

Nanopores

A versatile tool to study protein Dynamics

Schmid, Sonja; Dekker, Cees

DOI

[10.1042/EBC20200020](https://doi.org/10.1042/EBC20200020)

Publication date

2021

Document Version

Accepted author manuscript

Published in

Essays in biochemistry

Citation (APA)

Schmid, S., & Dekker, C. (2021). Nanopores: A versatile tool to study protein Dynamics. *Essays in biochemistry*, 65(1), 93-107. <https://doi.org/10.1042/EBC20200020>

Important note

To cite this publication, please use the final published version (if applicable).
Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights.
We will remove access to the work immediately and investigate your claim.

Nanopores – a Versatile Tool to Study Protein Dynamics

Sonja Schmid, Cees Dekker

*Department of Bionanoscience, Kavli Institute of Nanoscience, Delft University of Technology,
Van der Maasweg 9, 2629 HZ Delft, The Netherlands.*

Correspondence: s.schmid@tudelft.nl

ABSTRACT

Proteins are the active working horses in our body. These biomolecules perform all vital cellular functions from DNA replication and general biosynthesis to metabolic signaling and environmental sensing. While static 3D structures are now readily available, observing the functional cycle of proteins – involving conformational changes and interactions – remains very challenging, e.g., due to ensemble averaging. However, time-resolved information is crucial to gain a mechanistic understanding of protein function. Single-molecule techniques such as FRET and force spectroscopies provide answers but can be limited by the required labelling, a narrow time bandwidth, and more. Here, we describe electrical nanopore detection as a tool for probing protein dynamics. With a time bandwidth ranging from microseconds to hours, it covers an exceptionally wide range of timescales that is very relevant for protein function. First, we discuss the working principle of label-free nanopore experiments, various pore designs, instrumentation, and the characteristics of nanopore signals. In the second part, we review a few nanopore experiments that solved research questions in protein science, and we compare nanopores to other single-molecule techniques. We hope to make electrical nanopore sensing more accessible to the biochemical community, and to inspire new creative solutions to resolve a variety of protein dynamics – one molecule at a time.

INTRODUCTION

Proteins are the working horses in our body [1]. They convert energy into function, and form complex dynamic protein-protein interaction networks. Protein function relies crucially on structured domains and structural flexibility [2] that are both encoded in the peptide chain and its post-translational modifications (PTMs; see glossary). Also natively unfolded parts that may obtain a specific 3D arrangement only upon binding to their interaction partners play an important role [3]. The protein world offers an inexhaustible wealth of active and passive dynamic nanoscale phenomena ranging from the extreme precision during DNA replication [4] (polymerases, helicases), mechanical force generation (myosin [5], flagellar motor [6], dynein, kinesin [7], proteasome [8]), to other energy conversion (ATPsynthase [9], ion pumps [10], bacterio-rhodopsin [11], light harvesting complexes [12]), as well as sensing and signalling (von Willebrandt factor [13], tip-link cadherins [14,15], piezo protein [16,17], kinases, isomerases, countless PTM regulators [18–21]). These are just a few examples of the wide variety of protein functions that rely critically on conformational dynamics and protein-protein interactions. However, this key aspect – the functional dynamics that a single protein performs – is challenging to observe experimentally. Established structural biology techniques such as X-ray diffraction or cryo-electron microscopy [22] have outstanding spatial resolution but are blind for dynamic effects that are happening in solution, while NMR [23], EPR [24], SAXS [25], and SANS [26,27] suffer from ensemble averaging.

Fortunately, various single-molecule techniques have taken on the challenge to resolve protein dynamics in real time at room-temperature: most prominently, single-molecule fluorescence techniques (e.g., smFRET [28,29]), single-molecule force spectroscopies (e.g. AFM [30], magnetic [31,32] or optical [33,34] tweezers), and – more recently – electrical nanopore detection [35]. None of these techniques can compete with the 3D Angström resolution of cryoEM or x-ray crystallography. But in exchange for some spatial resolution, they resolve dynamic processes performed by just one molecule or one functional entity – and notably in real-time at room temperature and in solution. The fundamental advantage of time-resolved single-molecule approaches is that different functional states can be distinguished in a molecular ensemble, and that following one molecule over time allows one to resolve the timescales involved, to quantify kinetic rate constants, and ultimately to uncover the energetic driving forces that control protein function. In short, time-resolved single-molecule techniques provide a direct view on how proteins perform function, which constitutes the essential ingredient to move from static protein structures to understanding dynamic protein function at the nanoscale.

The scope of this review

So why, as a protein fan, should you care about nanopores? This is the central question in this review. Well, you should care specifically if you are interested in protein dynamics, i.e., in pushing the boundaries beyond static structural biology. The unrivalled time range accessible within one nanopore experiment is perfectly suitable to study protein dynamics, in most cases even label-free (as reviewed

below). Moreover, nanopores have already proven their utility in many DNA and RNA sequencing applications performed outside traditional nanopore-centric labs [36–40], and portable handheld nanopore sequencing devices have been realized and marketed [41]. Building on the ongoing success of nanopore technology in DNA/RNA sequencing [42,43], we review here the next step: nanopore solutions to research questions in protein science.

We target this review at scientists who are interested in original quantitative biochemistry, and protein function in particular, while we simultaneously hope to stimulate enthusiasm for proteins among nanopore experts. In the first part of this brief review, we introduce in a nutshell the basic concept of nanopores. We describe typical experiments, various pore designs, the characteristics of the electrical nanopore signals - all from a protein sensing perspective. In the second part, we highlight a few creative nanopore applications that contributed to our understanding of protein function, and we compare electrical nanopore sensing to other single molecule techniques. Clearly, by zooming in on a few selected recent findings on proteins, we pass over many seminal contributions that have shaped the nanopore field. We refer the inclined reader to excellent reviews on the general rise of nanopore technology [44–47], and the chronology from first electrophysiology experiments to DNA sequencing [48] and its commercialization.

WHAT ARE NANOPORES?

A nanopore is a most simple and elegant sensor: it literally consists of a hole in a membrane that can sense molecules in solution one by one, even label-free (**Figure 1A**). The insulating membrane separates two compartments filled with an electrolyte (i.e., a conducting buffer solution). Depending on the experiment, the pore diameter can range from sub-nanometer size to several tens of nanometers. When a voltage is applied between both compartments, an ionic current flows through the nanopore, which can be measured with an amplifier. In the most basic sensing scheme, an analyte – such as a single protein – reaches the pore (by diffusion or additional driving forces described below), where it blocks the flow of ions leading to a characteristic resistive pulse with a current blockade ΔI , and an event duration Δt that signals the residence time of the protein in the pore (**Figure 1B**). The magnitude of the ionic current blockage depends on the size (molecular weight) and even on the shape of the analyte [49]. The event duration varies greatly for proteins, depending on the specific solid-state or biological pore structure, possible interactions with the pore walls, and the dominating driving force to the nanopore: while ‘near-ballistic’ translocation events happen within microseconds [50], specific trapping conditions offer much longer observation times (**Fig 1C**). The development of such new long-term sensing schemes was a major step forward, making nanopores more interesting for protein science than ever. We describe several specific examples in the second part of this review, to illustrate how nanopores catch protein functional features that are inaccessible to other methods.

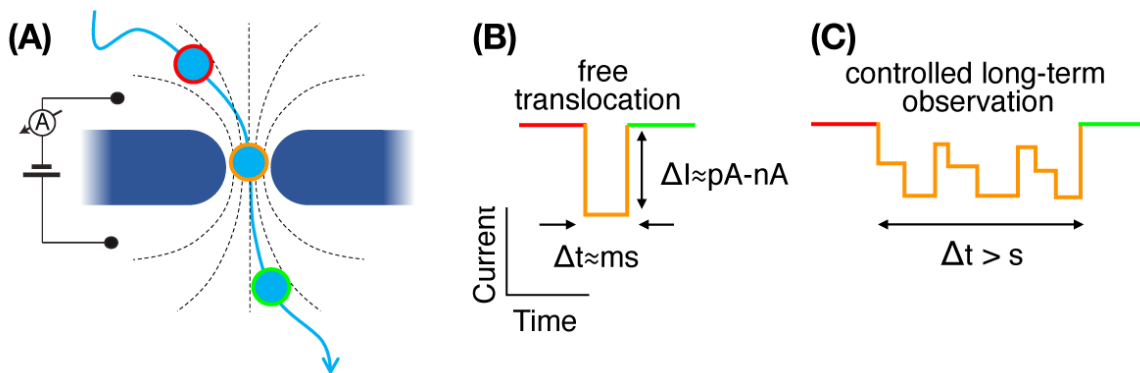


Figure 1: Nanopores in a nutshell. (A) Voltage is applied across an insulating membrane (dark blue) with a nanopore immersed in buffer, causing a measurable ionic current through the pore. A particle (light blue) approaches the nanopore (red position), partially blocks it (orange position), and subsequently leaves the pore again (green position). Electric field lines are illustrated as dashed lines. (B, C) Schematic nanopore current signals. The position color code of (A) is used to refer to the particle trajectory: a baseline current (red, green) is observed before and after the particle induced current blockade (orange). (B) A resistive pulse caused by free particle translocation provides only a short observation time (orange). (C) Creative experiment designs achieve much longer observation times (orange).

The nanopore zoo

Two classes of nanopores are typically distinguished: solid-state nanopores made by nanofabrication, and self-assembled biological nanopores. Solid-state nanopores have the advantage that they can be made at will, with a wide range of sizes and material properties. Glass nanopipets are the most affordable version of solid-state nanopores (**Figure 2A, left**): a pipet puller is used to produce a glass pipet that narrows down to a small nanopore at its end. While nanopipets have been used in various insightful experiments, their application is limited by their inherent asymmetry and the set material. Cleanroom-fabricated solid-state chips (**Fig. 2A, center**) offer more design flexibility. They often use a $\sim 5 \times 5 \times 0.3$ mm silicon chip as a substrate for a much thinner free-standing silicon nitride membrane (several nm to hundreds of nm thick), which is symmetrically accessible. Standard chips with thinned-down silicon nitride membranes are commercially available [51]. Recently, their high-frequency noise and thus signal-to-noise performance was significantly improved by adopting glass instead of silicon as the main substrate material, offering better dielectric and capacitive properties and hence lower noise [35]. Pores can be drilled by diverse techniques, each with their pros and cons: e.g., by controlled dielectric breakdown [52–56], by laser etching [56], using an electron beam in an SEM [57], a focused ion beam in a helium ion microscope [58,59], or a TEM [60] – listed in order of increasing cost, but also in approximate order of control and capacity. Beyond silicon nitride, various other membrane materials have been investigated, including atomically thin 2D materials [61], such as graphene [62], hexagonal boron nitride [63], or molybdenum disulfide [64] (displayed in **Fig 2a, right**). Semiconducting 2D materials have been investigated regarding additional detection strategies, such as sensing in-plane currents through the 2D membrane [65]. In addition, 2D materials attracted great attention regarding

DNA sequencing, because the atomic thickness at the nanopore should provide the ultimate spatial precision for reads along a translocating ssDNA strand. Until today, however, these delicate high-end solid-state sensors suffer from low reproducibility, added noise sources, and prohibitive fabrication cost – all still preventing DNA sequencing applications for the end user. Instead, protein nanopores won the race for nanopore-based DNA sequencing [66,67], admittedly by playing several clever tricks, including additional helper proteins, as described in part 2 of this review.

