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Disentangling a Complex Biomolecular World with Single-Molecule Resolution

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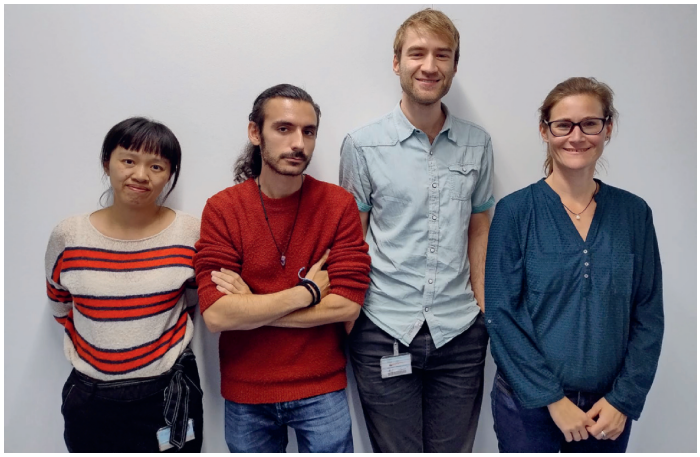
Abstract: Biological systems rely on a complex and precisely controlled mix of biomolecules to sustain life as we know it. In addition to their compositional heterogeneity, individual biomolecules undergo dynamic rearrangements to fulfil their cellular function: they move, reversibly interact, and alternate between multiple conformations. Disentangling these compositional and dynamic complexities of biological systems poses a formidable challenge to established ensemble techniques. In this review, we discuss two single-molecule techniques – nanopore recordings and single-molecule Förster Resonance Energy Transfer (smFRET) measurements – and highlight their powerful abilities to unravel mixtures and resolve biomolecular dynamics with the ultimate resolution of single molecules. Applications range from identifying the vast sequence space populated by nucleic acids and stoichiometries observed in small messenger molecules, to detecting time-varying conformations and interactions of large multi-domain proteins. This non-exhaustive review aims to introduce non-expert readers to the unique benefits of single-molecule experiments, which can overcome ensemble and time averaging as well as dynamic range limitations, and therefore offer unique, quantitative descriptions of the intriguingly complex biomolecular mechanisms found within and around us.

Keywords: Biomolecules · Conformational change · Dynamics · FRET · Nanopore · Single-molecule technique



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Prof. Dr. Sonja Schmid is the principal investigator of the Biomolecular Nano Dynamics Lab. Schmid obtained her PhD from TU Munich (DE) for her studies on the functional mechanism of chaperone proteins in life and disease by single-molecule fluorescence/FRET. During her postdoctoral studies at TU Delft (NL), she pioneered a novel single-molecule trap based on nanopore technology, enabling the label-free study of proteins and their conformations. In 2021, she joined Wageningen University as a tenure-track assistant professor, and later accepted an offer from the University of Basel to join the Department of Chemistry in 2024. Since her independent career, Schmid has received the Young Fluorescence Investigator Award 2024 from the BPS Fluorescence Subgroup, as well as several Dutch and Swiss grants.

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1. Biomolecules Exist in a Plethora of Different Compositions and Time-Varying States

The molecular building blocks of life include a gigantic variety of biomolecules, ranging from small-molecular cofactors and metabolites, to lipids, sugars and other large macromolecules, such as nucleic acids (DNAs, RNAs), and proteins.[1–9] Minutely regulated ratios of individual biomolecular species are necessary for proper biological function,[10–20] but often difficult to detect. Some of this compositional complexity of biomolecules is captured in David Goodsell's famous paintings (Fig. 1A). Deciphering and understanding these multi-species mixtures requires detailed investigations. Although great progress has been made in such 'biomolecular bookkeeping',[21–28] immense challenges remain.[29] Metaphorically speaking, the quest is to quantitatively identify the ingredients of an immensely complex fruit salad (Fig. 1B), including a myriad of different species, notably very rare ones next to very abundant ones. Disentangling such a complex mixture is still a daunting task for established techniques that rely on ensemble averaging. In exaggerated metaphoric terms, ensemble averaging is like blending the fruit salad into a smoothie to extract an average, from which it is difficult to deduce the precise recipe with rare and abundant ingredients. In reality, established techniques, such as mass spectrometry, have the sensitivity to identify molecular species, but often lack the dynamic range to quantify highly heterogenous samples,[29,30] leaving, for example, a large 'dark proteome' unknown.[31] Here, single-molecule techniques have a fundamental advantage, namely their ability to literally count individual molecules (fruits in the metaphoric picture Fig. 1B). By achieving the ultimate resolution of single molecules, these techniques can identify individual molecules regardless of the presence or absence of abundant other species, allowing them to bypass dynamic range limitations and more, as discussed in sections two and three.

Next to these compositional complexities, life critically relies on the dynamic, time-varying behaviour of biomolecules. Particularly fascinating and of vital importance are the structural dynamics of bio-macromolecules, such as proteins and RNAs, where a given molecule dynamically changes its conformation (*i.e.* 3D shape) and/or its interaction partners (Fig. 1C[32–36]). Specific examples include chemically or thermally driven conformational changes (*e.g.* dihydrofolate reductase,[37] multi-domain chaperone proteins[33]), transient structural switches during molecular interactions (*e.g.* 7SK noncoding RNAs interacting with the transcription factor protein complex

