suggested two alternative mechanisms to explain how the discontinuous lagging strand synthesis would keep pace with that of the leading strand: 1) the lagging strand synthesis or primase activity would halt the advance of the leading strand transiently [99,130,160,161]. 2) The lagging-strand DNApol synthesizes DNA faster than the leading strand polymerase [145]. Interestingly, the prevailing deterministic view of a coordinated synthesis of the two strands was challenged recently by *in vitro* single-molecule fluorescence (TIRF) studies with the reconstituted *E.coli* replisome [95]. This study presented clear evidence showing that instead of a deterministic coupling, the two strands could replicate autonomously, Fig. 6. The observed coordination would be the outcome of the stochastic behavior of the DNApols at each strand, which start, stop, and move at variable rates.

The emerging picture coming out from *in vitro* single-molecule studies in prokaryotes is that of a stochastic, dynamic replisome in which protein–protein and protein-DNA associations are continually broken and reformed. Interestingly, recent *in vitro* [162,163] and *in vivo* [164] single-molecule studies of *S. cerevisiae* replisome operation showed that in eukaryotes, the lagging strand DNApols, and other subunits of the replisome, also present dynamic exchange but associate more stably with the replisome than their prokaryotic counterparts, Fig. 7. These pioneer works point to relevant differences between the operational dynamics of prokaryotic and eukaryotic replisomes and forecast exciting discoveries in the near future.

6. Summary and outlook

In the last 60 years, the combination of biochemical, structural, genetic, and more recently, single-molecule approaches has provided a solid understanding of the molecular mechanisms underlying the complex choreography of the replisome components during replication of the double helix of the DNA. We have identified the leading dancers, their looks and their roles. We have also begun to realize the stochastic nature and the high adaptability of the replisome machinery, which changes its composition and operation mode continuously. This property would play an essential role in coping with constraints associated with the various stages of DNA replication and would ensure robust replication under varying conditions.

