Zero-mode waveguides and nanopore-based sequencing technologies accelerate single-molecule studies

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Single-molecule technologies can provide detailed information regarding molecular mechanisms and interactions that cannot easily be studied on the bulk scale; generally, individual molecular behaviors cannot be distinguished, and only average characteristics can be measured. Nevertheless, the development of the single-molecule sequencer had a significant impact on conventional in vitro single-molecule research, featuring automated equipment, high-throughput chips, and automated analysis systems. However, the utilization of sequencing technology in in vitro single-molecule research is not yet globally prevalent, owing to the large gap between highly organized single-molecule sequencing and manual-based in vitro single-molecule research. Here, we describe the principles of zero-mode waveguides (ZMWs) and nanopore methods used as single-molecule DNA sequencing techniques, and provide examples of functional biological measurements beyond DNA sequencing that contribute to a global understanding of the current applications of these sequencing technologies. Furthermore, through a comparison of these two technologies, we discuss future applications of DNA sequencing technologies in in vitro single-molecule research.

Key words: DNA sequencer, single-molecule measurements, zero-mode waveguides, nanopore

Introduction

Recent developments in single-molecule detection techniques using electron multiplying charge-coupled devices (EMCCD) and scientific complementary metal–oxide–semiconductor (sCMOS) cameras have revealed the behavior of individual biomolecules by labeling proteins, nucleotides, and other biomolecules. Although the study of these behaviors has contributed to the understanding of enzymatic functions, their bulk-scale study has been restricted by the averaging of individual behaviors among tons of molecules [1].

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Single-molecule Förster resonance energy transfer (smFRET) is a powerful biophysical tool used to measure the distances of single molecules, such as biomolecules, in the order of 1–10 nanometers. The detection of the energy transfer efficiency between intra- or intermolecularly linked donor and acceptor dye pairs within a biomolecule at the single-molecule level, allows for the estimation of their distances at each detection time frame. Moreover, smFRET signal variations can provide kinetic information and molecular heterogeneity, including that for protein-DNA interactions, chromatin remodeling, and protein translation [2–5].

To elucidate these biomolecular functions, it has generally been necessary to collect at least hundreds to thousands of data points in various conditions to obtain a suitable statistical confidence level due to the stochastic behavior of individual molecules. It is feasible to compare peak values under certain conditions by optimizing the number of histogram bins according to Sturge's Rule, even if the number of events is approximately 100 or less. However, when comparing, for instance, the histograms of 100 out of 3,000 events to that of all 3,000 events, a different profile is observed in the lower frequency regions [6]. Therefore, at least a few thousand data points are required to comprehensively investigate such events in low frequency regions.

Nevertheless, the emergence of high-throughput, fully automated single-molecule sequencers able to overcome this artifact limitation has advanced the single-molecule field tremendously. Pacific Biosciences (PacBio) developed a highly functional DNA sequencing single-molecule real-time (SMRT) chip, capable of collecting more than 8 million single-molecule data points in a single experiment [7]. Although this DNA sequencing system allows for long reads and short turnaround times [7,8], it is highly reliant on the fluorescent labeling of nucleotide substrates to detect individual bases. Accordingly, Oxford Nanopore Technologies released its first portable nanopore sequencing device in 2014, actualizing DNA sequencing without necessitating fluorescent labeling [9,10]. In particular, the Oxford Nanopore MinION and GridION sequencers are equipped with identical flow cell types, containing 2,048 individual nanopores, capable of sequencing exceedingly long, non-amplified reads much more affordably and quickly than previously possible [7].

Moreover, PacBio provides comprehensive analytical applications for de novo assembly, variant identification, RNA analysis, and detection of epigenetic modifications, while Oxford Nanopore provides access to cutting-edge algorithms, including modified base-calling, such as 5mC, 6mA, and CpG, from raw signal data.

These single-molecule sequencers are integrated with high-throughput chips and automated equipment and analysis systems to overcome the previously mentioned artifacts; however, manual-based approaches are still predominantly utilized in the laboratory. Therefore, it is important to minimize the large gap between the highly organized single-molecule sequencing and manual-based approaches.

As an example, Chen et al. customized a commercial zero-mode waveguide (ZMW)-based DNA sequencer for use as a versatile single-molecule fluorescence detection instrument. This customized system provides long fluorophore lifetimes with a good signal-to-noise ratio (SNR) and low spectral cross-talk [11]. Additionally, they used a ribosomal translation assay to show real-time fluidic delivery during data acquisition, demonstrating that it is possible to follow the conformation and composition of thousands of single biomolecules simultaneously through four spectral channels [11].