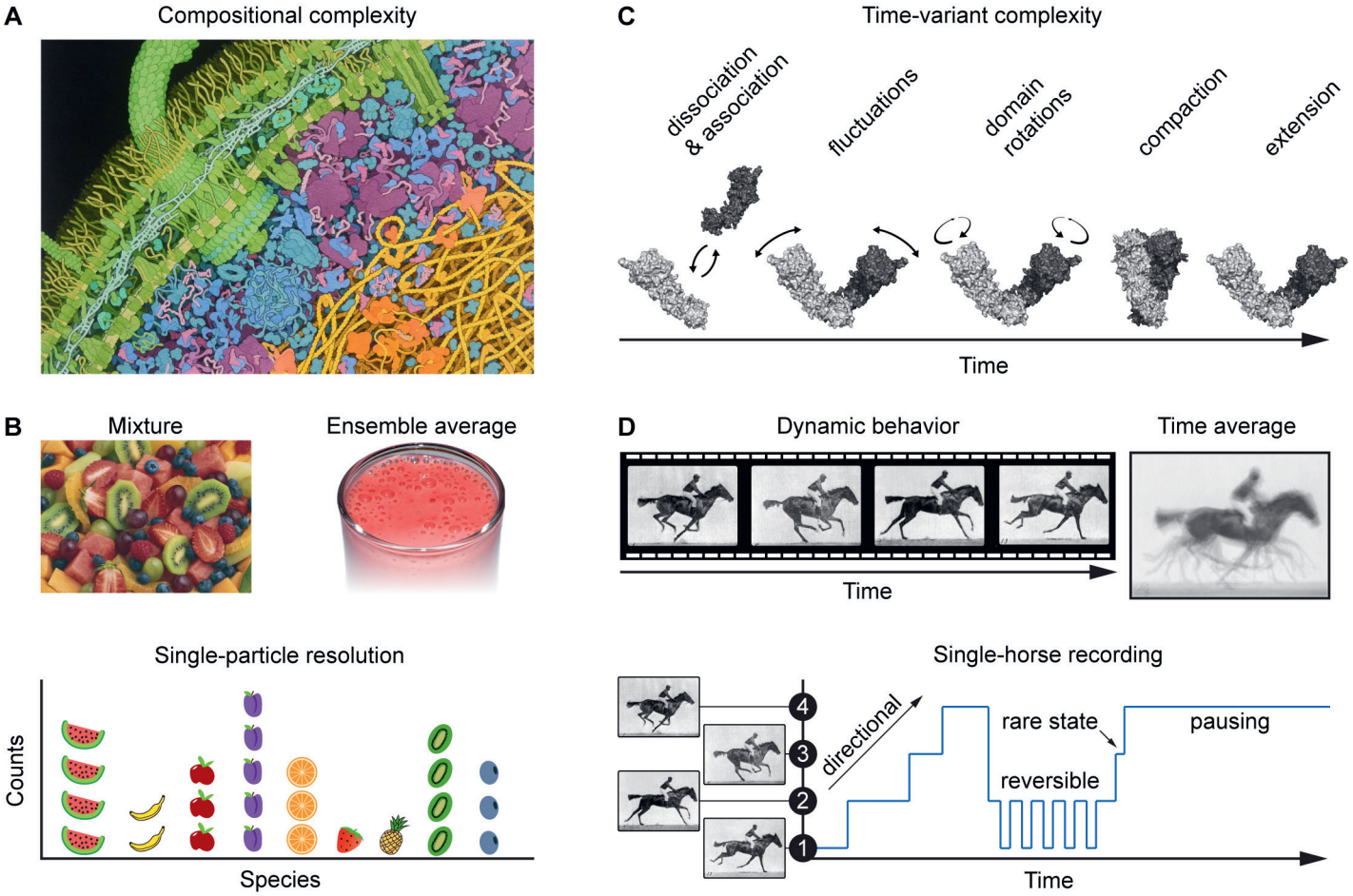

Fig. 1. **Compositional and time-variant complexity of biomolecules studied by ensemble and single-molecule approaches – a metaphoric comparison**. A) The biological cell is a crowded environment composed of a complex mixture with proteins (purple, blue, orange), DNA (yellow), lipids, sugars, and more in the membrane (green), and many other molecules. Illustration of *Escherichia Coli* by David S. Goodsell.[45] B) A complex fruit salad serves as a macroscopic metaphor for compositional complexity: an ensemble average (here: a fruit smoothie) reflects the composition of a mixture only partially, whereas single-particle resolution offers the fundamental ability to count the individual ingredients of the mixture (here: the fruit pieces). C) Individual biomolecules transform over time: illustrated time-variant complexity of a protein, including transient interactions, thermal fluctuations, rotations, and conformational changes. D) The historic study of the horse gallop[44] presents an instructive analogy for dynamics studies at the molecular level. Single-horse recordings were necessary to fully understand the gallop, including key events, their timing, and their sequence. Such information is lost through time averaging (or ensemble averaging), whereas time-resolved single-horse recordings directly reveal the underlying mechanisms. Examples of key features include directional and reversible processes, rare states, or pause states, or rare and decisive states that are easily missed by ensemble averaging. The same holds true at the molecular level, where time-resolved single-molecule recordings provide unique kinetic and energetic insights revealed by direct observations.

P-TEFb[38]), ultrafast intramolecular reconfigurations (*e.g.* intrinsically disordered proteins[39]), and slow misfolding processes (*e.g.* in multi-domain titin proteins[40]), *etc*. Moreover, even the kinetic rates of conformational dynamics themselves can vary over time (*e.g.* in ribozymes[41]), and such dynamic regime changes often affect catalytic rates, which is referred to as dynamic disorder (*e.g.* fluctuating turnover rates of cholesterol oxidase).[42] Taken together, dynamic biomolecular processes occur on various timescales from nanoseconds to minutes, depending on the high or low energy barriers involved, causing long or short residence times in individual states, respectively. The higher the energy barrier to cross, the more rate-limiting a functional process is.