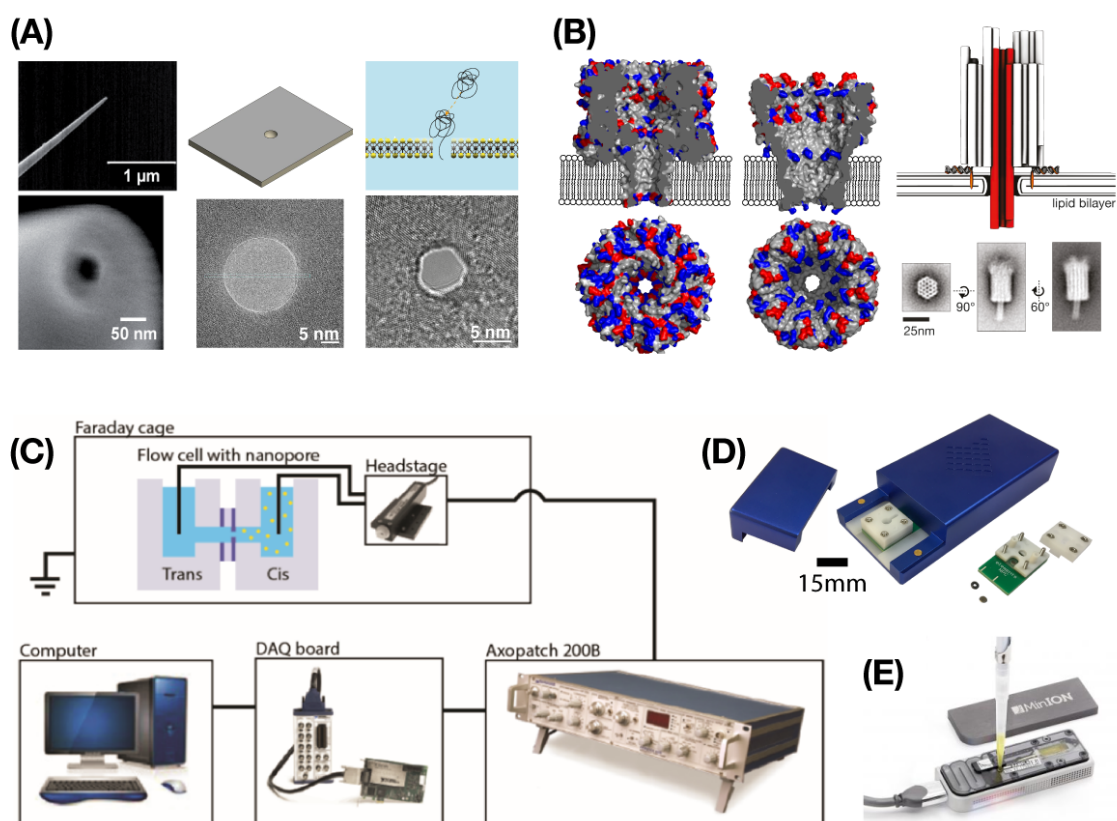


Figure 2: Various nanopores and instrumentation. (A) Three prominent examples of solid-state nanopores: (from left to right) glass pipet pulled to yield a sub-50nm pore; glass chips with a TEM-drilled 20nm pore in a silicon nitride membrane; nanopore in the 2D-material molybdenum disulfide. (B) Three representative biological nanopores – two protein pores (cut-open view in top panels, and top view in bottom panels; colors encode the charge distribution at neutral pH: positive, blue; negative, red; neutral, gray): the toxin α -hemolysin from *Staphylococcus aureus* (pdb: 3ANZ [68]); the commonly used M2 mutant of MspA from *Mycobacterium smegmatis* (pdb: 1UUN [69], mutated); an early synthetic DNA-origami pore with cartoon and TEM images. (C) A standard laboratory nanopore setup, with two-compartment flowcell, amplifier headstage, Axopatch 200B amplifier (Molecular Devices), DAQ board digitizer (National Instruments), computer for control & recording. (D) More compact integrated instrumentation: the Nanopore Reader (elements). (E) The portable, and highly parallelized Minion DNA sequencer (Oxford Nanopores) with 512 channels. Figure sources: panel (A) left [70], center [71], right [72]; panel (B) right [73]; panel (C) [74]; panel (D) adapted from [75]; panel (E) [76].

Biological nanopores [77] differ from their solid-state brothers, in two fundamental ways: (i) a lipid bilayer [78,79] or a block-copolymer membrane [80] serves as the insulating barrier between the buffer compartments, and (ii) the pores come with fixed self-assembled 3D structures and sizes, providing advantageous atomic precision and reproducibility. The α -hemolysin pore shown in **Figure 2B left** is

the most famous and widely studied pore protein. However, many other pores have been described [81,82], ranging in size from ~1nm to ~10nm in diameter (e.g. CsgG [83] and PlyAB [84], respectively). In many cases, these protein pores were engineered (with truncations, fusions, point mutations) to serve a specific sensing task [85]. For example, the charges in the pore mouth of MspA were mutated to facilitate the translocation of highly negatively charged ssDNA strands: **Figure 2B, center** shows the charge distribution of the widely used M2-MspA mutant [86]. In addition, entirely synthetic nanopores have been rationally designed using DNA-origami technology (**Fig. 2B, right**). Lipid membrane insertion of these highly charged, ion permeable structures is achieved by attaching hydrophobic anchor molecules, such as cholesterol or porphyrins that spontaneously insert into lipid bilayers [87]. DNA-origami has also been used to control membrane insertion of peptide assemblies [88]. And most recently, a synthetic proteinaceous potassium channel was demonstrated, indicating that protein nanopores with 'custom-made' specifications may become available in the future [89]. Two (manageable) bottlenecks in biological nanopore experiments are the stability of the fragile membrane, and the requirement to insert precisely one pore for single-molecule reads. Hybrid strategies combining solid-state scaffolds for lipid bilayers and biological nanopore sensors have been proposed as an improvement, but classical free-standing lipid bilayer approaches [78,79] still prevail in the literature.

The driving force by which the analyte reaches the pore depends on the analyte's net charge, as well as on pore properties. It is often dominated by electrophoresis (i.e., essentially electrostatic forces acting on the analyte's net charge, which is reduced by charge screening in solution) as in the case of highly negatively charged nucleic acids. However, depending on the nanopore itself, electro-osmosis can instead be the main driving force, especially for less charged analytes such as proteins. Electro-osmosis is a phenomenon arising at charged pore walls, when the applied voltage moves the screening counter-ions uni-directionally along the field lines, dragging water molecules along, and thus causing substantial hydrodynamic flows [84,90]. Such electro-osmosis is very useful for protein sensing, as it allows one to drive proteins to the nanopore regardless of their net charge.

Instrumentation for nanopore experiments is commercially available and affordable. The general experimental setup is similar for solid-state and biological nanopores (**Fig. 2C**). In both cases, an insulating membrane separates two buffer compartments. A few tens to hundreds of millivolts are usually applied across the pore using chloridized silver electrodes, resulting in typical currents in the range of 10 pA to several nA, depending on the pore and the conductivity of the buffer (the electrolyte) used. This current signal is amplified by a low-noise patch clamp amplifier, digitized, and controlled and recorded through a computer. This all-electrical sensing scheme lends itself to miniaturization, which has led to very compact [51] (**Fig 2D**) and highly parallelized handheld devices (**Fig 2E**). Furthermore, megahertz amplifiers have been realized using on-chip architectures [91,92].

Characteristics of nanopore signals

Protein-induced nanopore signals arise, because the protein reduces the density of the mobile charge carriers (ions) that occupy the nanopore, leading to a characteristic reduction in electrical conductance. The sensitivity of nanopores for the translocation of proteins and even for their shape and orientation was experimentally demonstrated by various groups [93,94]. Mayer and coworkers showed that near-spherical proteins like streptavidin create uni-modal current blockades, while disk-like immunoglobulin G caused a broad blockade distribution, related to the orientation of the protein in the pore (**Fig 3A**). A different perspective on nanopore sensitivity was given by Bayley and coworkers who showed that human and bovine thrombins are readily distinguished, despite 86% sequence identity [95].

High signals and low noise are needed to resolve protein kinetics. We recently compared the signal-to-noise ratio and individual noise sources in solid-state and biological nanopores [35], and hence we give here only a brief example of how noise scales with time resolution. This is important for every technique, since the detection of protein kinetics requires both a high enough signal-to-noise ratio and a wide enough time bandwidth (defined by the shortest and longest resolvable time interval) [96]. The noise level depends on the chosen low-pass filter frequency and thus time resolution, which is exemplified in **Figure 3B**. The signal-to-noise ratio furthermore scales with the electrolyte conductivity, i.e., the ionic strength of the buffer. As we will see in part 2, already at physiological to moderate salt concentrations that are suitable for proteins, nanopores provide ample SNR to detect protein function.

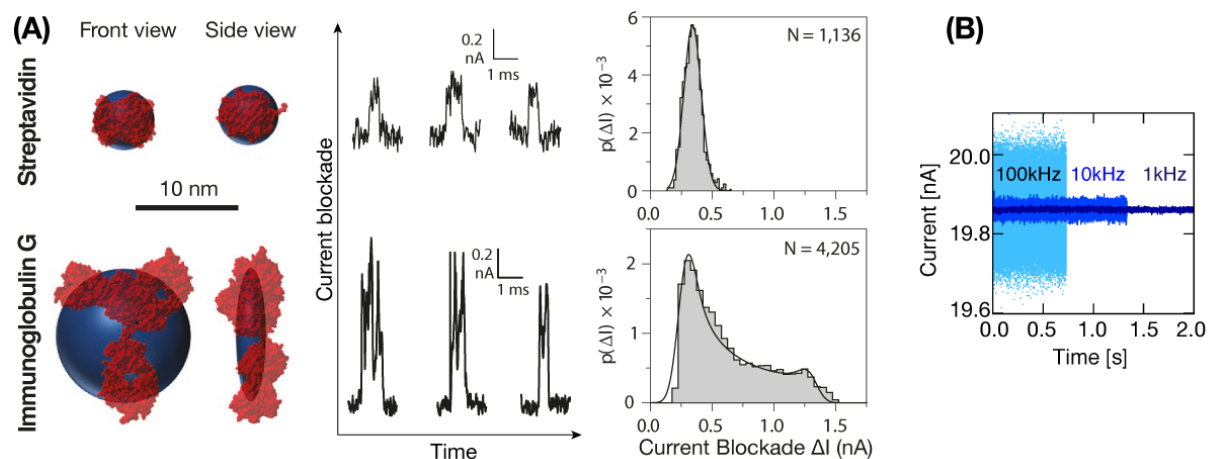


Figure 3: Nanopore signal characteristics. (A) Current blockades are protein size and shape dependent. Left: front and side view of streptavidin and Immunoglobulin G. Center: protein translocation events for streptavidin (top) and IgG (bottom). Right: corresponding event histograms. All adapted from Ref. [93]. (B) The noise dependence on low-pass filter frequency: current snippets of a 30nm silicon nitride pore in 1M KCl low-pass filtered at 100, 10, 1kHz as indicated.