Herein, we describe the principles of the ZMW and nanopore methods previously used as single-molecule DNA sequencing techniques. Moreover, the applications of these techniques beyond DNA sequencing are also demonstrated. These applications have unique refinements over their basic principles, and some utilize actual sequencing platforms. These applications contribute to the global understanding of how these sequencing technologies are currently being applied. Furthermore, through a comparison of ZMW and nanopore technologies, we discuss the future applications of DNA sequencing technology in in vitro single molecule research in the future.

ZMWs for Single-molecule Fluorescence Imaging

Single-molecule Fluorescence Imaging Using Total Internal Reflection Fluorescence Microscopy and ZMWs

Single-molecule fluorescence imaging visualizes the real-time behavior of individual biomolecules in an aqueous solution. The technique complements bulk experiments by providing detailed information on molecular mechanisms and interactions, which is generally difficult, if not impossible, to obtain using conventional methods [12–14]. Considering the very weak fluorescence of a single molecule, it is essential to reduce the background fluorescence by restricting the excitation volume. This is generally achieved by using total internal reflection fluorescence microscopy (TIRFM) [15,16]. In a conventional TIRFM setup, fluorophores are excited by an evanescent field instead of direct illumination, the former being generated by the total reflection of incident light at the interface between glass and water (Figure 1a). The evanescent field decays exponentially with increasing distance from the glass surface: the field is capable of exciting fluorophores near the surface (100–200 nm thick) while avoiding the excitation of fluorophores away from the surface [15,16]. Fluorophores in solution must be in the pico- to nanomolar concentration range; when the concentration increases to several tens of nM, the fluorophores are always present in the excitation volume. This results in high levels of background fluorescence that interfere with single-molecule fluorescence detection (Figure 1b). Unfortunately, most biological systems involving binding and catalysis require the molecules to be in the micro- to millimolar concentration.
range. For instance, the median Michaelis constant \( (K_m) \) value for 5,194 enzymes is 130 μM, where ~60% of the \( K_m \) values range from 10−1,000 μM [17].

Figure 1  Single-molecule fluorescence imaging of molecular interactions using total internal reflection microscopy and zero-mode waveguides. (a, b) Top panel, Single-molecule fluorescence imaging of molecular interactions using total internal reflection microscopy (TIRFM). Molecule A is a virtual ligand for molecule B. Molecule A is anchored on a glass substrate, and molecule B with a fluorophore is present in a solution. The total reflection of incident light generates an evanescent field (100−200 nm thickness) at the interface between the glass and water to excite fluorophores. Bottom panel, When the concentration of fluorophores (molecule B) is less than several tens of nM, individual fluorescent spots of molecule B can be detected upon binding to molecule A (a). At higher concentrations of fluorophores (molecule B), fluorescent spots are hardly observed due to high background fluorescence (b). The fluorescence images were obtained in the presence of 20 pM (a) and 1 μM Cy3-tRNA\textsuperscript{fMet} (b). mRNA molecules complexed with ribosomes were immobilized on a glass surface. (c) Top panel, Single-molecule imaging of molecular interactions using zero-mode waveguides (ZMWs). ZMWs are nanoapertures (typically ~100 nm diameter) in a thin metal film deposited on a glass substrate. When light enters the ZMWs from the bottom, a tightly confined evanescent field (20−30 nm thickness) is generated inside the ZMWs, enabling single-molecule fluorescence imaging in the presence of several μM of fluorophores in a solution. Bottom panel, The fluorescence image was obtained in the presence of 1 μM Cy3-tRNA\textsuperscript{fMet}. mRNA molecules complexed with ribosomes were immobilized onto the bottom of the ZMWs.

Single-molecule fluorescence imaging using zero-mode waveguides (ZMWs) overcomes these concentration restrictions by optically limiting the background excitation [18]. ZMWs are subwavelength-diameter apertures (typically ~100 nm) in a thin metal cladding film deposited on a glass substrate (Figure 1c). Aluminum is generally used for the metallic overlayer due to its desirable optical properties [19]; however, alternative cladding materials, such as gold [20] and an intermetallic compound of aluminum and gold [21] have also been employed. It is worth mentioning that the metals can affect the photophysical properties of fluorophores in ZMWs [21−23]. When light enters the ZMWs from the bottom (glass side), the subwavelength apertures prevent the light from propagating, thereby creating a tightly confined evanescent field inside the ZMWs. Moreover, the intensity of this evanescent field decays substantially faster with depth than TIRFM (20−30 nm thick) (Figure 1c). The excitation volume is limited to three dimensions in the atto- to zeptoliter range. Thus, this technique enables single-molecule investigations at micro- to millimolar concentrations of fluorescent molecules, i.e., under physiologically relevant conditions [18,24−26].