At present, the understanding of biomolecular dynamics lags far behind its 3D structural counterpart, which is reflected by, *e.g*. the lack of a dynamics repository of comparable magnitude as the 3D structure databank, Protein Data Bank (PDB).[43] While several established techniques exist to study molecular dynamics (*e.g.* nuclear magnetic and electron spin resonance techniques, hydrogen-deuterium exchange mass spectrometry, *etc*.), dynamic changes remain challenging to detect. Simulations form an alternative approach but are limited in time and require experimental validation. Experimental challenges include information loss from ensemble averaging, arising from the unsynchronized stochastic nature of intra- and intermolecular dynamics, and from time averaging, as explained in Fig. 1D by the historic example of the horse gallop. The 'mechanism' of the horse gallop (timing and sequence of events) was only deciphered by first motion pictures[44] – *i.e.* by time-resolved single-horse recordings – whereas all relevant information is lost in time-averaged acquisitions. The same holds at the molecular level, where time-resolved single-molecule recordings can directly reveal individual events underlying biomolecular function, such as directional or reversible processes, rare but decisive states, pausing, *etc.* (see Fig. 1D plot), which would be lost by ensemble or time averaging. Ultimately, these data give unique access to the thermodynamics and kinetics of biomolecules with broad relevance in the biomedical and biotechnological areas.

In this review, we shine a light on two time-resolved single-molecule techniques used in our own research, namely label-free nanopore detection and Förster resonance energy transfer (FRET) experiments. We briefly introduce both techniques and highlight their ability to unravel biomolecular complexity using examples selected from our own and other groups' works with no claim to comprehensiveness. We discuss the strengths and limitations of these techniques and conclude with an outlook on the current capacity and future potential of single-molecule techniques to decipher the biomolecular world in and around us.

2. Nanopores Resolve Biomolecular Complexity in a Label-Free Way

Nanopores are small holes designed to accommodate and study single molecules, with a diameter ranging from one to tens of nanometers. They gained popularity in the life sciences due to commercialized single-molecule DNA sequencers,[46] but their application range is much broader. Nanopores are categorized into solid-state nanopores and biological nanopores. The former are built by drilling a nanoscopic hole into a thin solid-state membrane, *e.g.* silicon nitride, graphene, or hexagonal boron nitride,[47–50] or by laser-pulling of glass capillaries.[51,52] Biological nanopores consist either of a transmembrane protein, pore-forming peptides, or a designed DNA-origami structure,[53,54] inserted into an insulating free-standing lipid bilayer (Fig. 2A) or polymer membrane.[55,56] The insulating membrane with the solid-state or biological nanopore separates two buffer reservoirs, each connected to an electrode. Voltage application across the membrane drives an ionic through-pore current in the pA to nA range that is detected with dedicated amplifiers. Single analyte molecules can be detected when they approach and partially block the nanopore, causing a drop in electrical conductivity and thus a resistive spike in the current signal (Fig. 2B), which is characteristic for the molecule's size, shape, charge, and other physicochemical properties.[57–60] Analytes generally approach the pore by diffusion, while charged molecules are also attracted or repelled by electrophoretic forces, depending on the applied voltage. Other relevant forces acting in nanopores include electro-osmotic forces (see nanopore trapping below), and dielectrophoretic forces at high voltage.[61] For a more detailed introduction on methodological and theoretical aspects of nanopore experiments, we refer to dedicated reviews on nano-fluidics[62] and solid-state nanopores,[47] protein nanopores,[63] comparison of biological and solid-state nanopores noise,[64] DNA or protein sequencing,[65,66] and nanopores beyond sequencing.[67]

2.1 *Counting Small Molecules in Mixtures Using Nanopores*

Nanopores offer a plethora of opportunities to build portable – and potentially wearable – detectors with single-molecule resolution. Small molecules, single atoms, distinct stoichiometries and even enantiomers of a given molecule can be sensitively detected one-by-one with adequately engineered nanopores.[68,69] For example, the stoichiometry of CRISPR second messengers, so-called cyclic oligo-adenylates (cOAs) was identified with protein nanopores. Produced by type III CRISPR-Cas, these cOAs trigger a nuclease response that protects bacterial populations against viral spread.[70–72] Questions remain about their mono- or poly-disperse enzymatic synthesis, since trimers to hexamers have been reported.[70,71,73] Nanopore experiments, illustrated in Fig. 2A, offered the resolution to detect single cOA molecules and identified their stoichiometry. Specifically, when single cOA molecules enter the pore, the unperturbed baseline current gets interrupted by short blockade events (Fig. 2B). In a first study, all four tested cOA stoichiometries could be detected (Fig. 2C), and the larger three could be reliably distinguished also in mixtures. Ultimately, unidentified enzymatically produced cOAs were assessed, originating from two different type III CRISPR-Cas variants of the same host bacterium. Unexpectedly, nanopore detection uncovered near identical cOA stoichiometries for both variants (Fig. 2D), hinting to functional redundancy as a possible evolutionary benefit in this CRISPR context.[74]

2.2 *Nanopore-Based DNA and Protein Sequencing*

The most widely recognized application of nanopore technology is commercialized DNA sequencing. Beyond the engineered protein nanopore, the key component of this highly optimized experiment is a motor protein (usually a helicase), which slowly drags the DNA single strand through the pore – slowly enough, in fact, to enable sufficient sampling and effective noise reduction by low-pass filtering, *etc*. The resulting nucleobase-specific through-pore current enables fast DNA sequencing with long reads. Notably, even portable devices powered by a smartphone were developed.[75] The next challenge in this field is single-molecule protein sequencing, which would be a game changer in protein research, promising the detection of post-translational modifications[76] and rare biomarkers currently missed with ensemble techniques. However, sequencing proteins is a daunting task given the much higher complexity of proteins compared to DNA, namely the 20 amino acids plus post-translational modifications, a 3D folded structure, no amplification method like DNA polymerase chain reaction, *etc*. Nevertheless, stunning progress has been achieved, with several academic groups and companies reporting first single-peptide reads,[56] full-length directional translocations of proteins through nanopores,[77] enzyme-free protein reads,[78] and protein discrimination.[79,80]

