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Synergistic perspectives—How single-molecule biophysics complement biochemical understanding

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Single-molecule biophysical techniques enable the study of biological systems by directly observing individual molecules and events, rather than measuring population averages. These approaches reveal transient states, rare events, conformational dynamics, and subpopulations that are often obscured in biochemical assays. The application of single-molecule methods has been limited by technical complexity and the need for specialized instrumentation. Recent standardization of protocols, methodologies, and platforms has lowered these barriers, enabling broader adoption and promoting interdisciplinary collaborations that facilitate integration into biochemical research. In this review, we discuss how ensemble biochemistry and single-molecule approaches are complementary, outline commonly used single-molecule techniques, and illustrate their relevance through two representative case studies: chromatin organization by SMC complexes and pathway choice during DNA double-strand break repair.

Keywords: biophysics; chromatin organization; DNA repair; DNA–protein interactions; single molecule

The value of single-molecule resolution

Biological systems are composed of individual molecules whose collective behavior gives rise to cellular function. Such complex systems are most often studied using ensemble assays, in which signals originating from many individual molecular events are measured simultaneously and reported as a population average (Fig. 1A). Ensemble approaches have been instrumental in identifying molecular components, defining pathways, and quantifying activity rates and remain central to biology research [1,2]. However, population averages mask differences between individual molecules, sometimes leading to distinct populations being observed as equal (Fig. 1A) [3]. In addition, rare or short-lived events that may be critical for biological function are missed [4]. Lastly, subpopulations or localized processes often do not measurably perturb the global equilibrium of a system. As a result, their signatures become diluted within ensemble signals and are difficult to detect.

Single-molecule approaches address these ensemble-average limitations by directly observing individual molecules and molecular events (Fig. 1B). This shift in perspective reveals stochastic dynamics, molecular heterogeneity, and nonequilibrium processes that are fundamental features of biological systems at the molecular scale [5].

Abbreviations
53BP1, p53-binding protein 1; AFM, Atomic force microscopy; ATP, Adenosine triphosphate; ATPase, Enzyme that hydrolyzes ATP; BARD1, BRCA1-associated RING domain protein 1; BRCA1, Breast cancer type 1 susceptibility protein; CtIP, CtBP-interacting protein; DNA, Deoxyribonucleic acid; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, Double-strand break; HR, Homologous recombination; Ku, Ku70 and Ku80 heterodimer; MP, Mass photometry; MRN, MRE11, RAD50 and NBS1 protein complex; MT, Magnetic tweezers; NHEJ, Nonhomologous end joining; OT, Optical tweezers; SMC, Structural maintenance of chromosomes; smFRET, single-molecule Förster (or Fluorescence) resonance energy transfer; TIRFM, Total internal reflection fluorescence microscopy.

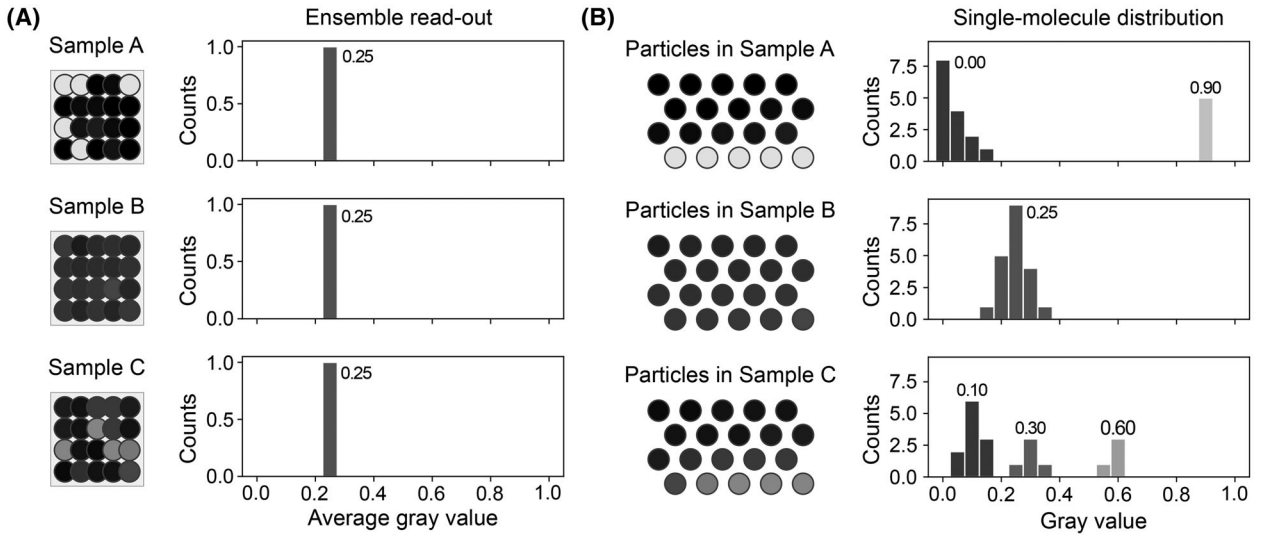

Fig. 1. Single-molecule techniques see beyond ensemble averages. (A) Three distinct populations yield the same average gray value in an ensemble read-out. (B) Single-molecule techniques reveal the distribution of gray values and their relative abundances within populations, enabling distinction of rare events and subpopulations. Note that any standard deviation in bulk experiments must come from experimental repetition, whereas single-molecule experiments reveal distributions directly even within the same experiment.

In this review, we introduce the basic principles underlying single-molecule biophysics and explain how they complement ensemble biochemical assays, focusing on systems probing DNA–protein interactions. Through selected examples, we illustrate how the combination of biochemical specificity and single-molecule resolution enables a deeper, more quantitative understanding of fundamental biological processes.

Quantifying individual molecular behavior

Single-molecule techniques provide access to biological information often hidden in ensemble biochemical measurements. In Table 1, we outline common experimental approaches and the main categories of information accessible through these single-molecule measurements. We refer the reader to suggested literature to explore the techniques in more detail.