WATCHING PROTEINS AT WORK USING NANOPORES

In this second part of the review, we showcase a few ground-breaking nanopore applications to protein science. We start with nanopore enzymology, the label-free real-time observation of protein enzymatic function.

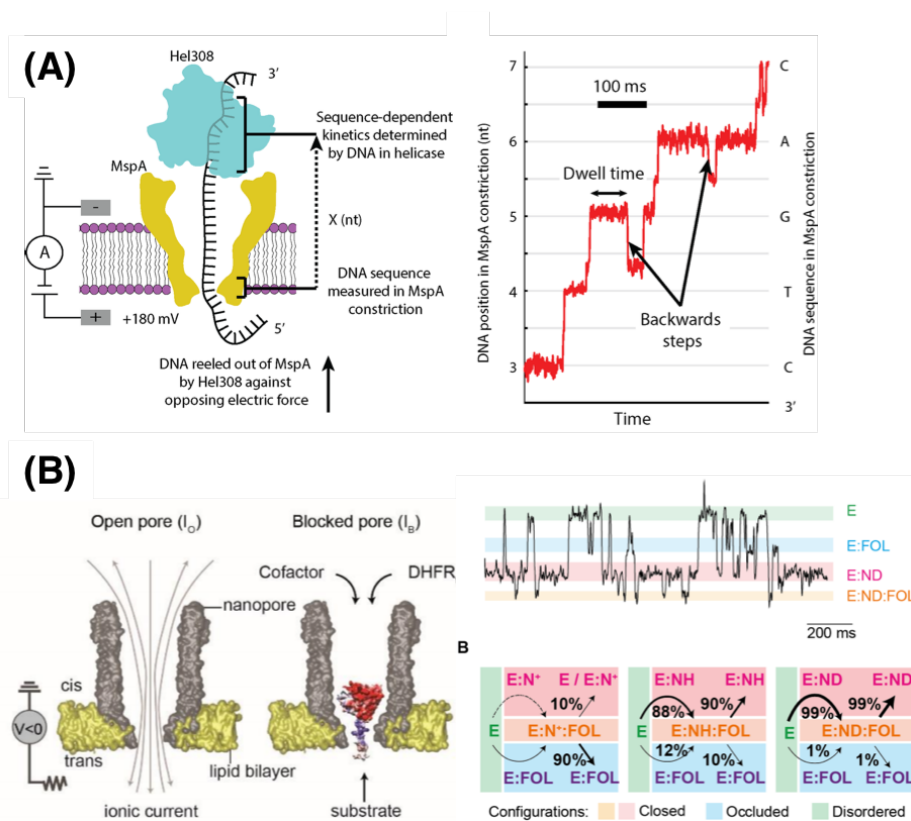


Figure 4: Live recording of protein function with nanopores. A) DNA processing by the helicase Hel 308 (cyan), detected using the MspA protein pore (yellow) as steps in the recorded nanopore current [97]. **B)** The functional cycle of dihydrofolate reductase (DHFR) directly observed by electro-osmotic trapping in the ClyA protein pore sensor resolves five functional states (four displayed) [98].

Nanopore enzymology

The Akeson and Gundlach labs developed a beautiful experiment that directly resolves ATP-dependent DNA processing by a motor protein, such as a polymerase [67,99] or a helicase [100], thereby achieving the central breakthrough to today's nanopore DNA sequencing. **Fig 4A** shows how the DNA's negative charge is exploited to trap the protein-DNA complex in the MspA pore protein under a positive voltage. In an ATP-dependent way, the helicase Hel308 reels in the ssDNA strand against the electrostatic pulling force (downwards in **Fig. 4A**). In this way, the helicase directly facilitates a slow DNA translocation through the protein nanopore sensor, leaving enough observation time to resolve half-basepair steps at millisecond time resolution, and notably all label-free. While the actual current signal at any given time is affected by approximately four neighbouring nucleotides along the DNA strand, individual base calling can still be achieved by post-hoc signal processing and pattern recognition algorithms. This combination of an ATP-driven motor protein plus a protein nanopore sensor has

become the basis for today's commercialized nanopore DNA or RNA sequencers. These experiments further illustrate that DNA/RNA-binding or processing proteins are convenient targets for nanopore experiments, as those nucleoprotein complexes can be 'grabbed' by the negative charge of the nucleic acid. The Gundlach lab appropriately termed this elegant nanopore force spectroscopy 'SPRINT', short for Single-molecule Picometer Resolution Nanopore Tweezers [100,101]. Regarding noise and resolution of DNA stepping, it benefits greatly from the extremely short distance between the speed-controlling helicase and the sensing pore constriction, in contrast to e.g. optical tweezers that involve micron-long handles to the distant micron-sized beads. Note that, unlike nucleic acids, peptides lack a uniform charge distribution, which is one reason why protein sequencing is a much bigger challenge for nanopore technology (see below).

Next, we consider a DNA-free all-protein system: dihydrofolate reductase (DHFR) studied with a ClyA protein nanopore (**Fig 4B**). The Maglia lab trapped such single protein complexes for tens of seconds to interrogate their functional dynamics [98]. A positively charged tail was attached to the DHFR to enhance (electro-osmotic with electrophoretic) trapping under negative voltage, and to orient the protein in a preferential direction [95,102]. In this way, tens to hundreds of dihydrofolate to tetrahydrofolate turnovers could be sensed at one DHFR molecule. And a total of five functional states were distinguished and deciphered from the label-free current recordings, simply by systematic substrate variation: \pm folate, \pm NADPH, etc. The result is the real-time observation of a single DHFR protein progressing through its functional cycle involving reversible and irreversible transitions that can now be distinguished. It is this kinetic connectivity between states — that is inaccessible from ensemble kinetics including time-correlation analysis — which is the key to revealing energetically driven functional processes. Interestingly, these single-molecule measurements revealed among other things that DHFR undergoes second-long catalytic pausing, related to an off-pathway that was linked to the tolerance of high NADP⁺ concentrations. The Achilles heel of this elegant label-free trapping approach lies in its applicability to small proteins only, set by the pre-determined size of the (already relatively large) protein nanopore lumen (≤ 7 nm diameter). To turn single protein trapping into a more generally applicable protein sensing tool, we recently developed the NEOtrap, the 'Nanopore Electro-Osmotic trap', by combining DNA origami and passivated solid-state nanopores that can be obtained at any size suitable for the target protein [103]. In brief, the origami structure is used to create strong electro-osmotic flows in a solid-state nanopore, which allows us to catch a protein, label-free, and hold it for several minutes at the most sensitive region of the nanopore. Alternatively, plasmonic trapping is being developed as an entirely optical way to trap and study single proteins [104,105]. Finally, many more approaches are possible. For example, bulk enzyme activity such as ubiquitination, was studied using nanopores by monitoring the time-dependent accumulation of product molecules [106]. And the kinetics of amyloid fibrillization was also observed in label-free solid-state nanopore experiments [49].

Transient protein-protein interactions

Specific protein-protein interactions (PPIs) have been studied at the single-molecule level using functionalized protein nanopores. For example, Movileanu and co-workers turned the FhuA β -barrel pore into a PPI sensor by fusing a Barnase domain plus an additional sensor peptide to the FhuA pore (Fig. 5A). Upon Barnase-Barstar interaction, the sensor peptide is pulled out of the pore, leading to a (at first counter-intuitive) higher conductance level of the bound state compared to the unbound state. In this way, the authors detected transient binding that is hardly accessible in bulk, and the specificity of this nanopore sensor was also shown in mammalian serum [107]. A similar strategy was applied earlier to detect kinase-substrate interactions and their kinetics [108,109]. Alternatively, protein pores have been decorated with aptamers, and other functional motifs turning them into specific sensors. Furthermore, protein interaction has been studied using DNA carriers and solid-state nanopores: e.g. directly for dCas9 interactions [110,111], or using epitope [112] or aptamer [113] functionalized DNA constructs as designed by the Keyser and Edel labs to specifically detect antibodies, avidin, etc. Lastly, also small molecules like sugars and single amino acids were detected by specific binders like glucose-binding protein that change their conformation upon ligand binding. Trace amounts thereof were even quantified in bodily fluids, including sweat, blood, saliva, and urine [114].

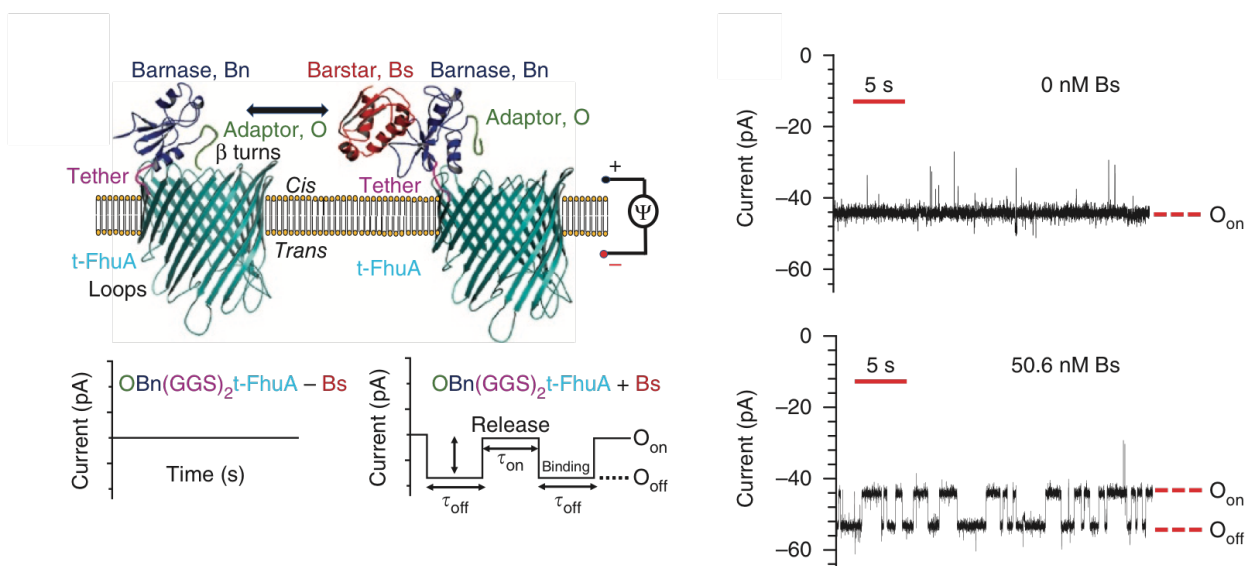


Figure 5: Single protein-protein interactions observed with nanopores. Barnase-Barstar interactions recorded using a FhuA fusion construct that serves as a specifically functionalized protein pore sensor [115]. Two-state kinetics appear upon Barstar addition due to transient binding events, leading to a detached sensor peptide (Adaptor O) and thus higher conductance than in the unbound state.