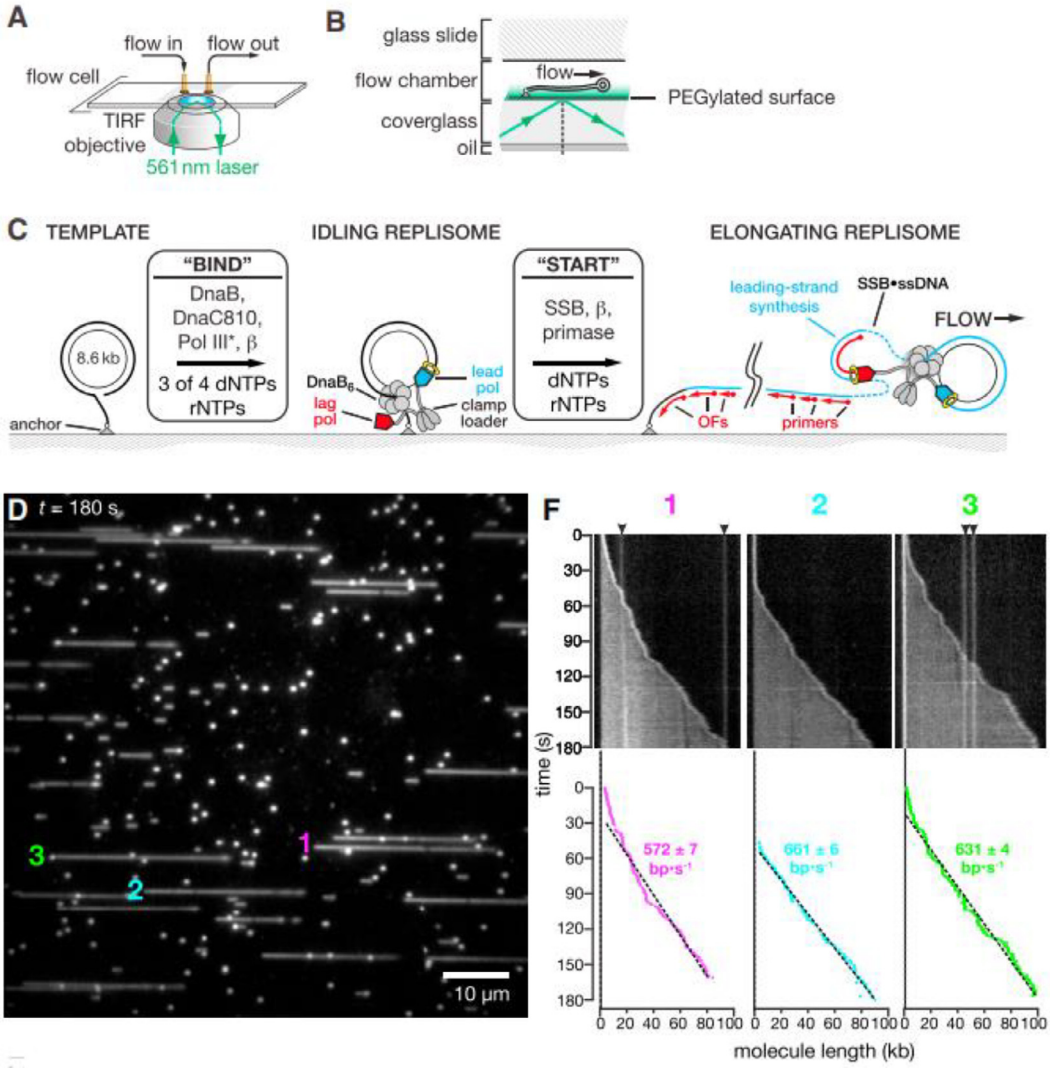

Fig. 6. Single-molecule TIRF assays to visualize leading- and lagging strand synthesis by the *E.coli* replisome. A) Schematic of a single-molecule TIRF microscope and flow-cell. TIRF microscopy use evanescent waves to excite only those molecules located within ~100 nm of the surface, substantially reducing the background fluorescence. B) Side-on view of the flow cell, showing surface-attached DNA, flow direction, the excitation beam (561 nm, green lines) and the evanescent wave range (green). C) Diagram of the rolling-circle assay to detect single-turnover replisome progressions. The template was adsorbed onto a cover-glass via biotin-streptavidin interaction. Upon assembly of a pre-initiation complex (BIND), replication was initiated (START) by introducing primase, clamp, SSB in the presence of all four dNTPs and rNTPs. D) dsDNA extension can be followed in real-time by stretching under flow (from left to right) in the presence of SYTOX Orange. The figure shows a representative field in which several circular template molecules (small foci at the start of reaction) are replicated to yield long products. F) Kymographs of three actively extending molecules (from D) showing the length of the replication product as a function of time. Bottom, linear fits to trajectories yield average rates of fork movement (magenta, cyan, and green traces). Adapted from [95]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

However, a complete understanding of the replisome operation will require defining the basic mechanistic, kinetic and dynamic processes that rule its operation at the molecular level and how these processes respond to external variables. Bottom-up *in vitro* single-molecule approaches, moving gradually from the study of individual components to increasingly complex replisomes, will help to set the path to addressing these challenges in combination with biochemical, structural and genetic advances. The assembly of robust reconstituted replisomes *in singulo* will be pivotal to exploring the effects of post-translational modifications, DNA roadblocks, DNA bound proteins, and disease-related mutations on the replisome operational dynamics.

In vitro single-molecule research will have to surmount several challenges and limitations to continue to play a relevant role in the study of the inner molecular workings driving DNA replication (and DNA metabolism, in general). Many of these challenges are currently the subject of intense research. One major limitation of some *in vitro* single-molecule technologies is their low throughput, which implies that acquisition of statistically significant results is very time consuming. Recent developments in acoustic force spectroscopy [165], multiplexing magnetic tweezers [166–168] and microfluidic systems [169] overcame this issue, at least partially, by allowing researchers to obtain increasingly large sets of high-resolution data. Furthermore, to elucidate the complex dynamics and mechano-chemical processes that govern the operation of a multi-nucleoprotein complex such as the replisome, it will be necessary to interrogate different variables of the system simultaneously. In this regard, the recent development of hybrid methods that combine single-molecule manipulation with fluorescence microscopy [103,170–175] will enable to correlate the real-time kinetics of DNA replication to the structural organization and/or to inter- or intramolecular structural changes of the replisome components. Progress in this area will be conditioned by advances in chemical methods that allow efficient fluorescent labeling of