One of the fields that has benefited significantly from the combination of biochemistry and single-molecule biophysics is understanding protein–DNA interactions. This field includes the study of DNA replication, transcription, chromosome organization, and DNA repair, all of which involve proteins dynamically engaging with, moving along, or remodeling DNA molecules. Understanding the precise conditions and mechanisms, governing these binding interactions is fundamental to deciphering how the genome folds and functions. Bulk biochemistry provides essential data, such as average binding affinities through concentration-dependent titration assays, but is inherently limited by ensemble averaging. Consequently, the specific topological nature of the interactions and the existence of different binding modes remain undetected.

Fluorescence-based single-molecule approaches can fill this gap by direct visualization of protein binding and molecular movements. For example, with total internal reflection fluorescence microscopy (TIRFM), the interaction of labeled proteins with DNA stretched on a surface can be visualized in real time, extracting parameters such as sequence preference, protein dwell time, and even conformational changes to the DNA [17,18]. By using either flow stretching or DNA curtains, DNA molecules can be immobilized on a surface, providing a platform for TIRFM to track protein–DNA interactions in real time [19–21]. Another step can be taken by labeling proteins in clever ways to distinguish dynamic switches between binding conformations with single-molecule Förster resonance energy transfer (smFRET) [22,23]. This type of kinetic information is fundamental to understand how proteins search for target sequences on DNA molecules, or how they assemble into complexes once bound to DNA.

Fluorescently labeling proteins is not always convenient or necessary. Label-free techniques like mass photometry (MP) can also provide conformational information and molecule-to-molecule variability. MP functions as a molecular scale: It determines the

Table 1. Single-molecule techniques and examples of biological information they can uncover.

Single-molecule techniques	Examples of biological information	Ref.
Atomic force microscopy (AFM)	• Molecular structure (shape, size) • Complex assembly and oligomerization • Molecular interactions • Conformational states • Event-based dynamics (high-speed AFM) • Mechanical properties (force spectroscopy) • Energy landscapes (transition states and barriers)	[6]
Magnetic tweezers (MT)	• Kinetics (rates, dwell times, pausing, processivity) • Intermediate states and sequential steps • Mechanical response to force and torque • Force-extension behavior (DNA, RNA, hybrids) • Event-based dynamics and variability • Conformational changes (nuclei acids, proteins, . . .) • Energy landscapes (transition states and barriers)	[7]
Mass photometry (MP)	• Molecular mass • Stoichiometry and oligomerization • Sample heterogeneity and integrity • Binding/dissociation dynamics	[8,9]
Nanopores	• Molecular structure (shape, size) • Translocation dynamics • Event-based dynamics (binding and dissociation) • Conformational changes • Event-based dynamics and variability	[10]
Optical tweezers (OT)	• Kinetics (rates, dwell times, pausing, processivity) • Intermediate states and sequential steps • Mechanical response to force and torque • Force-extension behavior (DNA, RNA, hybrids) • Event-based dynamics and variability • Conformational changes (nuclei acids, proteins, . . .) • Energy landscapes (transition states and barriers)	[11,12]
Single-molecule FRET (smFRET)	• Molecular interactions (colocalization) • Conformational changes • Intermediate states and sequential steps • Kinetics (residence times, transition rates) • Event-based dynamics and variability	[13]
Single-molecule localization microscopy (SMLM)	• Spatial organization and heterogeneity • Molecular colocalization and complex assembly • Molecular dynamics (live-cell SMLM)	[14]
TIRF microscopy	• Spatial organization (position, diffusion) • Molecular colocalization • Molecular dynamics (binding, dissociation) • Kinetics (residence times, association rates) • Intermediate states and sequential steps • Event-based dynamics and variability	[15,16]

molecular mass of particles in solution from scattered light [8]. This parameter reveals protein–protein and protein–DNA complex stoichiometry, enabling rapid identification of oligomeric states in heterogeneous assemblies [9].

In cells, the DNA is constantly being pulled and twisted by molecular motors such as helicases, polymerases, and chromatin remodelers. To mimic such forces, typically on the piconewton scale, single-molecule force spectroscopy techniques, including optical tweezers (OT), magnetic tweezers (MT), and atomic force microscopy (AFM), can pull on individual DNA molecules. They thus enable controlled application of force while monitoring DNA extension or protein activity in real time [24,25]. The resulting force-responses can be used to determine the mechanical properties of DNA [26]. By making changes to the environment of the measurement, for example, by exchanging buffer conditions or protein availability, the effect of these changes can be quantified. Magnetic

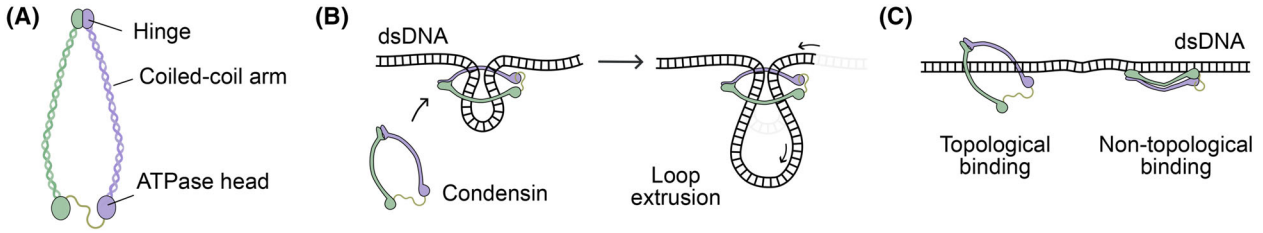

Fig. 2. SMC complexes. (A) Cartoon representation of a SMC complex. (B) SMC complexes can extrude loops from dsDNA. (C) Reported topological and nontopological binding modes of SMC.

tweezers provide the additional dimension of introducing torque on DNA, so the effect of proteins on supercoiling can be studied [27]. In some cases, force can also be used to study the stability of protein–protein or protein–DNA configurations, providing structural insight [28,29]. A primary example of how these techniques complement bulk biochemistry is in nucleosome topology. Biochemistry is used to assemble and estimate degree of nucleosome formation, while single-molecule force measurements can determine the exact unwrapping forces of inner and outer turns [30].