A glance at protein sequencing

As a future perspective for nanopore technology, protein sequencing is now emerging as a new hot topic [116]. Clearly, a single-molecule protein-sequencing device comparable to nanopore DNA sequencers would have a disruptive effect in the molecular life sciences. Yet, this nascent field has to deal with impressive challenges: no uniform charge along the peptide, twenty diverse building blocks

(amino acids), robust folds, etc. Nevertheless, many creative routes are currently being pursued, and specifically reviewed in [116–118]. Here, we briefly sketch a few current strategies, which fall into two main categories: *de novo* sequencing and fingerprinting. The latter aims at protein identification based on prior knowledge, which is e.g. pursued by Meller and coworkers who couple solid-state nanopores as a delivery system to confocal fluorescent detection [119]. Fluorescent tags are then attached to specific amino acids in unfolded peptides, in an attempt to read their number and sequence order. Brinkerhoff and Dekker exploit Akeson and Gundlach’s successful DNA-sequencing strategy described above (**Figure 4A**) and hook up a short peptide to the ssDNA strand, which is then slowly ratcheted through the MspA protein pore, with the potential to enable *de novo* sequencing of short peptides [120]. An encouraging proof-of-concept study by Oukhaled, Aksimentiev, Behrends and co-workers showed that under special conditions, a majority of all twenty amino acids could be distinguished using the aerolysin protein pore [121]. Early reports [122,123] of the unfolding and ratcheting of multi-domain proteins through a α -hemolysin protein pore by the proteasome ClpXP complex is inspiring strategies for long peptide reads. Such ideas are pursued by several groups, where also other unfolding motors are considered, e.g. SecYEG [124,125]. Besides sequencing or identification through fingerprinting, direct detection of post-translational modifications with single-molecule resolution would be another very important progress in biotechnology. Under specific conditions, phosphorylations and glycosylations were already detected in a label-free way [106,126,127], thereby paving the way for more general implementations.

Single-molecule techniques side by side

How do nanopores compare to other single-molecule techniques? In **Figure 6**, we compare various popular single-molecule techniques that can sense the time evolution of a single protein: high-speed AFM, optical and magnetic tweezers, surface immobilized smFRET, and nanopores. All of these techniques can detect kinetic connectivity, i.e., distinguish a transition from state A to state B, from the inverse transition B to A. This is a crucial difference to other time-resolved techniques that detect only direction-less fluctuations and mean life-times of states, such as FCS. This kinetic connectivity is absolutely necessary if the goal is to understand protein systems with more than two kinetic states, and in particular non-equilibrium steady-states. We further see from **Figure 6** that protein dynamics occur on a broad range of timescales, where the ultimately rate-limiting steps in protein function depends on underlying processes that can be orders of magnitudes faster, which is sometimes called the hierarchy of dynamics [128]. A broad time bandwidth is therefore key to uncover the origin of protein function, energetically and quantitatively. This is exactly where nanopores excel with their broad electrical bandwidth, and new experimental strategies that permit to effectively use a large part of it to generate information. Note that the actually informative bandwidth as shown in published work (white boxes in **Figure 6**) is narrower than the purely technical bandwidth (white lines) for all techniques. Depending on the technique, it is in practice limited by drift, protein stability (affected by surface interactions, force application, labelling, reactive oxygen species), photo-bleaching and other photo-physics, or baseline stability. Altogether, nanopores stand out in their ability to detect the broad-range dynamics of proteins,

which notably is achieved in solution, without the need of surface immobilization nor artificial labelling, and using affordable instrumentation.

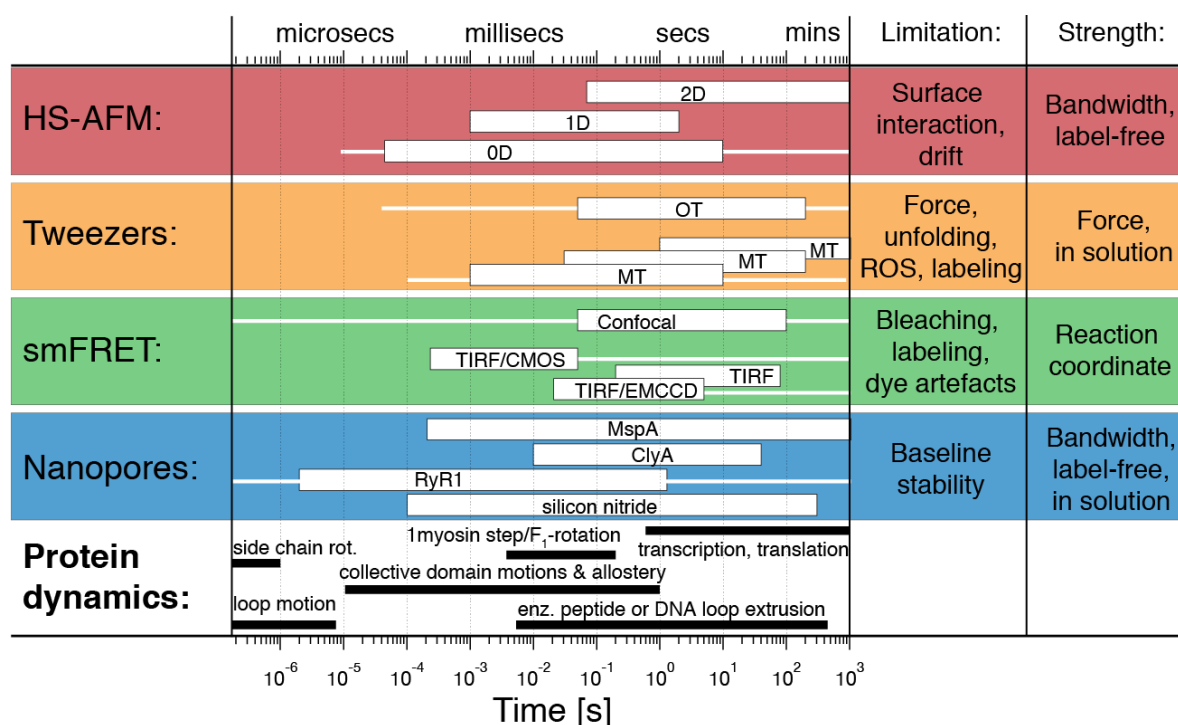


Figure 6: Timescales of protein functional dynamics and experimentally accessible bandwidths for their observation. White boxes represent experiments reported in the literature, thin white lines indicate the technical detector bandwidth: High-speed AFM (HS-AFM) in 2D imaging mode, 1D line scans, or 0D ‘on-spot’ detection, as indicated [129]. Optical (OT) [130,131], and magnetic tweezers (MT) [132–136] probing protein function (not unfolding, ROS: reactive oxygen species). Surface immobilized smFRET in confocal [137], or TIRF mode with CMOS [138] or EMCCD [2] detectors. Protein [98,139,140] and solid-state nanopores (own work). Examples of protein dynamics [141–143] controlling function [5,131,144] occurring in the microsecond to minutes range. Faster dynamics, as well as ensemble studies and mechanical unfolding experiments are not considered in this figure.

CONCLUSION

In this brief review, we introduced nanopore experiments, and discussed their great potential for protein science based on specific examples demonstrating the unique benefits of this technique, including (i) the label-free detection of protein function and protein interactions, (ii) at the single-molecule level, (iii) time-resolved with a bandwidth spanning currently up to seven orders in time in a single experiment, and (iv) all of this achieved with relatively cheap instrumentation compared to other single-molecule techniques. For DNA- or RNA-binding proteins, nanopore force-spectroscopy is a most convenient label-free technique with sub-basepair resolution for motor proteins stepping along nucleic acid strands. All-protein systems can be studied as well, e.g. by electro-osmotic trapping in protein or solid-state nanopores. Although these label-free experiments lack a predetermined spatial reaction coordinate (set by labelling in other techniques) dissecting individual functional states can be achieved by systematic experiments. Furthermore, transient interactions can be detected label-free and with high specificity,

even in bodily fluids. With all these techniques, protein scientists are well equipped to reach beyond the static structure era, and elucidate the dynamic character of these vital biomolecules. Furthermore, a lot can be expected from the emerging community that works towards protein sequencing, where biochemists and biophysicists meet with solid-state physicists, theoreticians, organic chemists, biomedical and nano-engineers to create a flourishing environment for ground-breaking interdisciplinary research.

SUMMARY

- **Nanopore detection is an affordable, label-free, single-molecule technique that is independent of photo-bleaching, surface immobilization, or mechanical unfolding of proteins.**
- **Long-term observation of single proteins can be achieved by electro-osmotic trapping, DNA association, or specific protein interactions.**
- **Protein functional states can be directly recognized in label-free current recordings.**
- **Electrical nanopore detection provides access to a broad time bandwidth of μ s to hours matching the protein functional time range.**
- **Thousands of protein functional cycles can be observed on one single molecule at sub-millisecond resolution.**

GLOSSARY

1D, 2D, 3D	1-, 2-, 3-dimensional
ATP	Adenosine triphosphate
cryoEM	cryo electron microscopy
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
EPR	electron paramagnetic resonance
FCS	fluorescence correlation spectroscopy
FRET	Förster resonance energy transfer
NMR	nuclear magnetic resonance
RNA	ribonucleic acid
SANS	small angle neutron scattering
SAXS	small angle x-ray scattering
smFRET	single-molecule FRET
SEM	scanning electron microscope
TEM	transmission electron microscope
TIRF	total internal reflection fluorescence

CONFLICTS OF INTEREST

The authors declare that there are no competing interests associated with the manuscript.

ACKNOWLEDGEMENTS

We thank Allard Katan and Richard Janissen for their comments on high-speed AFM and magnetic tweezers, respectively, and Sabina Caneva and Wayne Yang for comments on 2D materials.

FUNDING

SS acknowledges the Postdoc.Mobility fellowship no. P400PB_180889 by the Swiss National Science Foundation. CD acknowledges the ERC Advanced Grant LoopingDNA (no. 883684) and The Netherlands Organization of Scientific Research (NWO/ OCW) as part of the NanoFront and Basyk programs.