Application of ZMWs to DNA and RNA Sequencing

In the earliest work using ZMWs, Levene et al. visualized the enzymatic incorporation of fluorescently labeled nucleotides into DNA at the single-molecule level [18]. This approach has been applied to SMRT sequencing: a core PacBio technology (Figure 2). In SMRT sequencing, single φ29 DNA polymerase molecules (\( K_m^{\text{dNTP}} = 11−300 \) nM [27−

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29]), anchored in a flow chip (SMRT Cell) at the bottom of ZMWs, are immersed with DNA fragments and phospholinked fluorescent dNTPs, subsequently allowing for continuous observation of DNA synthesis (Figure 2a). The DNA sequence is determined by detecting fluorescence from the binding of correctly base-paired fluorescent dNTPs in the polymerase molecule [8] (Figure 2b). The time traces also contain kinetic information, i.e., the duration of each nucleotide incorporation event (interpulse duration). This information has been used to detect the positions and types of DNA base modifications such as N6-methyladenine, 5-methylcytosine and 5-hydroxymethylcytosine [30] (Figure 2c). Moreover, SMRT sequencing is also compatible with direct RNA sequencing by using reverse transcriptase, enabling the detection of base modifications and secondary structures of RNA [31].

![Figure 2](image)

**Figure 2** Schematic illustration of single-molecule real-time (SMRT) sequencing. (a) Single DNA polymerase molecules complexed with template DNA are immobilized at the bottom of ZMWs and immersed in dNTPs with different spectrally-separable fluorophores (indicated in red, blue, yellow, and green). (b) Schematic events of the fluorescent dNTP incorporation with time traces of fluorescence intensities from the ZMW. (1) A fluorescent nucleotide (dCTP) associates with the template in the polymerase active site. (2) The incorporation causes an increase in the fluorescence signal on the corresponding channel. (3) The fluorophore is cleaved from the nucleotide and diffuses out of the ZMW, thus ending the fluorescence pulse. (4) The polymerase translocates to allow the incorporation of the next dNTP. (5) The cognate dNTP (dATP) binds to the active site initiating the subsequent pulse. These figures were adapted from [8] with permission from the American Association for the Advancement of Science. (c) Detection of modified bases during SMRT sequencing. The template DNA strands contain a methylated (top) or unmethylated (bottom) adenine. The letters above the fluorescence pulses indicate the identity of the nucleotide incorporated into the nascent strand. Dashed arrows indicate the interpulse duration before incorporation of the cognate thymine. This figure was adapted from [30] with permission from Springer Nature.

**Application of ZMWs to the Investigation of Biological Systems**

ZMWs have been applied to numerous biomolecular *in vitro* single-molecule studies, aiding in the elucidation of molecular interactions [26,32–68,70–76] and enzymatic reactions [8,18,20,31,76,77]. However, the successful implementation of ZMWs in *in vitro* single-molecule experiments necessitates the circumvention of several barriers, preventing the widespread use of ZMWs.
The inherent difficulty of ZMW fabrication poses a major practical limitation, although various fabrication methods have been presented [24,25,78–81]. Generally, collaboration with nanofabrication specialists is required to manufacture custom-designed ZMWs. For instance, etching the glass surface of ZMWs can improve the SNR over that of the conventional non-etched ZMWs [32,82,83]. Accordingly, the high efficiency of the etched ZMWs has been demonstrated during the study of the molecular mechanism of the bacterial chaperonin GroEL, an essential molecular chaperone in Escherichia coli [33,35]. GroEL, which is capped by the co-factor GroES, forms a double-ring structure and assists protein folding in its central cavity. It is well established that GroES binds to two rings of GroEL alternatively, i.e., an asymmetric GroEL-GroES complex is formed during the reaction cycle, whereas a symmetric GroEL-(GroES)\(_2\) complex is not [84]. Using single-molecule fluorescence imaging incorporating etched ZMWs, Sameshima et al. directly observed and characterized these symmetric complexes during the reaction cycle [35]. Additionally, the presence of more than 200 nM of fluorescently labeled GroES in solution was required to achieve saturating amounts of the symmetric GroEL-(GroES)\(_2\) complex. The first GroES that interacts with GroEL does not always dissociate from the symmetric complex before dissociating the second GroES molecule; therefore, the dissociation of GroES molecules from this complex can occur in random order. Additionally, two cycles are present in the chaperonin reaction: an asymmetric cycle (GroEL ↔ asymmetric complex) and a symmetric cycle (asymmetric complex ↔ symmetric complex) [35,84]. Unfortunately, the application of ZMWs is limited when imaging fibrous protein filaments (such as actin and microtubules) and motor proteins (such as myosin and kinesin) because the cylindrical ZMW geometry does not allow the insertion of fibrous proteins. To overcome this problem, linear ZMWs (LZMWs) have been developed, which comprise nanoslits in a thin metal layer deposited on a glass substrate [85,86]. The slits are narrower than the wavelength of visible light (100–200 nm width) in one dimension and are ~100 μm long. Yokokawa et al. demonstrated that LZMW single-molecule fluorescence imaging can be performed in 10-fold higher fluorophore concentrations (up to 1 μM) than in TIRFM [86]. Additionally, they successfully observed fluorescent ATP molecules interacting with kinesin as it traversed microtubules immobilized in LZMWs [86].