Force spectroscopy becomes particularly powerful to understand DNA–protein interactions when combined with fluorescence microscopy, as it allows simultaneous manipulation and visualization of protein–DNA systems [11]. As mentioned for TIRFM based approaches, visualizing protein–DNA interactions in real time reveals parameters such as sequence preference, and protein dwell time or mobility. By combining with force spectroscopy, the preference or resistance for certain DNA topologies can be revealed. For example, proteins can lose specificity when DNA is overstretched, or resist looping when there is too much force on the DNA. Force-fluorescence hybrid approaches are also particularly useful for studying complex biological processes that are otherwise difficult to access, such as protein interactions with single-stranded DNA [31–34], which is highly flexible and tends to collapse in solution, or interactions involving Holliday junctions, or other dynamic DNA structures [35–37].

Case studies: Insights gained from single-molecule approaches

Chromatin organization by SMC proteins

Structural maintenance of chromosomes (SMC) protein complexes are key players in chromatin organization. They are found in archaea, bacteria, and eukaryotes and possess a distinctive ring-like structure [38,39] (Fig. 2A). Although structure and composition vary between classes and species, the rings always include two SMC monomers with long, flexible coiled-coil regions. These are joined at one end by dimerized hinge domains and at the other end by a non-SMC bridge protein between their ATPase domains [38,40,41]. Eukaryotic cells have three classes of SMC complexes—cohesin, condensin, and SMC5/6—each exhibiting distinct roles in genome organization and DNA repair [39,40].

Before single-molecule techniques became prevalent, conventional biochemical assays revealed important characteristics, such as the structural architecture [42–45] and ATPase activity [46–48]. However, a mechanistic insight into the molecular mechanism underlying its function in chromatin organization remained lacking. It was hypothesized that SMC proteins play a role in genome organization by using their ATPase domains for motor activity, thereby enabling loop extrusion, where a small DNA loop is enlarged via active DNA translocation [41,49–51] (Fig. 2B). Although bulk biochemistry provided evidence for ATPase activity [47,48], evidence of loop extrusion was provided later by single-molecule work, where DNA flow stretching combined with fluorescent imaging showed single condensin molecules performing loop extrusion in real-time [51]. This direct observation was only possible through single-molecule work, resolving a long-standing debate in the field.

Although loop extrusion has been generally accepted as an organizational principle, the precise molecular steps remain unresolved [52]. Several models have been proposed including the Brownian ratchet [53], segment capture [54], and scrunching model [41,55]. An important yet unresolved aspect in loop extrusion is the loading of SMCs onto the DNA. This can either be topological, where the SMC protein encircles the DNA, pseudotopological where a growing DNA loop enters the SMC complex, or nontopological in which case DNA associates externally [56] (Fig. 2C). To study the DNA-binding mode of SMC complexes,

pull-down assays have been used to selectively copurify SMC complexes bound to circular DNA. The DNA is then cut to linearize it, hypothesizing that if the SMC complex binds topologically, it slips off the DNA, whereas it would remain associated with the linearized DNA if bound nontopologically, which is then detected by comigration on gel electrophoresis, or Southern blot. Using these biochemical assays, topological association somewhere within the SMC complex was found for condensin and SMC5/6 [57,58], whereas both topological and nontopological binding was shown for cohesin [59]. However, these works were not sufficient to deduce loading mechanism of SMC complexes. A single-molecule DNA flow-stretching experiment in combination with fluorescent imaging was designed to specifically address this gap at the single-molecule level. In these assays, the authors introduced a roadblock on the DNA, which was bigger than the ring size of the SMC complexes, and reasoned that a topologically bound protein would not be able to bypass it. However, they observed that both cohesin and condensin were able to traverse this roadblock, pointing toward a nontopological model. These direct observations in single-molecule observations thus contradicted previous ensemble measurements [60]. To provide further mechanistic insight into the loading, single-molecule techniques were also used to study the conformational changes within the SMC complexes. By using high-speed AFM, the dynamic protein conformation of the SMC complex was revealed [55,61]. One study showed the condensin complex cycling between an open and collapsed conformation [55], which aligns with another study where dynamic switching between open-arm and closed-ring state was observed in the cohesin complex [61].

Supercoiling by SMC complex condensin had long been observed in biochemical assays [62–64], and decades later, single-molecule measurements showed the mechanistic details of the process. The unique torque measurement capabilities of single-molecule magnetic tweezers measurements showed that all three SMC complexes induced negative supercoiling, while compensatory positive supercoils formed outside the extruded loop [65]. Furthermore, AFM and DNA flow-stretch experiments detected a preferential binding of SMCs to supercoiled DNA at the stem of an extruded loop, while generating supercoils [66].

Overall, these findings shed light on the intricate mechanisms by which SMC complexes efficiently organize DNA. Our current mechanistic understanding is the result of integrating insights from both biochemical and single-molecule studies.

DNA double-strand break repair: Pathway choice between NHEJ and HR

DNA in cells is constantly engaged in essential biological processes, such as replication and transcription, and is therefore susceptible to damage. In addition, exposure to endogenous metabolic byproducts or external agents, such as ionizing radiation, can also damage DNA. Among the different types of DNA lesions, double-strand breaks (DSBs), where both strands of DNA are severed, are particularly toxic [67,68]. Failure to properly repair these lesions can lead to mutations and inappropriate genome rearrangements underlying various human diseases [69–72]. To maintain genome integrity, eukaryotic cells rely on two main pathways to repair DSBs: nonhomologous end joining (NHEJ) and homologous recombination (HR) (Fig. 3). NHEJ is a fast process that directly