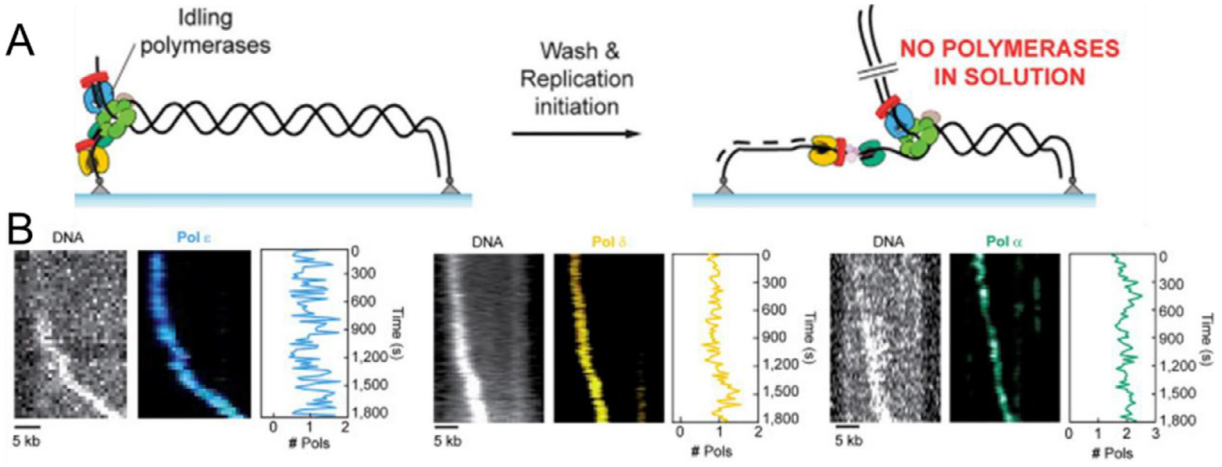

Fig. 7. Multicolor single-molecule TIRF assays to visualize simultaneously DNA synthesis and protein dynamics of the *S. cerevisiae* replisome. A) Schematic representation of the pre-assembly replication assay. A DNA molecule containing a premade replication fork at one end is attached at both ends to the surface of the flow cell of a TIRF microscope. Upon preassembly of the replisome, the flow cell is washed to remove the excess of DNApols and other replisome components and replication is initiated. B) Kymographs showing the advance of the replication fork and the stability and stoichiometry of eukaryotic DNApols. DNA was stained with SYTOX orange and Pol ε (blue), Pol δ (yellow), and Pol α-primase (green) were labeled fluorescently. As DNA synthesis proceeds, the leading strand appears as a diffraction-limited spot that moves along the template in one direction (left). All three DNA polymerases co-localize with the leading-strand spot during replication of thousands of nucleotides (center). This observation is consistent with a stable interaction of the DNApols with the replisome. The stoichiometry of each DNApol (right) was obtained by dividing the intensity at the fork by the intensity of a single polymerase. Adapted from [162]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins without affecting their function. Also, the combination of optical tweezers with precise temperature control systems [101], will help to define the crucial role of temperature on the real-time kinetics and mechano-chemistry of DNA replication. Another drawback of single-molecule manipulation methods is that manipulation is often restricted to a specific spatial coordinate. New optical tweezers set ups with multiple, freely adjustable optical traps will undoubtedly help to overcome this issue [176]. Ultimately, characterization of the real-time kinetics, dynamics and mechano-chemistry of individual replisomes in the context of molecular crowding characteristic of living cells will require transfer single-molecule position and force detection techniques to *in vivo* conditions. To this end, non-invasive approaches can be envisaged that make use of molecular force probes that change state as a function of the force applied to them [177,178] and/or spectroscopically stable fluorophores that change their emission spectra as a function of mechanical force [179,180]. As stated above, many of these challenges are currently the subject of significant interdisciplinary research, because 'the future is now'.

CRediT authorship contribution statement

Rebeca Bocanegra: Writing - original draft, Visualization. **Ismael Plaza G.A.:** Writing - original draft. **Carlos R. Pulido:** Writing - original draft. **Borja Ibarra:** Writing - original draft, Writing - review & editing, Visualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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