Single-molecule fluorescence imaging is hampered by unwanted adsorption of the fluorescently labeled molecules; therefore, appropriate surface passivation of ZMWs is required. Conventionally, aluminum-based ZMW passivation involves the covalent attachment of polyvinylphosphonic acid and polyethylene glycol, which drastically avoids unwanted protein adsorption [87,88].

Researchers who are familiar with optics can obtain images with good SNR using ZMWs. Typically, epifluorescence and confocal microscopy are adopted for ZMW-based imaging. Moreover, Zhao et al. demonstrated that a dark-field configuration displays diminished background and noise and enhanced SNR, and that the SNR remains unperturbed in the presence of background fluorescent molecules at micromolar concentrations [46]. Using this setup, they successfully probed weak DNA–protein and protein–protein interactions [46]. Nevertheless, PacBio SMRT Cells that utilize high density ZMW arrays suppress nonspecific adsorption by incorporating biotinylated polyethylene glycol-modified glass surfaces [8] and polyvinylphosphonic acid-coated aluminum surfaces [87]. Furthermore, the PacBio sequencer is a fully automated single-molecule imaging system: it allows real-time delivery of reagents, simultaneous four-color detection, excellent SNR, and low spectral crosstalk by using a specialized optical setup [8,11,89], downstream data processing, and statistical analysis. The recently released PacBio Sequel IIe monitors single-molecule events from up to eight million individual ZMWs at timescales ranging from milliseconds to a day ([https://www.pacb.com/technology/hifi-sequencing/sequel-system/](https://www.pacb.com/technology/hifi-sequencing/sequel-system/)). Thus, this PacBio platform has significant potential to dramatically improve the throughput of conventional single-molecule experiments.

PacBio has provided custom SMRT Cells and sequencers to a small number of researchers, allowing for the high-throughput study of single-molecule real-time dynamics in biological systems [11,34,37–45,47,48,50–53,56–65,67–72,74–76,90] (Figure 3a and b). For instance, Uemura et al. employed this sequencer to visualize ribosomal mRNA translation at physiologically-relevant (μM) concentrations. Briefly, bacterial ribosomes, engaged with mRNA and initiator tRNA (tMet-[Cy3]tRNA\(_{Met}\)), were immobilized in ZMWs and immersed in a solution containing aminoacylated tRNAs with different fluorophores (Phe-[Cy5]tRNA\(_{Phe}\) and Lys-[Cy2]tRNA\(_{Lys}\)) complexed with elongation factor Tu (EF-Tu) and GTP [34] (Figure 3c). Herewith, they observed the transit of tRNAs on single translating ribosomes and determined the number of tRNA molecules simultaneously bound to the ribosome at each codon of the mRNA. Ribosomes have three tRNA binding sites: an A site for aminoacyl-tRNAs, a P site for peptidyl-tRNAs, and an E site for the deacylated tRNA leaving the ribosome. They showed that ribosomes are briefly occupied by two tRNAs, and that deacylated tRNA release from the E site is uncoupled from A-site tRNA binding, which occurs rapidly after translocation. Nemashkalo et al. utilized the custom PacBio sequencer for real-time transcription observation, during which transcriptional pausing was observed without applying an external force [76]. Furthermore, Duss et al. successfully tracked transcription and ribosomal protein binding with nascent RNA transcripts [60,64]. These experiments demonstrate the capability of the PacBio sequencer to elucidate the mechanisms of complex multicomponent systems at the single-molecule level.
**Figure 3** Single-molecule investigation of biological systems using the PacBio sequencer. (a) Schematic drawing of the microscopic system for the custom PacBio RS system. Incident light is provided by a green (532 nm) and red (642 nm) laser, which are separated into ~75,000 beamlets to pararely illuminate ZMWs on the SMRT Cell that puts on a six-axis stage during data acquisition. The emitted light from the ZMWs is collected on four CMOS cameras. (b) Comparison of workflows for standard sequencing and single-molecule fluorescence imaging mode shows steps for users (square boxes) and instrument (ovals). Modifications made for the single-molecule fluorescence imaging mode reduce time to stage, alignment time, and exposure of components to laser illumination to allow for flexible single-molecule studies. The figures were adapted from [11]. (c) Monitoring the accommodation of tRNAs on the translating ribosome in ZMWs. The top panel provides a schematic representation of the experimental system. *E. coli* ribosomes engaged with biotin-modified mRNA and initiator tRNA (fMet-[Cy3]tRNA\(^{\text{Met}}\)) are immobilized on the glass surface of ZMWs via polyethylene glycol with biotin and neutravidin and immersed in a solution containing 200 nM fluorescent aminoacyl-tRNAs (Lys-[Cy2]tRNA\(^{\text{Lys}}\) and Phe-[Cy5]tRNA\(^{\text{Phe}}\)) complexed with elongation factor Tu (EF-Tu), and GTP. For simplicity, only the key components are explained in the figure. The bottom panel is representative of the time trace of the three fluorophores (Cy2, blue; Cy3, green; Cy5, red) attached to tRNAs during translation. Large stepwise changes in fluorescence intensity enabled the resolution of the entry and exit of single tRNA molecules. The arrows indicate the fluorescent signal corresponding to each codon on the mRNA. When the ribosome paused on the stop codon, tRNA sampling events are observed with short pulses [34].
Nanopore Sensing for Label-free Single Molecule Detection