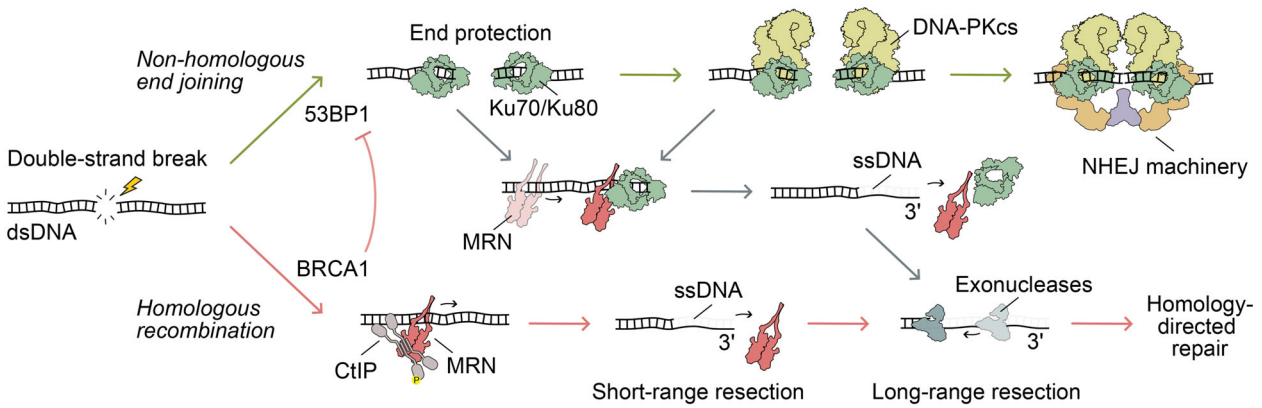

Fig. 3. Early steps of DNA double-strand break repair.

ligates DNA ends with minimal processing, whereas HR is a slower but more accurate pathway that requires a homologous DNA template [73,74]. The balance between these pathways must be tightly regulated, as it determines the trade-off between repair speed and fidelity. Ensemble cellular assays measuring cell survival and DNA damage sensitivity in repair-deficient mutants have established that both pathways play complementary roles in maintaining chromosomal integrity [75–78]. Structural and biochemical studies have elucidated the major protein complexes involved in both pathways and how they recognize and process the ends [79–84]. However, some aspects of DSB repair initiation remain unresolved, and single-molecule techniques are beginning to provide additional insights.

The cell cycle is one of the strongest inputs in the balance between NHEJ and HR. In G1, as HR is limited by the absence of a sister chromatid, NHEJ dominates. Shortly after a DSB forms, the abundant ring-shaped Ku70/Ku80 heterodimer (Ku) rapidly binds the exposed DNA ends with high affinity [85]. Once bound, Ku protects the DNA from nuclease activity and promotes classical NHEJ by recruiting the core NHEJ machinery, which includes DNA-PKcs [86,87] (Fig. 3). Concurrently, the multi-domain protein 53BP1 is recruited to the damaged chromatin where it protects the DNA ends from HR-associated nucleases [88,89].

In S/G2, repair regulators shift this balance. The complex formed by BRCA1-BARD1 counteracts 53BP1-dependent end protection, pushing repair toward HR [90–92]. The HR pathway is initiated through processing of the broken DNA ends, generating a 3′ overhang critical for homology search in downstream steps. This initial step, known as resection, starts with a minimal 5′-3′ processing mediated by the MRN complex together with CtIP [93]. BRCA1 also plays a structural role by forming a complex with CtIP and MRN at the DNA end, as shown by co-immunoprecipitation assays [94] and single-molecule super-resolution imaging in human cells [95]. Once the DNA ends undergo 5′-3′ resection by MRN, they are further processed by the exonucleases which extend the 3′ overhang (Fig. 3). Now, the ends are no longer compatible with classical NHEJ and repair becomes committed to a homology-based pathway.

However, even in S/G2 phase, when BRCA1-dependent HR is favored, binding of Ku to DSB ends is not prevented, nor is the subsequent recruitment of NHEJ machinery. As abundant Ku rapidly binds DNA ends upon DSB formation, the HR machinery must somehow overcome the Ku blocks at the DNA ends to initiate the HR pathway, starting with DNA end resection. Bulk cellular assays in which DSB ends were blocked with immobile Ku showed reduced DNA end resection and HR [96], supporting the idea that Ku had to be removed before HR initiation could occur. However, later single-molecule DNA curtains experiments, where multiple DNA molecules were monitored in the presence of fluorescently labeled proteins, revealed that Ku does not necessarily need to be removed before resection, but can instead be displaced during the resection process. This single-molecule study provided the first direct evidence that MRN can access and process Ku-bound DNA ends [97]. Specifically, it showed that MRN, either alone or in complex with CtIP, scans DNA by one-dimensional diffusion and, upon encountering a Ku-bound end, initiates endonuclease activity that promotes Ku eviction [97]. A later study, also using DNA curtains, revealed that the presence of Ku and DNA-PKcs at the DNA ends directly stimulates the MRN endonuclease activity, thereby promoting DNA end resection [98]. These findings contradict the earlier interpretation that Ku had to be removed before resection could initiate and instead suggest that Ku and DNA-PKcs actively contribute to resection initiation by stimulating end processing by the HR machinery: a level of mechanistic detail that could only be obtained through single-molecule approaches.

Single-molecule localization microscopy in human cells further confirmed that Ku removal specifically depends on MRN endonuclease activity, while being largely unaffected by MRN exonuclease activity [95]. More recently, smFRET experiments with *Xenopus* egg extract revealed that the MRN complex removes the NHEJ machinery from the DNA ends through an ATP-dependent translocase activity, a previously unrecognized function of MRN [99]. These transient mechanistic intermediates are masked in population-averaged read-outs.

Interestingly, while the examples discussed above demonstrate how NHEJ factors contribute to HR initiation, the opposite interplay has also been observed in human cells arrested in G1. In these cellular assays, limited MRN- and CtIP-dependent processing was required to remove protein blocks from DNA ends and promote NHEJ progression [100]. Together, ensemble-based and single-molecule observations strongly suggest that HR and NHEJ share common early intermediates that may remain partially reversible and mechanistically interconnected with pathway commitment emerging progressively as DNA end processing proceeds.

Historically, DSB repair pathway selection was viewed as a binary process based on a competition model, but there is now increasing evidence that repair initiation involves a coordinated and dynamic interplay between proteins from both pathways. Future studies combining biochemistry with direct single-molecule visualization of protein–DNA interactions will further reveal the mechanistic details of transient intermediates and clarify how this dynamic interplay regulates repair pathway balance across different chromatin contexts.