AUTHOR CONTRIBUTION

SS framed the concept of the review. Both authors wrote and discussed the manuscript.

REFERENCES

- 1 Berg, J.M., Tymoczko, J.L. and Stryer, L. (2002) *Biochemistry*.
- 2 Schmid, S. and Hugel, T. (2020) Controlling protein function by fine-tuning conformational flexibility. Spies, M., Faraldo-Gómez, J.D., Schlierf, M. and Craggs, T., Eds., *eLife*, eLife Sciences Publications, Ltd, **9**, e57180. <https://doi.org/10.7554/eLife.57180>.
- 3 Schuler, B., Soranno, A., Hofmann, H. and Nettels, D. (2016) Single-molecule fret spectroscopy and the polymer physics of unfolded and intrinsically disordered proteins. *Annual Review of Biophysics*, Annual Reviews, **45**, 207–231. <https://doi.org/10.1146/annurev-biophys-062215-010915>.
- 4 Burgers, P.M.J. and Kunkel, T.A. (2017) Eukaryotic dna replication fork. *Annual Review of Biochemistry*, **86**, 417–438. <https://doi.org/10.1146/annurev-biochem-061516-044709>.
- 5 Kodera, N., Yamamoto, D., Ishikawa, R. and Ando, T. (2010) Video imaging of walking myosin v by high-speed atomic force microscopy. *Nature*, **468**, 72–76. <https://doi.org/10.1038/nature09450>.
- 6 Nord, A.L., Gachon, E., Perez-Carrasco, R., Nirody, J.A., Barducci, A., Berry, R.M., et al. (2017) Catch bond drives stator mechanosensitivity in the bacterial flagellar motor. *Proceedings of the National Academy of Sciences*, **114**, 12952 LP – 12957. <https://doi.org/10.1073/pnas.1716002114>.
- 7 Lu, W. and Gelfand, V.I. (2017) Moonlighting motors: kinesin, dynein, and cell polarity. *Trends in Cell Biology*, **27**, 505–514. <https://doi.org/https://doi.org/10.1016/j.tcb.2017.02.005>.
- 8 Olivares, A.O., Baker, T.A. and Sauer, R.T. (2018) Mechanical protein unfolding and degradation. *Annual review of physiology*, Annual Reviews 4139 El Camino Way, PO Box 10139, Palo Alto, California 94303-0139, USA, **80**.
- 9 Junge, W. and Nelson, N. (2015) ATP synthase. *Annual Review of Biochemistry*, Annual Reviews, **84**, 631–657. <https://doi.org/10.1146/annurev-biochem-060614-034124>.
- 10 Dyla, M., Basse Hansen, S., Nissen, P. and Kjaergaard, M. (2019) Structural dynamics of p-type atpase ion pumps. *Biochemical Society Transactions*, **47**, 1247–1257. <https://doi.org/10.1042/BST20190124>.
- 11 Wickstrand, C., Nogly, P., Nango, E., Iwata, S., Standfuss, J. and Neutze, R. (2019) Bacteriorhodopsin: structural insights revealed using x-ray lasers and synchrotron radiation. *Annual Review of Biochemistry*, Annual Reviews, **88**, 59–83. <https://doi.org/10.1146/annurev-biochem-013118-111327>.
- 12 Romero, E., Novoderezhkin, V.I. and van Grondelle, R. (2017) Quantum design of photosynthesis for bio-inspired solar-energy conversion. *Nature*, **543**, 355–365. <https://doi.org/10.1038/nature22012>.
- 13 Butera, D., Passam, F., Ju, L., Cook, K.M., Woon, H., Aponte-Santamaría, C., et al. (2018) Autoregulation of von willebrand factor function by a disulfide bond switch. *Science Advances*, **4**, eaaq1477. <https://doi.org/10.1126/sciadv.aaq1477>.
- 14 Bartsch, T.F., Hengel, F.E., Oswald, A., Dionne, G., Chipendo, I. V, Mangat, S.S., et al. (2019) Elasticity of individual protocadherin 15 molecules implicates tip links as the gating springs for hearing. *Proceedings of the National Academy of Sciences*, **116**, 11048 LP – 11056. <https://doi.org/10.1073/pnas.1902163116>.
- 15 Oroz, J., Galera-Prat, A., Hervás, R., Valbuena, A., Fernández-Bravo, D. and Carrión-Vázquez, M. (2019) Nanomechanics of tip-link cadherins. *Scientific Reports*, **9**, 13306. <https://doi.org/10.1038/s41598-019-49518-x>.
- 16 Guo, Y.R. and MacKinnon, R. (2017) Structure-based membrane dome mechanism for piezo mechanosensitivity. Swartz, K.J., Ed., *eLife*, eLife Sciences Publications, Ltd, **6**, e33660. <https://doi.org/10.7554/eLife.33660>.
- 17 Moroni, M., Servin-Vences, M.R., Fleischer, R., Sánchez-Carranza, O. and Lewin, G.R. (2018) Voltage gating of mechanosensitive piezo channels. *Nature Communications*, **9**, 1096.

- <https://doi.org/10.1038/s41467-018-03502-7>.
- 18 Macek, B., Forchhammer, K., Hardouin, J., Weber-Ban, E., Grangeasse, C. and Mijakovic, I. (2019) Protein post-translational modifications in bacteria. *Nature Reviews Microbiology*, **17**, 651–664. <https://doi.org/10.1038/s41579-019-0243-0>.
- 19 Hirano, A., Fu, Y.-H. and Ptáček, L.J. (2016) The intricate dance of post-translational modifications in the rhythm of life. *Nature Structural & Molecular Biology*, **23**, 1053–1060. <https://doi.org/10.1038/nsmb.3326>.
- 20 Zamaraev, A. V., Kopeina, G.S., Prokhorova, E.A., Zhivotovsky, B. and Lavrik, I.N. (2017) Post-translational modification of caspases: the other side of apoptosis regulation. *Trends in Cell Biology*, **27**, 322–339. <https://doi.org/https://doi.org/10.1016/j.tcb.2017.01.003>.
- 21 Zhou, X.Z. and Lu, K.P. (2016) The isomerase pin1 controls numerous cancer-driving pathways and is a unique drug target. *Nature Reviews Cancer*, **16**, 463–478. <https://doi.org/10.1038/nrc.2016.49>.
- 22 Nogales, E. (2016) The development of cryo-em into a mainstream structural biology technique. *Nature Methods*, **13**, 24–27. <https://doi.org/10.1038/nmeth.3694>.
- 23 Alderson, T.R. and Kay, L.E. (2020) Unveiling invisible protein states with nmr spectroscopy. *Current Opinion in Structural Biology*, **60**, 39–49. <https://doi.org/https://doi.org/10.1016/j.sbi.2019.10.008>.
- 24 Jeschke, G. (2018) The contribution of modern epr to structural biology. Ubbink, M. and Perrakis, A., Eds., *Emerging Topics in Life Sciences*, **2**, 9–18. <https://doi.org/10.1042/ETLS20170143>.
- 25 Brosey, C.A. and Tainer, J.A. (2019) Evolving saxs versatility: solution x-ray scattering for macromolecular architecture, functional landscapes, and integrative structural biology. *Current Opinion in Structural Biology*, **58**, 197–213. <https://doi.org/https://doi.org/10.1016/j.sbi.2019.04.004>.
- 26 Trehwella, J. (2016) Small-angle scattering and 3d structure interpretation. *Current Opinion in Structural Biology*, **40**, 1–7. <https://doi.org/https://doi.org/10.1016/j.sbi.2016.05.003>.
- 27 Schlundt, A., Tants, J.-N. and Sattler, M. (2017) Integrated structural biology to unravel molecular mechanisms of protein-rna recognition. *Methods*, **118–119**, 119–136. <https://doi.org/https://doi.org/10.1016/j.ymeth.2017.03.015>.
- 28 Hellenkamp, B., Schmid, S., Doroshenko, O., Opanasyuk, O., Kühnemuth, R., Rezaei Adariani, S., et al. (2018) Precision and accuracy of single-molecule fret measurements - a multi-laboratory benchmark study. *Nature Methods*, **15**, 669–676. <https://doi.org/10.1038/s41592-018-0085-0>.
- 29 Lerner, E., Cordes, T., Ingargiola, A., Alhadid, Y., Chung, S., Michalet, X., et al. (2018) Toward dynamic structural biology: two decades of single-molecule förster resonance energy transfer. *Science, American Association for the Advancement of Science*, **359**, eaan1133. <https://doi.org/10.1126/science.aan1133>.
- 30 Ando, T. (2019) High-speed atomic force microscopy. *Current Opinion in Chemical Biology*, **51**, 105–112. <https://doi.org/https://doi.org/10.1016/j.cbpa.2019.05.010>.
- 31 De Vlaminc, I. and Dekker, C. (2012) Recent advances in magnetic tweezers. *Annual Review of Biophysics*, Annual Reviews, **41**, 453–472. <https://doi.org/10.1146/annurev-biophys-122311-100544>.
- 32 Kemmerich, F.E., Kasaciunaite, K. and Seidel, R. (2016) Modular magnetic tweezers for single-molecule characterizations of helicases. *Methods*, **108**, 4–13. <https://doi.org/https://doi.org/10.1016/j.ymeth.2016.07.004>.
- 33 Whitley, K.D., Comstock, M.J. and Chemla, Y.R. (2017) Chapter six - high-resolution optical tweezers combined with single-molecule confocal microscopy. In: Spies, M. and Chemla, Y.R.B.T.-M. in E., Eds., *Single-Molecule Enzymology: Nanomechanical Manipulation and Hybrid Methods*, Academic Press, 137–169. <https://doi.org/https://doi.org/10.1016/bs.mie.2016.10.036>.
- 34 Bustamante, C., Alexander, L., Maciuba, K. and Kaiser, C.M. (2020) Single-molecule studies of protein folding with optical tweezers. *Annual Review of Biochemistry*, Annual Reviews, **89**, 443–470. <https://doi.org/10.1146/annurev-biochem-013118-111442>.
- 35 Fragasso, A., Schmid, S. and Dekker, C. (2020) Comparing current noise in biological and solid-state nanopores. *ACS Nano*, American Chemical Society, **14**, 1338–1349. <https://doi.org/10.1021/acsnano.9b09353>.
- 36 Johnson, S.S., Zaikova, E., Goerlitz, D.S., Bai, Y. and Tighe, S.W. (2017) Real-time dna sequencing in the antarctic dry valleys using the oxford nanopore sequencer. *Journal of biomolecular techniques : JBT*, Association of Biomolecular Resource Facilities, **28**, 2–7. <https://doi.org/10.7171/jbt.17-2801-009>.
- 37 Zaaijer, S., Gordon, A., Speyer, D., Piccone, R., Groen, S.C. and Erlich, Y. (2017) Rapid re-identification of human samples using portable dna sequencing. Morris, A.P., Ed., *eLife*, eLife Sciences Publications, Ltd, **6**, e27798. <https://doi.org/10.7554/eLife.27798>.
- 38 Ho, J.K.I., Puniamoorthy, J., Srivathsan, A. and Meier, R. (2020) MinION sequencing of seafood in singapore reveals creatively labelled flatfishes, confused roe, pig dna in squid balls, and phantom crustaceans. *Food Control*, **112**, 107144. <https://doi.org/https://doi.org/10.1016/j.foodcont.2020.107144>.
- 39 Castro-Wallace, S.L., Chiu, C.Y., John, K.K., Stahl, S.E., Rubins, K.H., McIntyre, A.B.R., et al. (2017) Nanopore dna sequencing and genome assembly on the international space station. *Scientific Reports*, **7**, 18022. <https://doi.org/10.1038/s41598-017-18364-0>.
- 40 Quick, J., Loman, N.J., Duraffour, S., Simpson, J.T., Severi, E., Cowley, L., et al. (2016) Real-time, portable genome sequencing for ebola surveillance. *Nature*, **530**, 228–232. <https://doi.org/10.1038/nature16996>.
- 41 Quick, J., Quinlan, A.R. and Loman, N.J. (2014) A reference bacterial genome dataset generated on the