Fundamentals of Nanopore Sensing

The Oxford Nanopore sequencers have advanced significantly since they were first conceived in 1989 by Prof. Deamer from the University of California, Santa Cruz [91]. Supported by the $1,000 Genome Project launched by the U.S. National Institutes of Health in 2004, the fundamental nanopore technologies developed significantly, eventuating in the portable MinION nanopore sequencer. Currently, MinION features label-free and long-read sequencing of >4 megabases at a time (https://nanoporetech.com/products/comparison), and is capable of sequencing nucleotide modifications in addition to the four nucleotide bases [92].

The nanopore sequencer works on the principle of nanopore-based resistive sensing (nanopore sensing) [93], wherein the nanopores connect two buffer-filled fluidic chambers. Electrodes are inserted into both fluidic chambers (cis and trans) and a voltage is applied, resulting in ionic flow in the nanopore (Figure 4a) measured as an electrical current (Figure 4b). Under the applied voltage conditions, biological molecules in the fluidic chambers pass through the nanopores via driving forces such as electrophoresis [94], electroosmosis [95,96], and dielectrophoresis [97]. The occupancy of biological molecules in a pore results in a blockade current (Figure 4c and d). The blockade current frequency and dwell time correlates with the surface conditions, size, and structure of the biological molecules, thereby revealing the physical properties of the biological molecules. Fundamentally, nanopore sensing is explained by the volume exclusion effect, and the baseline and blockade current are determined as per equation (1) and (2), respectively.

\[
I_o = V \sigma \left( \frac{4 \pi h_{eff}}{\pi d^2} + \frac{1}{d} \right)^{-1}
\]

(1)

\[
\Delta I = V \sigma \left( \frac{4 \pi h_{eff}}{\pi d_m^2} + \frac{1}{d_m} \right)^{-1}
\]

(2)

Here, \( I_o \) denotes the open current, \( \Delta I \) the blockade current, \( V \) the applied voltage, \( \sigma \) the buffer conductivity, \( h_{eff} \) the effective pore thickness, \( d \) the pore diameter, and \( d_m \) the diameter of the molecule. These equations allow for an estimation of the nanopore geometry by obtaining the current trace while biological molecules with known dimensions pass through a pore [98]. Previous studies have predicted the effective contribution of factors other than volume exclusion, such as hydrophobicity and net charge, on the mechanisms underlying DNA sequence-dependent blockade currents [99–101]. Moreover, further details on the working principle of nanopore sensing has been reviewed elsewhere, and may be referred to for more information [93,102–104]. Generally, two nanopore classifications exist, namely biological and solid-state nanopores. Biological nanopores include pore-forming membrane proteins such as \( \alpha \)-hemolysin (aHL) [105], Mycobacterium smegmatis porin A (MspA) [106], and Curlin sigma S-dependent growth subunit G (CsgG) [107], among others. Additionally, biological nanopores embedded in lipid bilayer membranes produce a well-defined pore geometry, thereby offering noticeable reproducibility in detection and sensitivity. The application of some biological pores, such as MspA, in DNA sequencing necessitates the attachment of a motor protein to control the movement of DNA into the pores (Figure 4e). The current associated with the DNA sequence is depicted in Figure 4f.