Conclusions and perspectives

Single-molecule biophysics has transformed how we study biological systems by allowing direct observation of individual molecules and events, revealing heterogeneity, rare states, and dynamic processes that are often hidden in ensemble measurements. At the same time, ensemble biochemical assays remain essential for defining molecular components, measuring population-level behavior, and establishing the broader regulatory framework of biological systems. In other words, single-molecule methods reveal molecular-scale behavior, while ensemble assays reveal the system-level behavior. Together, these methods form a comprehensive and quantitative toolkit for dissecting molecular mechanisms in biological systems.

In the past, single-molecule biophysics was largely confined to specialized laboratories with strong backgrounds in physics. Many experiments relied on custom-built instruments that had to be built and maintained in-house. This required expertise in optics, laser systems, and access to mechanical workshops and specialized tools. As a result, even though single-molecule approaches offered unique insights, they remained difficult to implement in most laboratories. Over the past decade, this situation has changed. The standardization of protocols, methodologies, and platforms has lowered technical barriers and enabled broader adoption. A growing number of companies now provide commercially available instruments for single-molecule experiments, including optical tweezers, fluorescence imaging technologies, or nanofabricated devices. As a result, some single-molecule experiments are no longer restricted to specialized laboratories and are becoming part of standard experimental workflows by direct adoption. At the same time, more technically demanding single-molecule experiments are being effectively exploited through collaborations with dedicated single-molecule biophysics laboratories.

Looking forward, advances in molecule throughput, data analysis, and hybrid experimental platforms will enable more comprehensive and quantitative studies. Beyond fundamental research, single-molecule approaches can be incorporated into industrial applications, from drug screening to material characterization at the molecular scale. As methods continue to mature and become more accessible, their potential for clinical and industrial applications is expected to grow. In addition, extending single-molecule techniques to more complex and physiologically relevant environments, including living cells, will help bridge the gap between molecular mechanisms and cellular function.

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Author contributions

SdB, ELJ, and JME wrote the manuscript. SdB and ELJ drafted the figures.

References

1 Ross J (2008) From the determination of complex reaction mechanisms to systems biology. *Annu Rev Biochem* **77**, 479–494.
2 (2004) In praise of biochemistry. *Nat Struct Mol Biol* **11**, 107.
3 Van Oijen AM and Dixon NE (2015) Probing molecular choreography through single-molecule biochemistry. *Nat Struct Mol Biol* **22**, 948–952.
4 Miller H, Zhou Z, Shepherd J, Wollman AJM and Leake MC (2018) Single-molecule techniques in biophysics: a review of the progress in methods and applications. *Rep Prog Phys* **81**, 024601.
5 Qian H and Kou SC (2014) Statistics and related topics in single-molecule biophysics. *Annu Rev Stat its Appl* **1**, 465–492.
6 Dumitru AC and Koehler M (2023) Recent advances in the application of atomic force microscopy to structural biology. *J Struct Biol* **215**, 107963.
7 Dulin D (2024) An introduction to magnetic tweezers. In Single Molecule Analysis: Methods and Protocols (Heller I, Dulin D and Peterman EJG, eds), pp. 375–401. Springer US, New York, NY.
8 Asor R and Kukura P (2022) Characterising biomolecular interactions and dynamics with mass photometry. *Curr Opin Chem Biol* **68**, 102132.
9 Sonn-Segev A, Belacic K, Bodrug T, Young G, VanderLinden RT, Schulman BA, Schimpf J, Friedrich T, Dip PV, Schwartz TU *et al.* (2020) Quantifying the