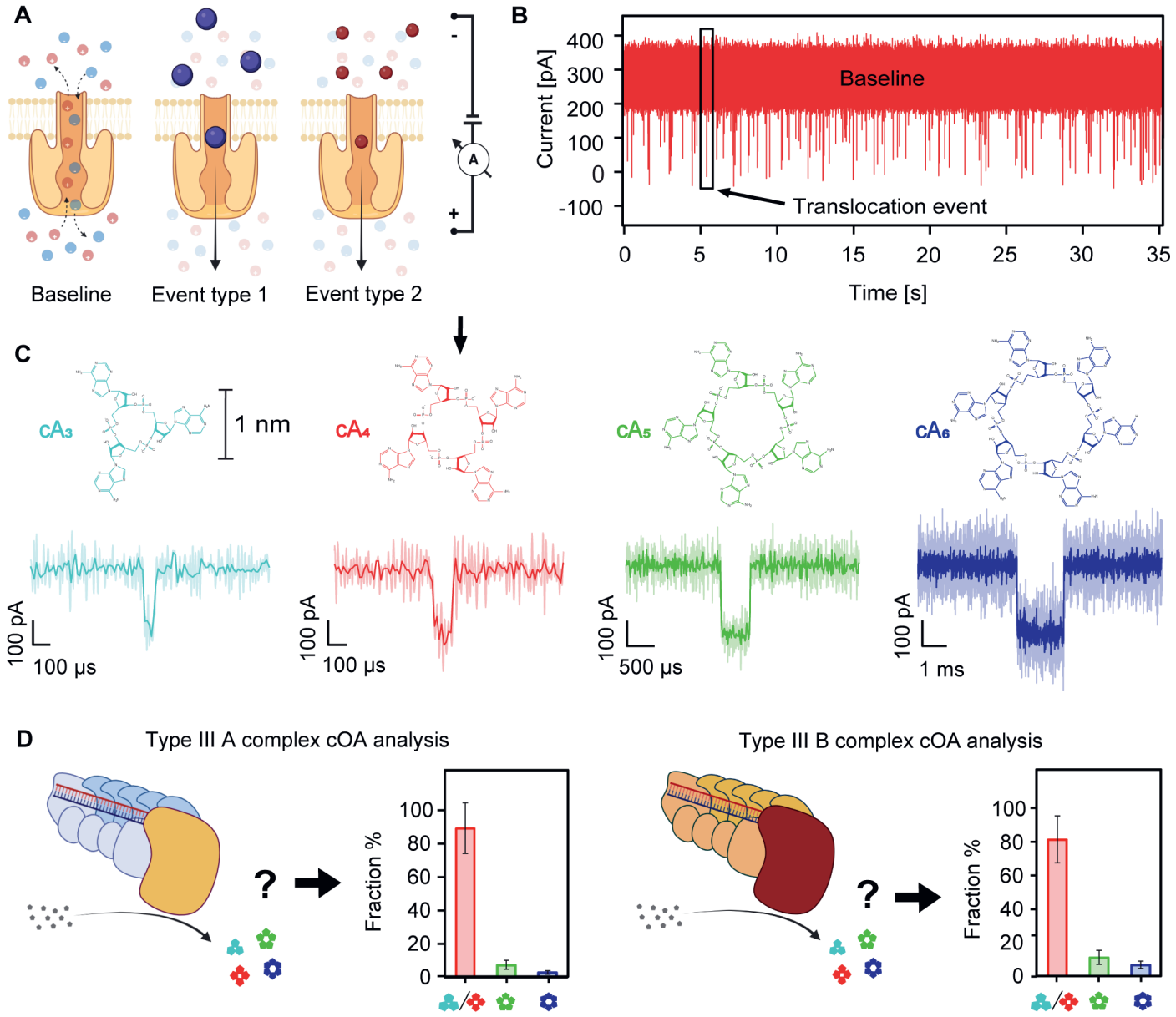

Fig. 2. **Basics of the protein nanopore experiment and its application to CRISPR second messengers.** A) Schematic nanopore experiment with lipid membrane, protein pore, ions, and voltage source. Left: open pore (no analyte) defining a baseline current; center and right: distinct analytes transiently block the pore, which partially blocks the current with specific characteristics (length, duration, *etc.*). B) Current recording showing short blockade events caused by a single tetrameric cOA that translocates through the alpha-hemolysin pore. A zoom view of the highlighted cA4 event is shown in C. C) Chemical structure and translocation events of single tri-, tetra-, penta-, or hexameric cOA molecules, termed cA3, cA4, cA5 and cA6, respectively. D) Cartoon of Type IIIA and IIIB CRISPR-Cas variants, each producing an initially unknown pool of cOA stoichiometries, which were identified using nanopore experiments and neural network-supported recognition (bar plots). In the initial study, cA3 and cA4 could not be distinguished, which is solved now in follow-up work to be published elsewhere.[74]

The strong biomedical need to identify rare species, especially biomarkers, in small volumes of patient samples has fuelled progress in the quest to sequence and/or identify proteins with single-molecule resolution, which will be exciting to follow in the coming years.[65,81,82]