Three Nanopore Sensing Approaches for Folded Protein Detection

Since nanopore sensing can analyze label-free single biological molecules with high sensitivity, its use has great potential, not only in DNA/RNA sequencing but also in other applications. Therefore, we reviewed three nanopore sensing approaches used to study tertiary protein structures: type 1 protein translocation through a large pore (\( d_{pore} > d_{protein} \)), type 2 protein translocation through a small pore (\( d_{pore} < d_{protein} \)), and type 3 protein trapping in a small pore (\( d_{pore} < d_{protein} \)); wherein \( d_{pore} \) and \( d_{protein} \) represent the nanopore and protein size, respectively. In type 1, protein translocations through a pore larger than molecular size provides the blockade current, thereby correlating protein size, fluctuations, and conformational changes [116–118]. In Figure 5a, the fractional blockade histograms for GFP and calmodulin correlate the mean blockade current with the protein gyration as well as the blockade current distribution with protein fluctuation. Moreover, protein conformational changes induced by protein-protein interaction [119], unfolding/folding [116,117,120], and DNA binding [121] can be characterized by observing the excluded volume change of a protein in a pore. For example, Freedman et al. demonstrated the translocation of three proteins (SAP97 PDZ2 and two mutants) through 15 nm solid-state nanopores under 200–800 mV. Accordingly, they found that by increasing the protein dipole moment with voltage,
Figure 4 Working principle of nanopore sensing. (a, b) Diagram of nanopore setup and the open-pore current ($I_o$) under applied voltage. A pore is immersed in electrolytes and electrodes are inserted in both cis- and trans-chambers. When voltage is applied across the pore, potassium and chloride ions flow through the pore. This flow can be electrically monitored using Patch-clamp measurement as the current increase to $I_o$ in b. (c, d) Diagram of DNA translocation and the blockade current. When biological molecules, such as DNA, are placed in a fluidic cell, DNA passes through the pore by electrophoresis. During DNA translocation, DNA blocks the ionic flow, resulting in the blockade current. The amount ($\Delta I$) and dwell time ($\Delta t$) of the blockade current provides physical properties of molecules which can be used for the fingerprint of molecules. Sequential numbers ((1)–(3)) describe the relationship with three stages of DNA translocation through the pore and current trace. (e–f) Schematic illustration of nanopore DNA sequencing using the MspA pore. MspA pore MspA–phi29 DNA polymerase complex is used to control DNA motion at single nucleotide steps. This controlled DNA motion allows the obtainment of nucleotide sequential current trace. Adapted with permission from [166].

a gradual protein unfolding, rather than a two-state transitional unfolding, results [117]. However, protein-sensing using large pores are restricted by the molecular transport speed. The protein translocation speed exceeds the typical time resolution of nanopore measurements, resulting in undetected protein translocation events [122]. To overcome this limitation, several approaches have been proposed to decelerate molecular translocation via surface modifications [69,123], additional external forces [124], surface interactions [125], buffer conditions [126,127], electroosmosis [128–130], and mechanical control [131,132]. In particular, Yusko et al. invented a lipid bilayer-coated solid-state nanopore (Figure 5b). These lipid-anchored ligands covalently bind to the target protein, thereby tethering the protein and decelerating its translocation speed by two orders of magnitude [123]. Moreover, this approach can estimate the volume, shape, surface charge, diffusion coefficient, and dipole moment of individual proteins [133].

When biological molecules pass through a pore that is smaller than its molecular size (type 2), the interaction between the biological molecules and the pore surface disrupts their tertiary structure; therefore, the structure-derived current trace is obtained [134–138]. For instance, Rodriguez et al. demonstrated the unfolding of thioredoxin (Trx) by using a DNA oligonucleotide leader to translocate it through a αHL pore, thereby revealing different C- and N-terminus unfolding pathways. This suggests that the protein unfolding rate is dependent on either N- or C-terminus-first co-translocation (Figure 5c) [134]. Solid-state nanopores are also used to facilitate high voltage conditions. In particular, this approach demonstrated that the electric field condition at the nanopore (25–900 mV) contributes to the folded, metastable,
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Figure 5  Protein tertiary structure detection using nanopore sensing. (a) Protein detection using a pore slightly larger than proteins. The fraction and the distribution of the blockade current for GFP and Calmodulin correlates with protein shape and flexibility. Adapted with permission from [118]. (b) Lipid bilayer coating on solid-state nanopore for protein detection. The anchored biotins (blue circle) in the lipid bilayer can specifically bind to streptavidin (large red) and delivers tethered proteins into a pore by two-dimensional diffusion. This strategy can slow down the protein translocation speed due to the viscous character of the lipid bilayer, and allows for the obtained of the volume, shape, surface charge, diffusion coefficient, and dipole moment. Adapted with permission from [133]. (c) Unfolding of Trx V5-C109-oligo(dC)30 by pulling into a αHL pore. The left shows that DNA oligonucleotide leader (oligo[dC]30) assists in threading the Trx protein into a αHL pore. The right presents the representative current trace while Trx unfolds and passes through a pore. Drawings on the top illustrate the unfolding/threading pathway of Trx V5-C109-oligo(dC)30 corresponding to the four current levels. Adapted with permission from [134]. (d) Calmodulin trapping on MspA pore. The diagram shows that three conformations of Calmodulin, including Apo-, divalent ion bound-, and target peptide bound-CaM, have different fitting conditions in a pore, allowing to distinguish each conformation. Adapted with permission from [146].

intermediate, and unfolded conformational states of cytochrome c during its translocation [139]. Furthermore, this approach can be extended to observe thermal conformational dynamics at the single-molecule level using the photothermal effect of silicon nitride membranes [140], water [141], and plasmonic nanostructures [142]. Accordingly, Yamazaki et al. used photothermal heating to measure the bulk melting temperature of tRNA [140].