heterogeneity of macromolecular machines by mass photometry. *Nat Commun* **11**, 1772.
10 Ritmejeris J, Chen X and Dekker C (2025) Single-molecule protein sequencing with nanopores. *Nat Rev Bioeng* **3**, 303–316.
11 Chua GNL and Liu S (2024) When force met fluorescence: single-molecule manipulation and visualization of protein–DNA interactions. *Annu Rev Biophys* **53**, 169–191.
12 Favre-Bulle IA and Scott EK (2022) Optical tweezers across scales in cell biology. *Trends Cell Biol* **32**, 932–946.
13 Ha T, Fei J, Schmid S, Lee NK, Gonzalez RL, Paul S and Yeou S (2024) Fluorescence resonance energy transfer at the single-molecule level. *Nat Rev Methods Primers* **4**, 21.
14 Khater IM, Nabi IR and Hamarneh G (2020) A review of super-resolution single-molecule localization microscopy cluster analysis and quantification methods. *Patterns* **1**, 100038.
15 Woodhouse M and Crickard JB (2023) DNA curtains to visualize chromatin interactions. *Methods* **217**, 36–42.
16 Hilario J and Kowalczykowski SC (2010) Visualizing protein–DNA interactions at the single-molecule level. *Curr Opin Chem Biol* **14**, 15–22.
17 Mashanov GI, Tacon D, Knight AE, Peckham M and Molloy JE (2003) Visualizing single molecules inside living cells using total internal reflection fluorescence microscopy. *Methods* **29**, 142–152.
18 Lee NK, Kapanidis AN, Koh HR, Korlann Y, Ho SO, Kim Y, Gassman N, Kim SK and Weiss S (2007) Three-color alternating-laser excitation of single molecules: monitoring multiple interactions and distances. *Biophys J* **92**, 303–312.
19 Ganguli AK, Alsamsam MN, Bagdonaitė U, Vu VT, Huang C-J, Kuzhir P, Zaremba M, Kopūstas A and Tutkus M Single-molecule DNA flow-stretch assays for high-throughput DNA–protein interaction studies. *FEBS Open Bio*
20 Greene EC, Wind S, Fazio T, Gorman J and Visnapuu M-L (2010) DNA curtains for high-throughput single-molecule optical imaging. *Methods Enzymol* **472**, 293–315.
21 Collins BE, Ye LF, Duzdevich D and Greene EC (2014) DNA curtains: novel tools for imaging protein-nucleic acid interactions at the single-molecule level. *Methods Cell Biol* **123**, 217–234.
22 Schärfen L and Schlierf M (2019) Real-time monitoring of protein-induced DNA conformational changes using single-molecule FRET. *Methods* **169**, 11–20.
23 Agam G, Gebhardt C, Popara M, Mächtel R, Folz J, Ambrose B, Chamachi N, Chung SY, Craggs TD, de Boer M *et al*. (2023) Reliability and accuracy of single-molecule FRET studies for characterization of structural dynamics and distances in proteins. *Nat Methods* **20**, 523–535.
24 Ros R, Eckel R, Bartels F, Sischka A, Baumgarth B, Wilking SD, Pühler A, Sewald N, Becker A and Anselmetti D (2004) Single molecule force spectroscopy on ligand-DNA complexes: from molecular binding mechanisms to biosensor applications. *J Biotechnol* **112**, 5–12.
25 Chaurasiya KR, Paramanathan T, McCauley MJ and Williams MC (2010) Biophysical characterization of DNA binding from single molecule force measurements. *Phys Life Rev* **7**, 299–341.
26 Bustamante C, Smith SB, Liphardt J and Smith D (2000) Single-molecule studies of DNA mechanics. *Curr Opin Struct Biol* **10**, 279–285.
27 Kriegel F, Ermann N and Lipfert J (2017) Probing the mechanical properties, conformational changes, and interactions of nucleic acids with magnetic tweezers. *J Struct Biol* **197**, 26–36.
28 Williams MC and Rouzina I (2002) Force spectroscopy of single DNA and RNA molecules. *Curr Opin Struct Biol* **12**, 330–336.
29 Löf A, Walker PU, Sedlak SM, Gruber S, Obser T, Brehm MA, Benoit M and Lipfert J (2019) Multiplexed protein force spectroscopy reveals equilibrium protein folding dynamics and the low-force response of von Willebrand factor. *Proc Natl Acad Sci* **116**, 18798–18807.
30 Daris LJC and Eeftens JM (2026) Insights from single-molecule force spectroscopy into chromatin topology. *Biophys Rev* **18**, 265–273.
31 Belan O, Barroso C, Kaczmarczyk A, Anand R, Federico S, O'Reilly N, Newton MD, Maeots E, Enchev RI, Martinez-Perez E *et al*. (2021) Single-molecule analysis reveals cooperative stimulation of Rad51 filament nucleation and growth by mediator proteins. *Mol Cell* **81**, 1058–1073.
32 Hormeno S, Wilkinson OJ, Aicart-Ramos C, Kuppa S, Antony E, Dillingham MS and Moreno-Herrero F (2022) Human HELB is a processive motor protein that catalyzes RPA clearance from single-stranded DNA. *Proc Natl Acad Sci USA* **119**, e2112376119.
33 Greenhough LA, Liang C-C, Belan O, Kunzelmann S, Maslen S, Rodrigo-Brenni MC, Anand R, Skehel M, Boulton SJ and West SC (2023) Structure and function of the RAD51B–RAD51C–RAD51D–XRCC2 tumour suppressor. *Nature* **619**, 650–657.
34 Greenhough LA, Galanti L, Liang C-C, Boulton SJ and West SC (2026) Cryo-electron microscopic visualization of RAD51 filament assembly and end-capping by XRCC3-RAD51C-RAD51D-XRCC2. *Science* **391**, eaea1546.