2.3 *Nanopore Traps to Capture and Study Proteins*

Furthermore, single-molecule traps have been built with protein and solid-state nanopores, enabling prolonged observations of a single protein, their conformational dynamics, and catalytic cycles. Protein pores have first been used in this way for single-molecule enzymology studies, where small enzymes were trapped for multiple seconds to resolve their catalytic cycle.[83,84] Typically, the electro-osmotic effect is used to trap a protein hydrodynamically inside a pore lumen. Electro-osmosis occurs in nanopores with substantial surface charge inside the pore, which attracts counter ions that break charge neutrality among the mobile charge carriers in the narrow pore (*cf.* electric double layer). These excess charges drive a directional water flow under an applied voltage, which can be used to trap functionally intact proteins,[37,83,84] regardless of their net charge.[85] For example, the enzyme guanylate kinase (Guk1) can be trapped and studied in the cytolysin A (ClyA) nanopore following this principle (Fig. 3A). Interestingly, the addition of nucleotide substrates (ATP, GMP) prolonged the residence time of the kinase inside the pore, likely indicating a structural change (Fig. 3B). Moreover, ATP and GMP caused a shift in the current blockades as well as larger current fluctuations, hinting at dynamic rearrangements during catalysis (Fig. 3C). Indeed, more extensive enzyme trapping experiments have previously provided direct observations of dihydrofolate

reductase (DHFR) catalysis, revealing a hierarchical order of substrate binding with mutually controlled affinities, serving a regulatory role in the cell.[37] Furthermore, the Abl kinase cycle was studied using ClyA traps. The dynamic changes between apo and ligand-bound states of the Abl kinase could be directly observed and the energy landscape mapped, providing mechanistic insights on Abl function and malfunction of a deficient mutant.[84] Other protein nanopore traps have also been described (*e.g.* PlyAB,[86] YaxAB[87]) – each with unique properties, such as different size and sensitivity.

The Nanopore Electro-Osmotic trap (NEOtrap) can hold and monitor even larger proteins (14 to >300 kDa).[88] The NEOtrap consists of a solid-state nanopore onto which a DNA-origami sphere is docked electrophoretically (Fig. 3D), which provides sufficient negative charge to induce strong electro-osmotic flow to hydrodynamically trap single proteins for up to many hours. It provides specific protein fingerprints depending on size, shape, protein flexibility and charge distribution, and it can distinguish distinct nucleotide-dependent conformations of a protein, as shown for the heat-shock protein Hsp90 (Fig. 3E-F). The ability to site-specifically functionalize the DNA origami[89] enables numerous additional sensing modalities as recently summarized.[90] Taken together, the label-free nature, broad range of accessible proteins, precise functionalization opportunities, and the physicochemical