Although biological molecules are predominantly investigated via translocation through a pore, significantly more conformational information can be obtained by trapping a protein on a pore (type3) [143–145]. Figure 5d shows the three conformational states of Calmodulin trapped on an MspA pore, resulting in different within-pore fitting conditions [146]. This produces distinct trapping time and blockade current levels for each stage and allows for the evaluation of the binding capacities of divalent ions to Calmodulin. In addition to using the type 3 approach for protein tertiary structure sensing, Avinash et al. investigated protein-protein interactions by synthesizing the 110-amino acid RNase barnase (Bn)-fused
ferric hydroxamate uptake component A (FhuA) that specifically captures and releases the 89-residue barstar. This allowed for current transition monitoring between the binding and unbinding states [147].

**Protein Enzyme Motion Along with Nucleic Acid Chain**

Nanopore sensing can be used for label-free observation of nucleic acid-binding protein activities at the single nucleotide level. This approach, named Single-Molecule Picometer Resolution Nanopore Tweezers (SPRNT), was developed by the Gundlac group at the University of Washington [148]. As illustrated in Figure 6a, a motor protein (phi29 DNA polymerase [DNAP]) pulls a single-stranded DNA through a sequence-reading membrane protein pore (MspA), allowing for single-base DNA position shift observations. Moreover, SPRNT provides 40 picometer resolutions with millisecond time resolutions for in some DNA sequences wherein a small DNA displacement results in a large current change [148]. By using these high SPRNT resolutions, Derrington et al. observed the individual hydrolysis cycles of Hel308, showing similar ATP-driven movement with the inchworm model [148]. Thereafter, Craig et al. comprehensively investigated Hel308 kinetics using SPRNT to elucidate the ATP hydrolysis mechanism. Accordingly, they proposed that the direction of Hel308 movement depends on the conformational change induced by the energy differences between ATP and ADP bound states [149].

**Amino Acids Identification and Sequencing**

Recently, growing attention has been paid to the development of protein and amino acid sequencing techniques using nanopore sensing [150–152]. Numerous studies have established that nanopore sensing can discriminate between peptide length and sequence by running short peptide chains through a biological nanopore [153–158], thereby identifying the associated protein. In particular, Lucas et al. found identical current trace profiles for nine different proteins following their digestion using trypsin protease, demonstrating the translocation of peptide fragments through a Fragaceatoxin C (FraC) pore (Figure 6b) [159]. Furthermore, Bakhshoo et al. showed that an aerolysin nanopore can identify three proteins (myoglobin, lysozyme, and cytochrome c) via their fragmentation into peptide chains using trypsin protease [160]. Additionally to protein identification, it has been proposed that the translocation of long peptides into a pore can be controlled by AFM tip manipulation [161] and enzyme activities [162–165]. One such approach successfully detected amino acid sequences by attaching a peptide chain to the tip of a DNA molecule (DNA-peptide) and pulling the chain through a pore using a motor protein (Figure 6c) [162,164,165]. Using a double Hel308-attached DNA-peptide, Brinkerhoff et al. increased the reading accuracy by repetitively rereading the peptide sequence. The first Hel308 pulls the nucleotides through the MspA nanopore, following which, the second Hel308 repeats the same process to initiate peptide rereading after the first Hel308 dissociation [162].

**Figure 6** Motor protein kinetics and peptide detections using nanopore sensing. (a) Single-Molecule Picometer Resolution Nanopore Tweezers (SPRNT). Motor protein (phi29 DNA polymerase [DNAP])-attached single-stranded DNA is tethered at the MspA pore and the activity of DNAP draws ssDNA through the pore, allowing DNA position movement at single nucleotide step. This yields stepwise-current trace associated with DNAP kinetics at single nucleotide levels. Adapted with permission from [167]. (b) Enzyme-assisted protein chain detection for protein identification. When digesting proteins into peptide chains using a protease, and running them into FraC nanopore, the resulting blockade current profiles provide the identity of proteins. Adapted with permission from [159]. (c) Peptide sequencing using MspA pore with motor protein control. The DNA helicase Hel308 is attached to ssDNA with peptide chains, which is bound by click linker. These Hel308/ssDNA-peptide chain complexes are inserted into a MspA pore. As Hel308 pulls ssDNA through a pore, the peptide chains move step by step through constriction of the MspA pore, resulting in current trace associated with the peptide sequence. Adapted with permission from [162].
Conclusion and Future Perspectives

The different measurement principles utilized by ZMW- and Nanopore-mediated single-molecule measurement techniques have resulted in the unique evolution of both techniques as DNA sequencing technologies. The requirement to determine unique DNA sequences from stochastic single-molecule data similarly challenged the advancement of these techniques, resulting in the development of highly accurate, high-throughput measurement systems and statistical algorithms that maximizes artifact elimination. Additionally to limiting the development of DNA sequencing technology, these challenges also commonly restrict human-based in vitro single molecule research.