- minion™ portable single-molecule nanopore sequencer. *GigaScience*, **3**. <https://doi.org/10.1186/2047-217X-3-22>.
- 42 Jain, M., Koren, S., Miga, K.H., Quick, J., Rand, A.C., Sasani, T.A., et al. (2018) Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nature Biotechnology*, The Author(s) SN -, **36**, 338 EP-. <http://dx.doi.org/10.1038/nbt.4060>.
- 43 Garalde, D.R., Snell, E.A., Jachimowicz, D., Sipos, B., Lloyd, J.H., Bruce, M., et al. (2018) Highly parallel direct rna sequencing on an array of nanopores. *Nature Methods*, **15**, 201–206. <https://doi.org/10.1038/nmeth.4577>.
- 44 Plesa, C. and Dekker, C. (2015) Data analysis methods for solid-state nanopores. *Nanotechnology*, **26**, 84003. <http://stacks.iop.org/0957-4484/26/i=8/a=084003>.
- 45 Edel, J.B. and Albrecht, T. (2013) Engineered nanopores for bioanalytical applications. Elsevier Science. <https://books.google.nl/books?id=eDeFUXIWn-MC>.
- 46 Noy, A. and Wanunu, M. (2017) Nanopores. *An Introduction to Single Molecule Biophysics*, CRC Press, 197.
- 47 Albrecht, T. (2019) Single-molecule analysis with solid-state nanopores. *Annual Review of Analytical Chemistry*, Annual Reviews, **12**, 371–387. <https://doi.org/10.1146/annurev-anchem-061417-125903>.
- 48 Deamer, D., Akeson, M. and Branton, D. (2016) Three decades of nanopore sequencing. *Nature Biotechnology*, **34**, 518–524. <https://doi.org/10.1038/nbt.3423>.
- 49 Yusko, E.C., Johnson, J.M., Majd, S., Prangio, P., Rollings, R.C., Li, J., et al. (2011) Controlling protein translocation through nanopores with bio-inspired fluid walls. *Nat Nano*, Nature Publishing Group, **6**, 253–260. <http://dx.doi.org/10.1038/nnano.2011.12>.
- 50 Plesa, C., Kowalczyk, S.W., Zinsmeister, R., Grosberg, A.Y., Rabin, Y. and Dekker, C. (2013) Fast translocation of proteins through solid state nanopores. *Nano Letters*, American Chemical Society, **13**, 658–663. <https://doi.org/10.1021/nl3042678>.
- 51 Niedzwiecki, D.J., Chou, Y.-C., Xia, Z., Thei, F. and Drndić, M. (2020) Detection of single analyte and environmental samples with silicon nitride nanopores: antarctic dirt particulates and dna in artificial seawater. *Review of Scientific Instruments*, American Institute of Physics, **91**, 31301. <https://doi.org/10.1063/1.5138210>.
- 52 Ying, C., Zhang, Y., Feng, Y., Zhou, D., Wang, D., Xiang, Y., et al. (2016) 3D nanopore shape control by current-stimulus dielectric breakdown. *Applied Physics Letters*, American Institute of Physics, **109**, 63105. <https://doi.org/10.1063/1.4960636>.
- 53 Waugh, M., Briggs, K., Gunn, D., Gibeault, M., King, S., Ingram, Q., et al. (2020) Solid-state nanopore fabrication by automated controlled breakdown. *Nature Protocols*, **15**, 122–143. <https://doi.org/10.1038/s41596-019-0255-2>.
- 54 Ying, C., Houghtaling, J., Eggenberger, O.M., Guha, A., Nirmalraj, P., Awasthi, S., et al. (2018) Formation of single nanopores with diameters of 20–50 nm in silicon nitride membranes using laser-assisted controlled breakdown. *ACS Nano*, **12**, 11458–11470. <https://doi.org/10.1021/acsnano.8b06489>.
- 55 Yanagi, I., Akahori, R. and Takeda, K. (2019) Stable fabrication of a large nanopore by controlled dielectric breakdown in a high-ph solution for the detection of various-sized molecules. *Scientific Reports*, **9**, 13143. <https://doi.org/10.1038/s41598-019-49622-y>.
- 56 Gilboa, T., Zrehen, A., Girsault, A. and Meller, A. (2018) Optically-monitored nanopore fabrication using a focused laser beam. *Scientific Reports*, **8**, 9765. <https://doi.org/10.1038/s41598-018-28136-z>.
- 57 Spinney, P.S., Howitt, D.G., Smith, R.L. and Collins, S.D. (2010) Nanopore formation by low-energy focused electron beam machining. *Nanotechnology*, IOP Publishing, **21**, 375301. <https://doi.org/10.1088/0957-4484/21/37/375301>.
- 58 Ananth, M., Stern, L., Ferranti, D., Huynh, C., Notte, J., Scipioni, L., et al. (2011) Creating nanohole arrays with the helium ion microscope. *Proc.SPIE*. <https://doi.org/10.1117/12.887497>.
- 59 Xia, D., Huynh, C., McVey, S., Kobler, A., Stern, L., Yuan, Z., et al. (2018) Rapid fabrication of solid-state nanopores with high reproducibility over a large area using a helium ion microscope. *Nanoscale*, The Royal Society of Chemistry, **10**, 5198–5204. <https://doi.org/10.1039/C7NR08406D>.
- 60 van den Hout, M., Hall, A.R., Wu, M.Y., Zandbergen, H.W., Dekker, C. and Dekker, N.H. (2010) Controlling nanopore size, shape and stability. *Nanotechnology*, IOP Publishing, **21**, 115304. <https://doi.org/10.1088/0957-4484/21/11/115304>.
- 61 Danda, G. and Drndić, M. (2019) Two-dimensional nanopores and nanoporous membranes for ion and molecule transport. *Current Opinion in Biotechnology*, **55**, 124–133. <https://doi.org/https://doi.org/10.1016/j.copbio.2018.09.002>.
- 62 Schneider, G.F., Kowalczyk, S.W., Calado, V.E., Pandraud, G., Zandbergen, H.W., Vandersypen, L.M.K., et al. (2010) DNA translocation through graphene nanopores. *Nano Letters*, American Chemical Society, **10**, 3163–3167. <https://doi.org/10.1021/nl102069z>.
- 63 Zhou, Z., Hu, Y., Wang, H., Xu, Z., Wang, W., Bai, X., et al. (2013) DNA translocation through hydrophilic nanopore in hexagonal boron nitride. *Scientific Reports*, The Author(s) SN -, **3**, 3287 EP-. <http://dx.doi.org/10.1038/srep03287>.
- 64 Liu, K., Feng, J., Kis, A. and Radenovic, A. (2014) Atomically thin molybdenum disulfide nanopores with high sensitivity for dna translocation. *ACS Nano*, American Chemical Society, **8**, 2504–2511.