35 Kaczmarczyk AP, Déclais A-C, Newton MD, Boulton SJ, Lilley DMJ and Rueda DS (2022) Search and processing of Holliday junctions within long DNA by junction-resolving enzymes. *Nat Commun* **13**, 5921.
36 Bragança S D, Aicart-Ramos C, Rivera-Calzada Á, Arribas-Bosacoma R, Dillingham MS, Llorca O and Moreno-Herrero F (2026) OT-curtains: an approach for studying protein interactions with DNA ends using optical tweezers and confocal fluorescence microscopy. *Nucleic Acids Res* **54**, gkag370.
37 Guo L, Bao Y, Zhao Y, Ren Z, Bi L, Zhang X, Liu C, Hou X, Wang MD and Sun B (2023) Joint efforts of replicative helicase and SSB ensure inherent replicative tolerance of G-quadruplex. *Adv Sci* **11**, 2307696.
38 Bürmann F and Löwe J (2023) Structural biology of SMC complexes across the tree of life. *Curr Opin Struct Biol* **80**, 102598.
39 Hoencamp C and Rowland BD (2023) Genome control by SMC complexes. *Nat Rev Mol Cell Biol* **24**, 633–650.
40 Oldenkamp R and Rowland BD (2022) A walk through the SMC cycle: from catching DNAs to shaping the genome. *Mol Cell* **82**, 1616–1630.
41 Higashi TL and Uhlmann F (2022) SMC complexes: lifting the lid on loop extrusion. *Curr Opin Cell Biol* **74**, 13–22.
42 Haering CH, Löwe J, Hochwagen A and Nasmyth K (2002) Molecular architecture of SMC proteins and the yeast Cohesin complex. *Mol Cell* **9**, 773–788.
43 Schleiffer A, Kaitna S, Maurer-Stroh S, Glotzer M, Nasmyth K and Eisenhaber F (2003) Kleisins: a superfamily of bacterial and eukaryotic SMC protein partners. *Mol Cell* **11**, 571–575.
44 Krepel D, Cheng RR, Di Pierro M and Onuchic JN (2018) Deciphering the structure of the condensin protein complex. *Proc Natl Acad Sci* **115**, 11911–11916.
45 Lammens A, Schele A and Hopfner K-P (2004) Structural biochemistry of ATP-driven dimerization and DNA-stimulated activation of SMC ATPases. *Curr Biol* **14**, 1778–1782.
46 Gomes MV, Landwerlin P, Diebold-Durand M-L, Shaik TB, Durand A, Troesch E, Weber C, Brillet K, Lemée MV, Decroos C *et al.* (2024) The cohesin ATPase cycle is mediated by specific conformational dynamics and interface plasticity of SMC1A and SMC3 ATPase domains. *Cell Rep* **43**.
47 Hirano M, Anderson DE, Erickson HP and Hirano T (2001) Bimodal activation of SMC ATPase by intra- and inter-molecular interactions. *EMBO J* **20**, 3238–3250.
48 Arumugam P, Gruber S, Tanaka K, Haering CH, Mechtler K and Nasmyth K (2003) ATP hydrolysis is required for cohesin's association with chromosomes. *Curr Biol* **13**, 1941–1953.
49 Alipour E and Marko JF (2012) Self-organization of domain structures by DNA-loop-extruding enzymes. *Nucleic Acids Res* **40**, 11202–11212.
50 Corsi F, Rusch E and Goloborodko A (2023) Loop extrusion rules: the next generation. *Curr Opin Genet Dev* **81**, 102061.
51 Ganji M, Shaltiel IA, Bisht S, Kim E, Kalichava A, Haering CH and Dekker C (2018) Real-time imaging of DNA loop extrusion by condensin. *Science* **360**, 102–105.
52 Moon K-W and Ryu J-K (2023) Current working models of SMC-driven DNA-loop extrusion. *Biochem Soc Trans* **51**, 1801–1810.
53 Higashi TL, Pobegalov G, Tang M, Molodtsov MI and Uhlmann F (2021) A Brownian ratchet model for DNA loop extrusion by the cohesin complex. *Elife* **10**, e67530.
54 Marko JF, De Los Rios P, Barducci A and Gruber S (2019) DNA-segment-capture model for loop extrusion by structural maintenance of chromosome (SMC) protein complexes. *Nucleic Acids Res* **47**, 6956–6972.
55 Ryu J-K, Katan AJ, van der Sluis EO, Wisse T, de Groot R, Haering CH and Dekker C (2020) The condensin holocomplex cycles dynamically between open and collapsed states. *Nat Struct Mol Biol* **27**, 1134–1141.
56 Barth R, Pradhan B, Kim E, Davidson IF, van der Torre J, Peters J-M and Dekker C (2023) Testing pseudotopological and nontopological models for SMC-driven DNA loop extrusion against roadblock-traversal experiments. *Sci Rep* **13**, 8100.
57 Taschner M and Gruber S (2023) DNA segment capture by Smc5/6 holocomplexes. *Nat Struct Mol Biol* **30**, 619–628.
58 Cuylen S, Metz J and Haering CH (2011) Condensin structures chromosomal DNA through topological links. *Nat Struct Mol Biol* **18**, 894–901.
59 Srinivasan M, Scheinost JC, Petela NJ, Gligoris TG, Wissler M, Ogushi S, Collier JE, Voulgaris M, Kurze A, Chan K-L *et al.* (2018) The Cohesin ring uses its hinge to organize DNA using non-topological as well as topological mechanisms. *Cell* **173**, 1508–1519.e18.
60 Pradhan B, Barth R, Kim E, Davidson IF, Bauer B, van Laar T, Yang W, Ryu J-K, van der Torre J, Peters J-M *et al.* (2022) SMC complexes can traverse physical roadblocks bigger than their ring size. *Cell Rep* **41**, 111491.
61 Kaur P, Lu X, Xu Q, Irvin EM, Pappas C, Zhang H, Finkelstein IJ, Shi Z, Tao YJ, Yu H *et al.* (2023) High-speed AFM imaging reveals DNA capture and loop extrusion dynamics by cohesin-NIPBL. *J Biol Chem* **299**, 105296.