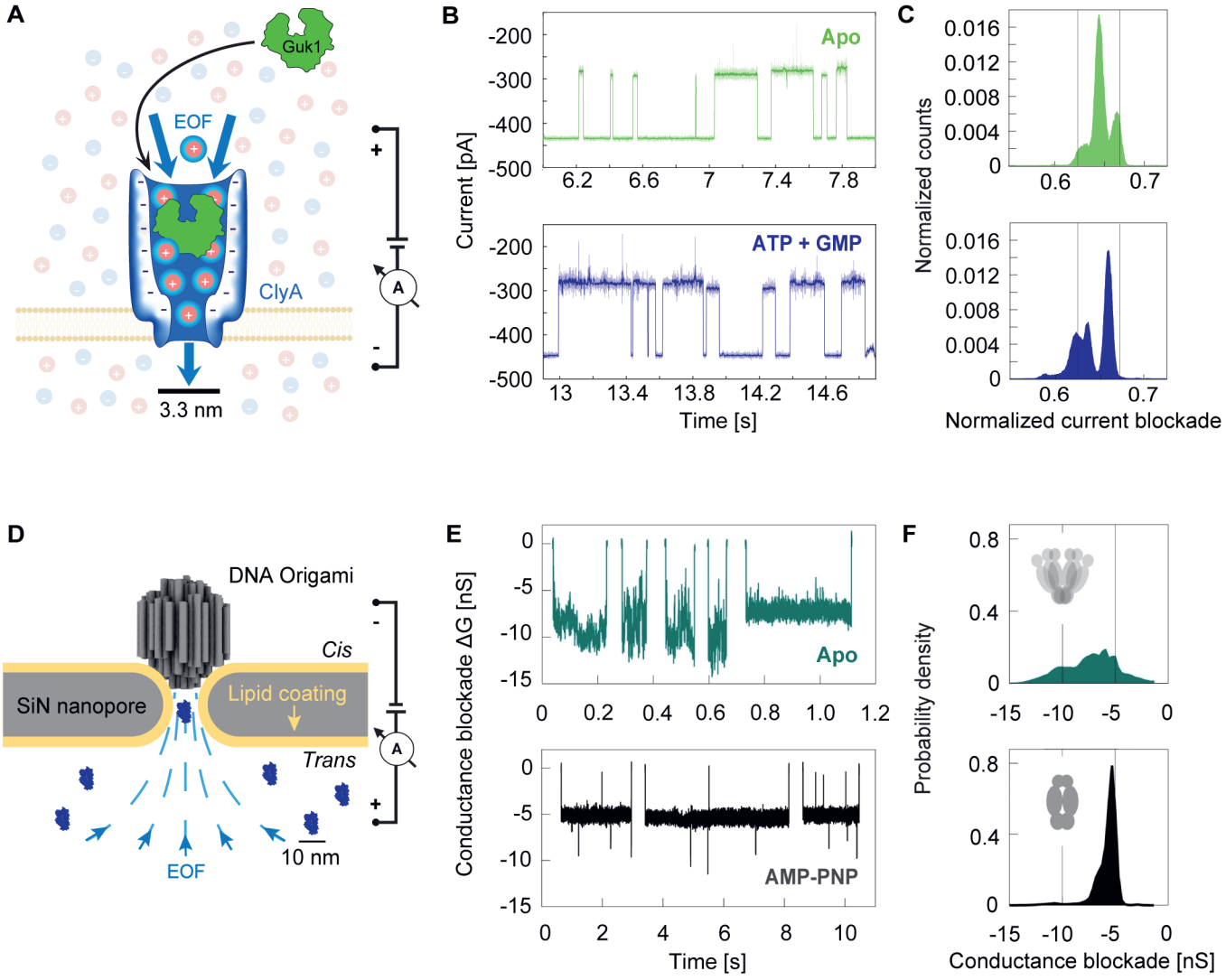

Fig. 3. **Nanopore traps to study folded proteins and their mechanisms**. A) Cartoon of a ClyA nanopore used to trap small proteins (*e.g.* Guk1, green) by electro-osmotic flow (EOF, see text for details). The negatively charged pore lumen and counter-cations with hydration shells are shown. B) ClyA current traces of the experiment in A) with Guk1 trapping events. Top: apo (no substrate). Bottom: with ATP and GMP substrates. All measured at –70 mV, in 0.5 M KCl, 1 mM nucleotide as indicated. C) Current histograms of the Guk1 trapping events in the conditions in B). Current blockades were normalized to the open pore current, corresponding counts were normalized to the total counts of the histogram. D) Cartoon of the NEOtrap composed of a silicon nitride (SiN) nanopore, surface-passivated with a lipid coating. The negatively charged and ion-permeable DNA-origami sphere generates EOF sufficient to trap proteins and nanoparticles. E) Individual trapping events of single Hsp90 proteins in units of conductance blockade ΔG (not to be confused with Gibbs free energy). Top: apo condition (no substrate). Bottom: with AMP-PNP bound, a non-hydrolysable ATP analog. In the former case, the protein is more flexible leading to less uniform events compared to the AMP-PNP-stabilized and compacted case. F) The histograms of >1000 trapping events as shown in E reveal the broad distribution under apo condition and a defined peak in the presence of AMP-PNP, respectively.[88]

understanding gained[85,91] render nanopore trapping a versatile approach to investigating unlabelled protein mixtures and to monitoring dynamic changes and enzymatic cycles in real time.

2.4 *Challenges and Considerations for Nanopore Studies*

The challenges of nanopore detection often arise from the fact that they are not 'one-size-fits-all' detectors. Instead, nanopore experiments need dedicated optimization for a given analyte or biomolecular question, namely regarding the choice of the nanopore (diameter, charge distribution, shape, *etc.*), the buffer (salinity, pH, crowders, denaturants, *etc.*), voltage (magnitude, constant, ramp, or alternating), and more. For biological nanopore recordings, membrane stability can be limiting (while polymer-based solutions exist[92]), whereas solid-state nanopores sometimes suffer from imperfect reproducibility which is normally solved by control experiments. Also, signal interpretation requires systematic control experiments to identify which biological state relates to a given current pattern. Interestingly, the atomic precision, reproducibility and (commercial) success of bottom-up engineered biological nanopores (mostly protein pores) have attracted much attention over recent years, in comparison to the top-down engineered solid-state nanopores that often require costly nano-fabrication. However, cost-effective solid-state nanopores, such as glass nano-pipets, also exist, offering practical solutions for a range of specialized experiments.[51,52,93] Overall, this creative field keeps innovating single-molecule biosensing at a fast pace, enabling ever more precise analyses – of which we could only highlight a small selection.

3. Single-Molecule FRET to Observe Biomolecular Dynamics in Real-Time

Förster resonance energy transfer (FRET) lends itself particularly well to study intra-molecular conformational changes of bio-macromolecules (proteins, DNA, RNA) that would be lost by ensemble averaging (Figs. 1C & 4A). For a typical single-molecule FRET experiment, two organic fluorophores – a so-called donor and (red-shifted) acceptor – are coupled to the biomolecule at specific points of interest. Distance changes between these two positions are then detected as anti-correlated changes in the donor and acceptor intensities[94] (Fig. 4B), or alternatively, as an altered fluorescence lifetime of the donor.[95] The physicochemical basis of this convenient FRET effect is a transition dipole interaction between fluorophores in close (nanoscale) proximity, where resonance is enabled by spectral overlap. The resulting non-radiative energy transfer is (i) highly distance-dependent ($\propto r^{-6}$) and (ii) optically detectable at the single-molecule level even at ambient temperatures and in aqueous solution, making FRET an ideal 'spectroscopic ruler' to measure nanometer conformational changes of bio-macromolecules in real-time.[96–99] A great asset of smFRET is its ability to detect biomolecular dynamics across a broad range of timescales, namely from nanoseconds to hours, using a range of different detection modalities.[100] Rapid transitions (*i.e.* frequent low-energy barrier crossings) can be revealed down to the nanosecond range by confocal detection of freely diffusing molecules using time-correlated single-photon counting (TCSPC). Slower ≥ milliseconds dynamics (*i.e.* rate-limiting high-energy barrier crossings) can be measured by highly-parallelized wide-field detection of surface-tethered biomolecules under total-internal reflection (TIR) excitation. Fig. 4C shows a single-molecule FRET trajectory of a single protein proceeding from an extended conformation (green), through several conformational changes (gray), to a compact conformation (red). The relevant states and kinetic rate constants can be readily extracted from such direct observations (Fig. 4D), providing a quantitative description of the conformational changes governing a protein's functional cycle. For more detailed information on smFRET, interested readers are referred to more comprehensive reviews, such as refs. [94], [101], and 102].

3.1 *The Broad Impact of smFRET in Resolving Biomolecular Mechanisms*

With its unique spatio-temporal resolution, smFRET has contributed to the elucidation of many biomolecular mechanisms. For example, intra-molecular ribosome dynamics preceding individual translation events were uncovered,[104] and the mechanisms of ribosomal stalling[105] and frameshifting[106,107] could be elucidated. smFRET also resolved a controversy in DNA transcription initiation by providing strong evidence for the scrunching model, where a stressed DNA intermediate supplies the driving force for promoter escape.[108] G-protein-coupled receptors (GPCRs) are a frequent target for drug design, and smFRET revealed oligomerization of GPCRs in live cell membranes,[109] as well as detailed conformational dynamics including previously missed states[110,111] relevant for GPCR targeted drug design.[112] In ion channel research, smFRET has revealed the dynamics of an ion selectivity filter,[113] and enabled the observation of constriction opening providing evidence for a helix-tilt model.[114] It also identified a previously missed intermediate state in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel, revealing a new molecular basis for cystic fibrosis.[115] In addition, smFRET led to the elucidation of temperature-dependent folding dynamics of intrinsically disordered proteins in live cells,[116] revealed five *meta*-stable conformations for the Diels-Alderase ribozyme,[117] and showed the reversibility of G-quadruplexes formed for the regulation of human DNA telomeres.[118] This small selection illustrates the broad range of applications of smFRET in resolving crucial functional steps that are otherwise missed by ensemble averaging.