- <https://doi.org/10.1021/nn406102h>.
- 65 Heerema, S.J. and Dekker, C. (2016) Graphene nanodevices for dna sequencing. *Nature Nanotechnology*, **11**, 127–136. <https://doi.org/10.1038/nnano.2015.307>.
- 66 Cherf, G.M., Lieberman, K.R., Rashid, H., Lam, C.E., Karplus, K. and Akeson, M. (2012) Automated forward and reverse ratcheting of dna in a nanopore at 5-Å precision. *Nature Biotechnology*, **30**, 344–348. <https://doi.org/10.1038/nbt.2147>.
- 67 Manrao, E.A., Derrington, I.M., Laszlo, A.H., Langford, K.W., Hopper, M.K., Gillgren, N., et al. (2012) Reading dna at single-nucleotide resolution with a mutant mspa nanopore and phi29 dna polymerase. *Nature Biotechnology*, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. SN -, **30**, 349 EP-. <http://dx.doi.org/10.1038/nbt.2171>.
- 68 Tanaka, Y., Hirano, N., Kaneko, J., Kamio, Y., Yao, M. and Tanaka, I. (2011) 2-methyl-2,4-pentanediol induces spontaneous assembly of staphylococcal α -hemolysin into heptameric pore structure. *Protein Science*, Wiley Subscription Services, Inc., A Wiley Company, **20**, 448–456. <https://doi.org/10.1002/pro.579>.
- 69 Faller, M., Niederweis, M. and Schulz, G.E. (2004) The structure of a mycobacterial outer-membrane channel. *Science*, **303**, 1189 LP – 1192. <https://doi.org/10.1126/science.1094114>.
- 70 Tiwari, P.B., Astudillo, L., Miksovska, J., Wang, X., Li, W., Darici, Y., et al. (2014) Quantitative study of protein–protein interactions by quartz nanopipettes. *Nanoscale*, The Royal Society of Chemistry, **6**, 10255–10263. <https://doi.org/10.1039/C4NR02964J>.
- 71 Buvac, V. (2019) Personal communication. Goepfert LLC.
- 72 Feng, J., Liu, K., Bulushev, R.D., Khlybov, S., Dumcenco, D., Kis, A., et al. (2015) Identification of single nucleotides in mos2 nanopores. *Nat Nano*, Nature Publishing Group, **10**, 1070–1076. <http://dx.doi.org/10.1038/nnano.2015.219>.
- 73 Langecker, M., Arnaut, V., Martin, T.G., List, J., Renner, S., Mayer, M., et al. (2012) Synthetic lipid membrane channels formed by designed dna nanostructures. *Science*, American Association for the Advancement of Science, **338**, 932–936. <https://doi.org/10.1126/science.1225624>.
- 74 Lee, J.S., Saharia, J., Bandara, Y.M.N.D.Y., Karawdeniya, B.I., Goyal, G., Darvish, A., et al. (2019) Stiffness measurement of nanosized liposomes using solid-state nanopore sensor with automated recapturing platform. *ELECTROPHORESIS*, John Wiley & Sons, Ltd, **40**, 1337–1344. <https://doi.org/10.1002/elps.201800476>.
- 75 Thei, F. (2019) Elements srs1. <https://elements-ic.com/nanopore-reader/>.
- 76 Regalado, A. (2014) Radical new dna sequencer finally gets into researchers' hands. <https://www.technologyreview.com/2014/09/17/171338/radical-new-dna-sequencer-finally-gets-into-researchers-hands/>.
- 77 Howorka, S. (2017) Building membrane nanopores. *Nat Nano*, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., **12**, 619–630. <http://dx.doi.org/10.1038/nnano.2017.99>.
- 78 Jetha, N.N., Wiggin, M. and Marziali, A. (2009) Forming an α -hemolysin nanopore for single-molecule analysis. In: Foote, R.S. and Lee, J.W., Eds., *Micro and Nano Technologies in Bioanalysis: Methods and Protocols*, Humana Press, Totowa, NJ, 113–127. https://doi.org/10.1007/978-1-59745-483-4_9.
- 79 Maglia, G., Heron, A.J., Stoddart, D., Japrun, D. and Bayley, H. (2010) Chapter 22 - analysis of single nucleic acid molecules with protein nanopores. In: Walter, N.G., Ed., *Single Molecule Tools, Part B: Super-Resolution, Particle Tracking, Multiparameter, and Force Based Methods*, Academic Press, 591–623. [https://doi.org/https://doi.org/10.1016/S0076-6879\(10\)75022-9](https://doi.org/https://doi.org/10.1016/S0076-6879(10)75022-9).
- 80 Morton, D., Mortezaei, S., Yemencioğlu, S., Isaacman, M.J., Nova, I.C., Gundlach, J.H., et al. (2015) Tailored polymeric membranes for mycobacterium smegmatis porin a (mspa) based biosensors. *J. Mater. Chem. B*, The Royal Society of Chemistry, **3**, 5080–5086. <https://doi.org/10.1039/C5TB00383K>.
- 81 Wang, S., Zhao, Z., Haque, F. and Guo, P. (2018) Engineering of protein nanopores for sequencing, chemical or protein sensing and disease diagnosis. *Current Opinion in Biotechnology*, **51**, 80–89. <https://doi.org/https://doi.org/10.1016/j.copbio.2017.11.006>.
- 82 Willems, K., Van Meervelt, V., Wloka, C. and Maglia, G. (2017) Single-molecule nanopore enzymology. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, The Royal Society, **372**. <https://doi.org/10.1098/rstb.2016.0230>.
- 83 Van der Verren, S.E., Van Gerven, N., Jonckheere, W., Hambley, R., Singh, P., Kilgour, J., et al. (2020) A dual-constriction biological nanopore resolves homonucleotide sequences with high fidelity. *Nature Biotechnology*. <https://doi.org/10.1038/s41587-020-0570-8>.
- 84 Huang, G., Willems, K., Bartelds, M., van Dorpe, P., Soskine, M. and Maglia, G. Electro-osmotic vortices promote the capture of folded proteins by plyab nanopores. *Nano Letters*, **0**, null. <https://doi.org/10.1021/acs.nanolett.0c00877>.
- 85 Ayub, M. and Bayley, H. (2016) Engineered transmembrane pores. *Current Opinion in Chemical Biology*, **34**, 117–126. <https://doi.org/https://doi.org/10.1016/j.cbpa.2016.08.005>.
- 86 Butler, T.Z., Pavlenok, M., Derrington, I.M., Niederweis, M. and Gundlach, J.H. (2008) Single-molecule dna detection with an engineered mspa protein nanopore. *Proceedings of the National Academy of Sciences*, **105**, 20647–20652. <https://doi.org/10.1073/pnas.0807514106>.

- 87 Göpfrich, K., Li, C.-Y., Ricci, M., Bhamidimarri, S.P., Yoo, J., Gyenes, B., et al. (2016) Large-conductance transmembrane porin made from dna origami. *ACS Nano*, American Chemical Society, **10**, 8207–8214. <https://doi.org/10.1021/acsnano.6b03759>.
- 88 Spruijt, E., Tusk, S.E. and Bayley, H. (2018) DNA scaffolds support stable and uniform peptide nanopores. *Nature Nanotechnology*, **13**, 739–745. <https://doi.org/10.1038/s41565-018-0139-6>.
- 89 Xu, C., Lu, P., Gamal El-Din, T.M., Pei, X.Y., Johnson, M.C., Uyeda, A., et al. (2020) Computational design of transmembrane pores. *Nature*, **585**, 129–134. <https://doi.org/10.1038/s41586-020-2646-5>.
- 90 Firnkes, M., Pedone, D., Knezevic, J., Döblinger, M. and Rant, U. (2010) Electrically facilitated translocations of proteins through silicon nitride nanopores: conjoint and competitive action of diffusion, electrophoresis, and electroosmosis. *Nano letters*, American Chemical Society, **10**, 2162–2167. <https://doi.org/10.1021/nl100861c>.
- 91 Chien, C.-C., Shekar, S., Niedzwiecki, D.J., Shepard, K.L. and Drndić, M. (2019) Single-stranded dna translocation recordings through solid-state nanopores on glass chips at 10 mhz measurement bandwidth. *ACS Nano*, American Chemical Society, **13**, 10545–10554. <https://doi.org/10.1021/acsnano.9b04626>.
- 92 Hartel, A.J.W., Shekar, S., Ong, P., Schroeder, I., Thiel, G. and Shepard, K.L. (2019) High bandwidth approaches in nanopore and ion channel recordings - a tutorial review. *Analytica Chimica Acta*, **1061**, 13–27. <https://doi.org/https://doi.org/10.1016/j.aca.2019.01.034>.
- 93 Yusko, E.C., Bruhn, B.R., Eggenberger, O.M., Houghtaling, J., Rollings, R.C., Walsh, N.C., et al. (2016) Real-time shape approximation and fingerprinting of single proteins using a nanopore. *Nature Nanotechnology*, Nature Publishing Group SN -, **12**, 360 EP-. <http://dx.doi.org/10.1038/nnano.2016.267>.
- 94 Waduge, P., Hu, R., Bandarkar, P., Yamazaki, H., Cressiot, B., Zhao, Q., et al. (2017) Nanopore-based measurements of protein size, fluctuations, and conformational changes. *ACS Nano*, American Chemical Society, **acsnano.7b01212**. <https://doi.org/10.1021/acsnano.7b01212>.
- 95 Soskine, M., Biesemans, A., Moeyaert, B., Cheley, S., Bayley, H. and Maglia, G. (2012) An engineered clyA nanopore detects folded target proteins by selective external association and pore entry. *Nano Letters*, **12**, 4895–4900. <https://doi.org/10.1021/nl3024438>.
- 96 Schmid, S. and Hugel, T. (2018) Efficient use of single molecule time traces to resolve kinetic rates, models and uncertainties. *The Journal of Chemical Physics*, **148**, 123312. <https://doi.org/10.1063/1.5006604>.
- 97 Craig, J.M., Laszlo, A.H., Nova, I.C., Brinkerhoff, H., Noakes, M.T., Baker, K.S., et al. (2019) Determining the effects of dna sequence on hel308 helicase translocation along single-stranded dna using nanopore tweezers. *Nucleic Acids Research*, **47**, 2506–2513. <https://doi.org/10.1093/nar/gkz004>.
- 98 Galenkamp, N.S. and Maglia, G. (2020) Substrate binding and turnover modulate the affinity landscape of dihydrofolate reductase to increase its catalytic efficiency. *bioRxiv*, 2020.04.14.040733. <https://doi.org/10.1101/2020.04.14.040733>.
- 99 Lieberman, K.R., Cherf, G.M., Doody, M.J., Olasagasti, F., Kolodji, Y. and Akeson, M. (2010) Processive replication of single dna molecules in a nanopore catalyzed by phi29 dna polymerase. *Journal of the American Chemical Society*, American Chemical Society, **132**, 17961–17972. <https://doi.org/10.1021/ja1087612>.
- 100 Derrington, I.M., Craig, J.M., Stava, E., Laszlo, A.H., Ross, B.C., Brinkerhoff, H., et al. (2015) Subangstrom single-molecule measurements of motor proteins using a nanopore. *Nat Biotech*, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., **33**, 1073–1075. <http://dx.doi.org/10.1038/nbt.3357>.
- 101 Laszlo, A.H., Derrington, I.M. and Gundlach, J.H. (2016) MspA nanopore as a single-molecule tool: from sequencing to sprnt. *Methods*, **105**, 75–89. <https://doi.org/https://doi.org/10.1016/j.ymeth.2016.03.026>.
- 102 Willems, K., Ruić, D., Lucas, F., Barman, U., Hofkens, J., Maglia, G., et al. (2020) Modeling of ion and water transport in the biological nanopore clyA. *bioRxiv*, 2020.01.08.897819. <https://doi.org/10.1101/2020.01.08.897819>.
- 103 Schmid, S. et al. (2020) *In preparation*.
- 104 Pang, Y. and Gordon, R. (2012) Optical trapping of a single protein. *Nano Letters*, American Chemical Society, **12**, 402–406. <https://doi.org/10.1021/nl203719v>.
- 105 Verschuere, D. V., Pud, S., Shi, X., De Angelis, L., Kuipers, L. and Dekker, C. (2019) Label-free optical detection of dna translocations through plasmonic nanopores. *ACS Nano*, American Chemical Society, **13**, 61–70. <https://doi.org/10.1021/acsnano.8b06758>.
- 106 Wloka, C., Van Meervelt, V., van Gelder, D., Danda, N., Jager, N., Williams, C.P., et al. (2017) Label-free and real-time detection of protein ubiquitination with a biological nanopore. *ACS Nano*, **11**, 4387–4394. <https://doi.org/10.1021/acsnano.6b07760>.
- 107 Thakur, A.K. and Movileanu, L. (2019) Single-molecule protein detection in a biofluid using a quantitative nanopore sensor. *ACS Sensors*, American Chemical Society, **4**, 2320–2326. <https://doi.org/10.1021/acssensors.9b00848>.
- 108 Harrington, L., Cheley, S., Alexander, L.T., Knapp, S. and Bayley, H. (2013) Stochastic detection of pim protein kinases reveals electrostatically enhanced association of a peptide substrate. *Proceedings of the National Academy of Sciences of the United States of America*, National Academy of Sciences, **110**,