Several aspects necessitate consideration to evaluate whether these DNA sequencer technologies are suitable for specific in vitro single-molecule studies. Therefore, Table 1 provides a summary of the advantages and disadvantages of ZMW- and Nanopore-mediated single-molecule techniques as well as their suitability for different applications.

Although ZMWs, which require fluorescent labeling and immobilization to the surface, allows for the monitoring of molecular behaviors that emit a fluorescent signal, it is not suitable for molecular counting. Contrastingly, Nanopore, which allows detection of any molecule passing through the pores, is suitable for molecular counting, although the continuous monitoring of molecular behaviors is not possible.

Table 1 Comparative summary of technologies for ZMWs and Nanopore

| Materials | Pore size (nm) | Sensing approach | Target biomolecules | Applications | Acquired information | Advantages | Disadvantages |
|-----------|---------------|------------------|--------------------|--------------|----------------------|------------|--------------|
| ZMWs      | Glass and metal (aluminum, gold, aluminum-gold) | ~150 | Fluorescence detection | Protein + nucleic acids + lipids | Probing molecular interactions including molecular recognition and enzymatic reactions under physiologically relevant conditions | On-Off event durations, Fluorescence intensity of individual spots (Number of molecules involved in the events, transition of molecular states under various conditions) | Number of spots (Number of events) | Quantitative determination of kinetic parameters of reactions under physiologically relevant conditions | Labeling of target molecule, immobilization of target molecule, Estimation of ZMWs, Appropriate surface selection |
| Nanopore  | Biological molecule (Protein, DNA, Oligo etc.) / Solid-state | 1–1900 | Electrical measurement | Protein + nucleic acids + Protein-DNA/RNA complexes + Vesicles | Biomarker detection, Concentration measurement, Molecular structure estimation | Current blockade (Molecular size, shape, surface properties, Enzymatic reaction) | Frequency of current blockade events (Molecular concentration) | Label-free detection, High-throughput, Separation of inorganic materials, Appropriate surface selection | Less resolution for mixture samples, Fabrication of nanopore |

Both techniques are generally dedicated to pure reconstruction systems; however, in the case of mixed samples, the traces obtained from different molecule types that pass through the Nanopore include distinct molecular information, thereby allowing the identification of the type of molecule by utilizing analysis methods such as deep learning. Contrastingly, ZMW is not suitable for identifying molecules from mixed samples, owing to the limited information obtained from fluorescence-labeled target molecules. The major limitation for users of both technologies is the obtention of fabricated chips and analysis algorithms. The barriers are relatively intensive to overcome, as they require a lot of time, human resources, and are expensive to fabricate in-house. DNA sequencing, however, has been boosted by constant and intense corporate competition, which has already succeeded in making fully automated sequencing commercially available.

Nevertheless, the barrier remains challenging when these technologies are applied beyond DNA sequencing. Although they are highly transferable into laboratory-based in vitro single-molecule studies, sequencing companies generally have not yet allowed open access to their elemental technologies contained in the sequencers, including the high-throughput chip, automated measurements, and analysis systems, owing to their profitability.

To overcome this barrier, Chen et al. collaborated with PacBio to customize a commercial ZMW-based DNA sequencer for use as a versatile single-molecule fluorescence detection instrument, thereby demonstrating the possibility to manually obtain 20 times more data on ribosome structure and composition through four spectral channels [39]. Therefore, upon public availability of the PacBio ZMW chip, it could be used for a wide range of single-molecule fluorescence imaging experiments, including smFRET and the four-color simultaneous detection system, thereby expanding the scope of in vitro single-molecule research. Similarly, if we could freely modify the MinION chip for customized use, the obtention of single trace data from a single pore could be drastically improved.

Furthermore, the analysis tools built into both sequencers are based on analyzing single-molecule traces with minimal artifacts, making them widely transferable to in vitro single-molecule studies.

This review paper discussed the possible applications of single-molecule sequencing technology beyond DNA sequencing. We expect that the application range of sequencing technologies will expand greatly in the future, leading to the advancement of in vitro single-molecule research.
Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: S.U; writing – original draft preparation: R.I., H.Y., and S.U.; writing – review and editing: R.I., H.Y., and S.U.; figure preparation: R.I., H.Y., and S.U.; supervision: S.U. All authors have read and agreed to the published version of the manuscript.

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