3.2 *Circumventing Photobleaching in smFRET*

A persistent limitation of smFRET trajectories is their finite duration, which is caused by photobleaching. An important avenue is therefore the development of fluorophores with better photo-stability to extend the observation time and thus increase the amount of information gained per labelled biomolecule.[119–121] Alternatively, the recent approaches DyeCycling[103] and REFRESH-FRET[122] target the fluorophore coupling strategy, rather than photo-stability. In short, the observation of FRET is decoupled from photobleaching by using reversible fluorophore binding, where continuous binding and dissociation enables fresh fluorophores from solution to replace bleached ones (Fig. 4E-G). Unwanted background from freely diffusing fluorophores can be suppressed using fluorogenicity,[122,123] or nanophotonics.[124] These new smFRET approaches have the potential to significantly prolong single-molecule observation times, enabling the discovery of new dynamic phenomena that are currently inaccessible, such as slow kinetic regime changes, as reported for exonucleases,[125] RNA polymerases pausing,[126] transcription initiation,[127] *etc*. The existence of static and dynamic disorder in proteins has been known since the advent of single-molecule technologies several decades ago, but technical limitations have so far prevented their in-depth investigation – limitations that these new smFRET approaches promise to overcome.

3.3 *Challenges and Considerations for smFRET Studies*

Beyond photo-bleaching, the need for labels coupled to the biomolecule of interest (Fig. 4A-B) represents a limitation of smFRET. The labelling positions define the observable motions, and are usually chosen based on prior structural information (*e.g.* from the PDB or AlphaFold) and by avoiding genetically conserved sites. For nucleic acids, custom synthesis and labelling are commercially available. For proteins, point mutations are