- E4417–E4426. <https://doi.org/10.1073/pnas.1312739110>.
- 109 Harrington, L., Alexander, L.T., Knapp, S. and Bayley, H. (2019) Single-molecule protein phosphorylation and dephosphorylation by nanopore enzymology. *ACS Nano*, American Chemical Society, **13**, 633–641. <https://doi.org/10.1021/acsnano.8b07697>.
- 110 Yang, W., Restrepo-Pérez, L., Bengtson, M., Heerema, S.J., Birnie, A., van der Torre, J., et al. Detection of crispr-dcas9 on dna with solid-state nanopores. *Nano Letters*, **0**, null. <https://doi.org/10.1021/acs.nanolett.8b02968>.
- 111 Weckman, N.E., Ermann, N., Gutierrez, R., Chen, K., Graham, J., Tivony, R., et al. (2019) Multiplexed dna identification using site specific dcas9 barcodes and nanopore sensing. *ACS Sensors*, **4**, 2065–2072. <https://doi.org/10.1021/acssensors.9b00686>.
- 112 Bell, N.A.W. and Keyser, U.F. (2016) Digitally encoded dna nanostructures for multiplexed, single-molecule protein sensing with nanopores. *Nature Nanotechnology*, **11**, 645–651. <https://doi.org/10.1038/nnano.2016.50>.
- 113 Sze, J.Y.Y., Ivanov, A.P., Cass, A.E.G. and Edel, J.B. (2017) Single molecule multiplexed nanopore protein screening in human serum using aptamer modified dna carriers. *Nature Communications*, **8**, 1552. <https://doi.org/10.1038/s41467-017-01584-3>.
- 114 Galenkamp, N.S., Soskine, M., Hermans, J., Wloka, C. and Maglia, G. (2018) Direct electrical quantification of glucose and asparagine from bodily fluids using nanopores. *Nature Communications*, **9**, 4085. <https://doi.org/10.1038/s41467-018-06534-1>.
- 115 Thakur, A.K. and Movileanu, L. (2018) Real-time measurement of protein–protein interactions at single-molecule resolution using a biological nanopore. *Nature Biotechnology*, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. SN -, **37**, 96 EP-. <https://doi.org/10.1038/nbt.4316>.
- 116 Restrepo-Pérez, L., Joo, C. and Dekker, C. (2018) Paving the way to single-molecule protein sequencing. *Nature Nanotechnology*, **13**, 786–796. <https://doi.org/10.1038/s41565-018-0236-6>.
- 117 Callahan, N., Tullman, J., Kelman, Z. and Marino, J. (2019) Strategies for development of a next-generation protein sequencing platform. *Trends in Biochemical Sciences*. <https://doi.org/https://doi.org/10.1016/j.tibs.2019.09.005>.
- 118 Asandei, A., Di Muccio, G., Schiopu, I., Mereuta, L., Dragomir, I.S., Chinappi, M., et al. (2020) Nanopore-based protein sequencing using biopores: current achievements and open challenges. *Small Methods*, John Wiley & Sons, Ltd, **n/a**, 1900595. <https://doi.org/10.1002/smt.201900595>.
- 119 Ohayon, S., Girsault, A., Nasser, M., Shen-Orr, S. and Meller, A. (2019) Simulation of single-protein nanopore sensing shows feasibility for whole-proteome identification. *PLOS Computational Biology*, Public Library of Science, **15**, e1007067. <https://doi.org/10.1371/journal.pcbi.1007067>.
- 120 Brinkerhoff, H. and Dekker, C. (2020) Single-molecule protein sequencing using biological nanopores. *Biophysical Journal*, **118**, 163a. <https://doi.org/https://doi.org/10.1016/j.bpj.2019.11.1005>.
- 121 Ouldali, H., Sarthak, K., Ensslen, T., Pigué, F., Manivet, P., Pelta, J., et al. (2020) Electrical recognition of the twenty proteinogenic amino acids using an aerolysin nanopore. *Nature Biotechnology*, **38**, 176–181. <https://doi.org/10.1038/s41587-019-0345-2>.
- 122 Nivala, J., Marks, D.B. and Akeson, M. (2013) Unfoldase-mediated protein translocation through an alpha-hemolysin nanopore. *Nat Biotech*, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., **31**, 247–250. <http://dx.doi.org/10.1038/nbt.2503>.
- 123 Nivala, J., Mulrone, L., Li, G., Schreiber, J. and Akeson, M. (2014) Discrimination among protein variants using an unfoldase-coupled nanopore. *ACS Nano*, **8**, 12365–12375. <https://doi.org/10.1021/nn5049987>.
- 124 Sachelar, I., Winter, L., Knyazev, D.G., Zimmermann, M., Vogt, A., Kuttner, R., et al. (2017) YidC and secyeg form a heterotetrameric protein translocation channel. *Scientific Reports*, **7**, 101. <https://doi.org/10.1038/s41598-017-00109-8>.
- 125 Knyazev, D.G., Kuttner, R., Zimmermann, M., Sobakinskaya, E. and Pohl, P. (2018) Driving forces of translocation through bacterial translocon secyeg. *The Journal of Membrane Biology*, **251**, 329–343. <https://doi.org/10.1007/s00232-017-0012-9>.
- 126 Rosen, C.B., Rodriguez-Larrea, D. and Bayley, H. (2014) Single-molecule site-specific detection of protein phosphorylation with a nanopore. *Nature Biotechnology*, Nature Publishing Group, **32**, 179–181. <https://doi.org/10.1038/nbt.2799>.
- 127 Restrepo-Pérez, L., Wong, C.H., Maglia, G., Dekker, C. and Joo, C. (2019) Label-free detection of post-translational modifications with a nanopore. *Nano Letters*, American Chemical Society, **19**, 7957–7964. <https://doi.org/10.1021/acs.nanolett.9b03134>.
- 128 Henzler-Wildman, K.A., Lei, M., Thai, V., Kerns, S.J., Karplus, M. and Kern, D. (2007) A hierarchy of timescales in protein dynamics is linked to enzyme catalysis. *Nature*, Nature Publishing Group, **450**, 913–916. <http://dx.doi.org/10.1038/nature06407>.
- 129 Heath, G.R. and Scheuring, S. (2018) High-speed afm height spectroscopy reveals μ s-dynamics of unlabeled biomolecules. *Nature Communications*, **9**, 4983. <https://doi.org/10.1038/s41467-018-07512-3>.
- 130 Desai, V.P., Frank, F., Lee, A., Righini, M., Lancaster, L., Noller, H.F., et al. (2019) Co-temporal force and fluorescence measurements reveal a ribosomal gear shift mechanism of translation regulation by

- structured mrnas. *Molecular Cell*, Elsevier, **75**, 1007-1019.e5.
<https://doi.org/10.1016/j.molcel.2019.07.024>.
- 131 Avellaneda, M.J., Franke, K.B., Sunderlikova, V., Bukau, B., Mogk, A. and Tans, S.J. (2020) Processive extrusion of polypeptide loops by a hsp100 disaggregase. *Nature*, **578**, 317–320.
<https://doi.org/10.1038/s41586-020-1964-y>.
- 132 Vanderlinden, W., Brouns, T., Walker, P.U., Kolbeck, P.J., Milles, L.F., Ott, W., et al. (2019) The free energy landscape of retroviral integration. *Nature Communications*, **10**, 4738.
<https://doi.org/10.1038/s41467-019-12649-w>.
- 133 Yang, X., Garnier, F., Débat, H., Strick, T.R. and Nadal, M. (2020) Direct observation of helicase–topoisomerase coupling within reverse gyrase. *Proceedings of the National Academy of Sciences*, **117**, 10856 LP – 10864. <https://doi.org/10.1073/pnas.1921848117>.
- 134 Janissen, R., Arens, M.M.A., Vtyurina, N.N., Rivai, Z., Sunday, N.D., Eslami-Mossallam, B., et al. (2018) Global dna compaction in stationary-phase bacteria does not affect transcription. *Cell*, Elsevier, **174**, 1188-1199.e14. <https://doi.org/10.1016/j.cell.2018.06.049>.
- 135 Löf, A., Walker, P.U., Sedlak, S.M., Gruber, S., Obser, T., Brehm, M.A., et al. (2019) Multiplexed protein force spectroscopy reveals equilibrium protein folding dynamics and the low-force response of von willebrand factor. *Proceedings of the National Academy of Sciences*, **116**, 18798 LP – 18807.
<https://doi.org/10.1073/pnas.1901794116>.
- 136 Seifert, M., Bera, S.C., van Nies, P., Kirchdoerfer, R.N., Shannon, A., Le, T.-T.-N., et al. (2020) Signatures and mechanisms of efficacious therapeutic ribonucleotides against sars-cov-2 revealed by analysis of its replicase using magnetic tweezers. *bioRxiv*, 2020.08.06.240325.
<https://doi.org/10.1101/2020.08.06.240325>.
- 137 Zosel, F., Mercadante, D., Nettels, D. and Schuler, B. (2018) A proline switch explains kinetic heterogeneity in a coupled folding and binding reaction. *Nature Communications*, **9**, 3332.
<https://doi.org/10.1038/s41467-018-05725-0>.
- 138 Fitzgerald, G.A., Terry, D.S., Warren, A.L., Quick, M., Javitch, J.A. and Blanchard, S.C. (2019) Quantifying secondary transport at single-molecule resolution. *Nature*, **575**, 528–534.
<https://doi.org/10.1038/s41586-019-1747-5>.
- 139 Laszlo, A.H., Derrington, I.M., Ross, B.C., Brinkerhoff, H., Adey, A., Nova, I.C., et al. (2014) Decoding long nanopore sequencing reads of natural dna. *Nature Biotechnology*, **32**, 829–833.
<https://doi.org/10.1038/nbt.2950>.
- 140 Hartel, A.J.W., Ong, P., Schroeder, I., Giese, M.H., Shekar, S., Clarke, O.B., et al. (2018) Single-channel recordings of ryr1 at microsecond resolution in cmos-suspended membranes. *Proceedings of the National Academy of Sciences*, **115**, E1789 LP-E1798. <https://doi.org/10.1073/pnas.1712313115>.
- 141 Henzler-Wildman, K. and Kern, D. (2007) Dynamic personalities of proteins. *Nature*, Nature Publishing Group, **450**, 964–972. <http://dx.doi.org/10.1038/nature06522>.
- 142 Ode, H., Nakashima, M., Kitamura, S., Sugiura, W. and Sato, H. (2012) Molecular dynamics simulation in virus research. *Frontiers in Microbiology*, **3**, 258. <https://doi.org/10.3389/fmicb.2012.00258>.
- 143 Bibow, S. and Hiller, S. (2019) A guide to quantifying membrane protein dynamics in lipids and other native-like environments by solution-state nmr spectroscopy. *The FEBS Journal*, John Wiley & Sons, Ltd, **286**, 1610–1623. <https://doi.org/10.1111/febs.14639>.
- 144 Ganji, M., Shaltiel, I.A., Bisht, S., Kim, E., Kalichava, A., Haering, C.H., et al. (2018) Real-time imaging of dna loop extrusion by condensin. *Science*, **360**, 102 LP – 105. <https://doi.org/10.1126/science.aar7831>.