62 Davidson IF, Barth R, Nagasaka K, Tang W, Wutz G, Horn S, Janissen R, Stocsits RR, Chlosta E, Bauer BW *et al.* (2025) Cohesin supercoils DNA during loop extrusion. *Cell Rep* **44**, 115856.
63 Martínez-García B, Dyson S, Segura J, Ayats A, Cutts EE, Gutierrez-Escribano P, Aragón L and Roca J (2022) Condensin pinches a short negatively supercoiled DNA loop during each round of ATP usage. *EMBO J* **42**, EMBJ2022111913.
64 Kimura K and Hirano T (1997) ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation. *Cell* **90**, 625–634.
65 Janissen R, Barth R, Davidson IF, Peters J-M and Dekker C (2024) All eukaryotic SMC proteins induce a twist of −0.6 at each DNA loop extrusion step. *Sci Adv* **10**, eadt1832.
66 Kim E, Gonzalez AM, Pradhan B, Van Der Torre J and Dekker C (2022) Condensin-driven loop extrusion on supercoiled DNA. *Nat Struct Mol Biol* **29**, 719–727.
67 Cannan WJ and Pederson DS (2016) Mechanisms and consequences of double-Strand DNA break formation in chromatin. *J Cell Physiol* **231**, 3–14.
68 Friskes A, Koob L, Krenning L, Severson TM, Koeleman ES, Vergara X, Schubert M, van den Berg J, Evers B, Manjón AG *et al.* (2022) Double-strand break toxicity is chromatin context independent. *Nucleic Acids Res* **50**, 9930–9947.
69 Bohgaki T, Bohgaki M and Hakem R (2010) DNA double-strand break signaling and human disorders. *Genome Integr* **1**, 15.
70 Aparicio T, Baer R and Gautier J (2014) DNA double-strand break repair pathway choice and cancer. *DNA Repair* **19**, 169–175.
71 De Alba EL, Salguero I, Giménez-Llorente D, Montes-Torres J, Fernández-Sanromán Á, Casajús-Pelegay E, Terrón-Bautista J, Barroso-González J, Bernal JA, Macintyre G *et al.* (2025) A comprehensive genetic catalog of human double-strand break repair. *Science* **390**, eadr5048.
72 Aguilera A and Gómez-González B (2008) Genome instability: a mechanistic view of its causes and consequences. *Nat Rev Genet* **9**, 204–217.
73 Mao Z, Bozzella M, Seluanov A and Gorbunova V (2008) Comparison of nonhomologous end joining and homologous recombination in human cells. *DNA Repair* **7**, 1765–1771.
74 Ensminger M and Löbrich M (2020) One end to rule them all: non-homologous end-joining and homologous recombination at DNA double-strand breaks. *Br J Radiol* **93**, 20191054.
75 Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A and Takeda S (1998) Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO J* **17**, 5497–5508.
76 Tiwari V and Wilson DM (2019) DNA damage and associated DNA repair defects in disease and premature aging. *Am J Hum Genet* **105**, 237–257.
77 Fan Y, Kuybu F, Cui H, Lammens K, Chen J-X, Kugler M, Jung C and Hopfner K-P (2025) Structural basis for DNA break sensing by human MRE11-RAD50-NBS1 and its regulation by telomeric factor TRF2. *Nat Commun* **16**, 8320.
78 Syed A and Tainer JA (2018) The MRE11–RAD50–NBS1 complex conducts the orchestration of damage signaling and outcomes to stress in DNA replication and repair. *Annu Rev Biochem* **87**, 263–294.
79 Frit P, Amin H, Zahid S, Barboule N, Hall C, Matharu G, Hardwick SW, Chauvat J, Britton S, Chirgadze DY *et al.* (2025) Structural and functional insights into the interaction between Ku70/80 and pol X family polymerases in NHEJ. *Nat Commun* **16**, 4208.
80 Chaplin AK, Hardwick SW, Stavridi AK, Buehl CJ, Goff NJ, Ropars V, Liang S, De Oliveira TM, Chirgadze DY, Meek K *et al.* (2021) Cryo-EM of NHEJ supercomplexes provides insights into DNA repair. *Mol Cell* **81**, 3400–3409.
81 Wilkinson OJ, Martín-González A, Kang H, Northall SJ, Wigley DB, Moreno-Herrero F and Dillingham MS (2019) CtIP forms a tetrameric dumbbell-shaped particle which bridges complex DNA end structures for double-strand break repair. *Elife* **8**, e42129.
82 Takeda S, Nakamura K, Taniguchi Y and Paull TT (2007) Ctp1/CtIP and the MRN complex collaborate in the initial steps of homologous recombination. *Mol Cell* **28**, 351–352.
83 Dynan WS and Yoo S (1998) Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids. *Nucleic Acids Res* **26**, 1551–1559.
84 Chen S, Vogt A, Lee L, Naila T, McKeown R, Tomkinson AE, Lees-Miller SP and He Y (2023) Cryo-EM visualization of DNA-PKcs structural intermediates in NHEJ. *Sci Adv* **9**, eadg2838.
85 Walker JR, Corpina RA and Goldberg J (2001) Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* **412**, 607–614.
86 Zahid S, Seif El Dahan M, Iehl F, Fernandez-Varela P, Le Du M-H, Ropars V and Charbonnier JB (2021) The multifaceted roles of Ku70/80. *Int J Mol Sci* **22**, 4134.

87 Fell VL and Schild-Poulter C (2015) The Ku heterodimer: function in DNA repair and beyond. *Mutat Res Rev Mutat Res* **763**, 15–29.
88 Shibata A and Jeggo PA (2020) Roles for the DNA-PK complex and 53BP1 in protecting ends from resection during DNA double-strand break repair. *J Radiat Res (Tokyo)* **61**, 718–726.
89 Kilgas S, Swift ML and Chowdhury D (2024) 53BP1-the 'Pandora's box' of genome integrity. *DNA Repair* **144**, 103779.
90 Dai L, Dai Y, Han J, Huang Y, Wang L, Huang J and Zhou Z (2021) Structural insight into BRCA1-BARD1 complex recruitment to damaged chromatin. *Mol Cell* **81**, 2765–2777.
91 Buis J, Stoneham T, Spehalski E and Ferguson DO (2012) Mre11 regulates CtIP-dependent double-strand break repair by interaction with CDK2. *Nat Struct Mol Biol* **19**, 246–252.
92 Isono M, Niimi A, Oike T, Hagiwara Y, Sato H, Sekine R, Yoshida Y, Isobe S-Y, Obuse C, Nishi R *et al.* (2017) BRCA1 directs the repair pathway to homologous recombination by promoting 53BP1 dephosphorylation. *Cell Rep* **18**, 520–532.
93 Rotheneder M, Stakyte K, van de Logt E, Bartho JD, Lammens K, Fan Y, Alt A, Kessler B, Jung C, Roos WP *et al.* (2023) Cryo-EM structure of the Mre11-Rad50-Nbs1 complex reveals the molecular mechanism of scaffolding functions. *Mol Cell* **83**, 167–185.
94 Chen L, Nievera CJ, Lee AY-L and Wu X (2008) Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair. *J Biol Chem* **283**, 7713–7720.
95 Whelan DR and Rothenberg E (2021) Super-resolution mapping of cellular double-strand break resection complexes during homologous recombination. *Proc Natl Acad Sci U S A* **118**, e2021963118.
96 Shao Z, Davis AJ, Fattah KR, So S, Sun J, Lee K-J, Harrison L, Yang J and Chen DJ (2012) Persistently bound Ku at DNA ends attenuates DNA end resection and homologous recombination. *DNA Repair* **11**, 310–316.
97 Myler LR, Gallardo IF, Soniat MM, Deshpande RA, Gonzalez XB, Kim Y, Paull TT and Finkelstein IJ (2017) Single-molecule imaging reveals how Mre11-Rad50-Nbs1 initiates DNA break repair. *Mol Cell* **67**, 891–898.e4.
98 Deshpande RA, Marin-Gonzalez A, Barnes HK, Woolley PR, Ha T and Paull TT (2023) Genome-wide analysis of DNA-PK-bound MRN cleavage products supports a sequential model of DSB repair pathway choice. *Nat Commun* **14**, 5759.
99 Sathyanarayana P, Wu Y, Walter JC and Loparo JJ (2025) The NHEJ machinery is translocated off DNA ends to enable resection. *bioRxiv*.
100 Quennet V, Beucher A, Barton O, Takeda S and Löbrich M (2011) CtIP and MRN promote non-homologous end-joining of etoposide-induced DNA double-strand breaks in G1. *Nucleic Acids Res* **39**, 2144–2152.