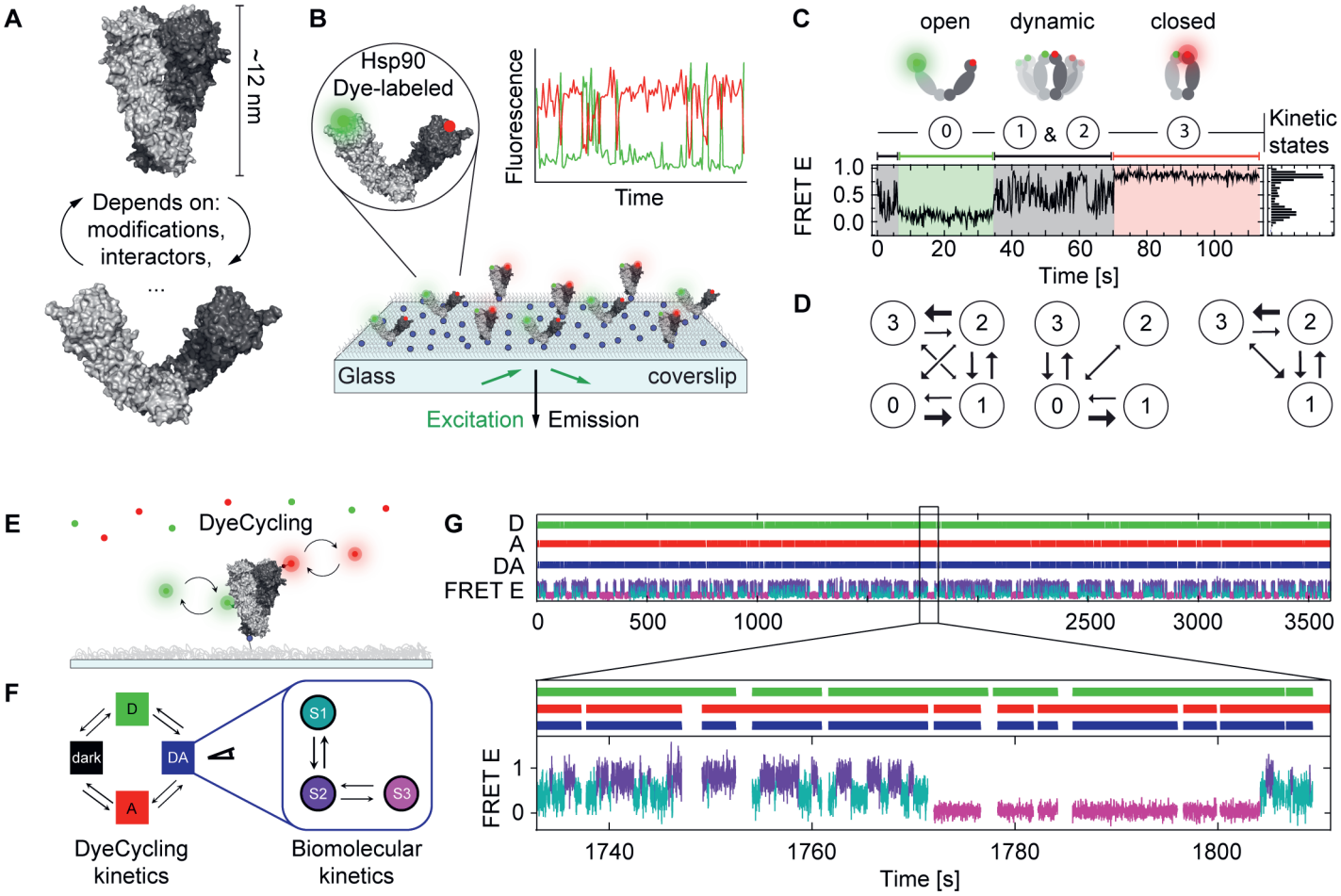

Fig. 4. **Watching single molecules in action using smFRET**. A) Protein function involves dynamic changes that are not captured by static structures. B) Schematic smFRET experiment in TIRF-mode: fluorophore-labelled proteins (green: donor, red: acceptor) are tethered to a passivated coverslip. Their individual movements are captured by the anticorrelated fluorescence trajectories, resolving nanoscale conformational changes in single proteins in real-time. C) SmFRET trajectory of an individual chaperone protein. The timing and order of various opening and closing events are directly observed *via* the FRET efficiency (FRET E). D) Specific kinetic models with quantitative rate constants (arrows) are obtained, supporting or dismissing hypothesized mechanisms. E) The DyeCycling approach to smFRET uses reversible fluorophore (dye) binding to circumvent photobleaching. F) Each 'cycler' (reversible fluorescent probe) binds to and dissociates from the biomolecule of interest independently. With both cyclers bound simultaneously, the kinetics of the biomolecule can be observed (here: a hypothetical 3-state system). G) Simulation of DyeCycling, illustrating the principle of continuously replaced dyes, extending the total observation time of a single biomolecule.[103]

inserted to achieve site-specific labelling, either by thiol-specific coupling to a single cysteine,[99] or by more involved genetic code expansion (mostly using the amber stop codon).[128,129] Although a number of suitable click chemistries exist, some positions are not suitable for labelling, either for preparative reasons (no or low yield), photophysical reasons (*e.g.* quenching, restricted mobility), or interference with the biological activity (revealed by control experiments). Next, while conventional smFRET is limited to detecting one inter-dye distance in 3D space, it also offers a clear 1D reaction coordinate. Multi-color smFRET reporting on multiple distances simultaneously is possible[110] at the cost of extra complication and usually reduced data yield. The sensitivity of FRET lies at *ca.* 2–9 nm for typical fluorophores, which is rarely limiting since it fits well with relevant molecular distances, and strategies for >10 nm exist, too.[130,131] Ultimately, data analysis involves discarding artefacts, standardized corrections,[98] and kinetic rate extraction, which can be time-consuming unless automated.[132–134] Analysis software packages are shared by many groups (see Table 1 in Ref. [101]), but software centralization – similar to that in the super-resolution field – is less advanced, notwithstanding several successful unification efforts.[98,101,135] Lastly, specialized smFRET setups can be purchased commercially or built individually to meet specific needs, while the components have much improved overall (*e.g.* switchable lasers, low-noise cameras with high quantum yields and large chip size, *etc.*), vastly facilitating data collection. Altogether, smFRET provides a versatile toolbox for studying biomolecular dynamics across broad timescales. It offers unique insights into biomolecular function by complementing structural biology with the detection of kinetic processes, rare states, and intermediates typically missed by ensemble averaging.

4. Conclusion and Outlook

Biomolecular complexity – including complex mixtures and time-variant dynamic effects (Fig. 1) – challenges the resolving power of traditional techniques relying on ensemble and/or time averaging. As discussed in the introduction, single-molecule techniques have the ability to capture compositional and dynamic variations and resolve hidden information of rare species and intermediate states in complex biomolecular samples, and this review summarizes a range of examples thereof. We first introduced nanopore recordings, highlighting their label-free capacity and sensitivity. The versatility of nanopore experiments is evident from the large variety of applications discussed, including the identification of molecular mixtures of small CRISPR second messengers,[74] commercialized nanopore DNA sequencing,[46] latest innovations toward nanopore protein sequencing,[29] and nanopore traps to study intact folded proteins[37,84,88] and reveal

enzyme catalysis in real-time.[37] Since there is no 'one-size-fits-all' in nanopore experiments, the challenges lie in finding and engineering the right conditions for a given question, as discussed. In the second part, we introduced smFRET experiments that allow the direct observation of intra-molecular conformational changes as well as inter-molecular interactions of biomolecules.[94–102] We highlighted the impact of smFRET on the elucidation of a wide variety of biomolecular mechanisms, including transcription,[108] translation,[104–107] GPCR signalling,[109–112] and ion channel function,[113–115] *etc*. We discussed ongoing efforts to bypass the fundamental photobleaching limit in smFRET to achieve unprecedented long-term observations for the study of static and dynamic disorder in proteins.[103,122] Ultimately, we summarized the challenges and practical considerations associated with employing smFRET.

Ongoing developments of nanopore and smFRET techniques include improvements in sensitivity, throughput, spatial and temporal resolution, and the integration with additional detection modalities (force, microfluidics, electro-optical combination, *etc*.). Such developments have the potential to contribute to a range of future innovations. For instance, nanopore-based single-molecule proteomics could revolutionize personalized medicine; single-molecule dynamics data may advance rational drug design from structure-based to integrative structure- and dynamics-based design; and portable nanopore devices capable of discriminating biomarkers in complex patient samples could operate directly on the wrist or in implants.

Overall, single-molecule techniques build upon and complement conventional ensemble techniques. On the one hand, when applied to heterogeneous mixtures, they can bypass dynamic range limitations encountered with conventional techniques by offering single-molecule counting. On the other hand, time-resolved single-molecule trajectories provide unique mechanistic insights that complement static 3D structures, uncovering rare but decisive states and revealing the kinetics and thermodynamics underlying biomolecular mechanisms. Given the unique insights offered, single-molecule techniques have become indispensable for elucidating the complex biomolecular world in both fundamental and applied research and hold great potential for future innovations.

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Author Contributions

B. V. drafted the Introduction and smFRET sections; D. F. and W. T. drafted the Nanopore section; W. T. drafted the Conclusion. S. S. conceived the overall structure and streamlined the manuscript in consultation with all authors. All authors reviewed and approved the final manuscript.

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