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Recent advances in nanopore sequencing

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Abstract

The prospect of nanopores as a next-generation sequencing (NGS) platform has been a topic of growing interest and considerable government-sponsored research for more than a decade. Oxford Nanopore Technologies recently announced the first commercial nanopore sequencing devices, to be made available by the end of 2012, while other companies (Life, Roche, IBM) are also pursuing nanopore sequencing approaches. In this paper, the state of the art in nanopore sequencing is reviewed, focusing on the most recent contributions that have or promise to have NGS commercial potential. We consider also the scalability of the circuitry to support multichannel arrays of nanopores in future sequencing devices, which is critical to commercial viability.

Keywords

DNA sequencing; nanopore sensing; next-generation sequencing technologies

1. Introduction

Personalized genomic medicine is a growing movement within the medical and government institutions of the United States that aims to transform healthcare through the gathering and analysis of patient's genomic data. Objectives of genomic medicine include earlier diagnosis, more effective prevention and treatment of disease, and avoidance of drug side effects, all based on signatures in the genome [1]. The future of genomic medicine depends largely on the development of new methods to cheaply sequence full genome DNA. The cheapest sequencing now costs \$18k per genome from a high-throughput sequencing center, with the potential to reach \$1k this year from commercial vendors [2]. These prices include usage and analysis costs, but not the one-time larger instrument cost. To reduce instrument and usage costs, nanopores are being pursued as an NGS platform that *in principle* could eliminate the needs for sample amplification, the use of enzymes and reagents used for catalytic function during the sequencing operation, and optics for detection of sequencing progress, some or all of which are required by the sequencing-by-synthesis methods that presently control the market [3]. Nanopore sensors are purely electrical, and can detect DNA in concentrations/volumes that are no greater than what is available from a blood or saliva sample [4]. Additionally, nanopores promise to dramatically increase the read-length of sequenced DNA, from 450 bases (Roche/454) to greater than 10k bases. Longer read lengths cut the costs and errors associated with shorter read lengths, for which the analysis must piece together the fragments to reconstruct an estimate of the original sequence.

While nanopores have long promised to push the total cost of genome sequencing to the prized \$1000-mark and below [4, 5], only recently has the promise begun to come to life. In

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February, 2012, Oxford Nanopore Technologies presented a \$900, single-use device that is USB-enabled, hand-held and can sequence a complete bacteriophage genome [6]. Meanwhile, several other companies (Life, Roche with IBM, Electronic BioSciences, NABsys, Genia) are investing in nanopore sequencing approaches. In university labs, research in support of nanopore sequencing technology continues to progress, with advances in the sensing of nucleotides, the necessary control of DNA motion through the nanopore as it is sequenced, and the development of peripheral components including integrated patch-clamp amplifier technology. We review the most recent advances in these areas of active research and commercial development, focusing on those that have occurred since the review on the topic in 2008 [4]. We also consider the scalability of nanopore sequencing, in terms of the costs associated with circuitry to support multichannel nanopore arrays, where the array size ultimately determines the cost of sequencing.

2. The performance of single nanopores for sequencing

Nanopores are a single-molecule sequencing approach [7], and so we begin by considering the performance of individual nanopores for sequencing individual DNA molecules. A nanopore is a nano-scale opening that forms naturally as a protein channel in a lipid membrane (the biological pore) [8], or is engineered by drilling or etching the opening in a solid-state substrate (the solid-state pore) [9]. Using a sensitive patch-clamp amplifier, nanopore devices monitor ionic current through a single pore that separates two chambers labeled *cis* and *trans* (Fig. 1A). Voltage is applied across the membrane, creating an ionic current through the nanopore that is filtered, sampled and recorded for analysis.

Both biological and solid-state nanopore instruments are research tools for single-molecule science [10], providing a technique for analyzing the structure and composition of DNA or RNA molecules [11–13], and for probing interactions between DNA and DNA-binding proteins [14–23]. With a nanopore instrument, individual DNA molecules are captured and driven through the pore by electrophoresis, with each capture event detected as a temporary shift in the ionic current. The current shift is caused by the DNA attenuating or boosting the channel conductance (depending on the salt concentration [24]), and the amplitude and duration of the shift are used to characterize the molecule. For example, using the biological pore α -hemolysin, one can detect individual ssDNA passing through the pore as current attenuation events. Specifically, with 1 μ M ssDNA in the *cis* chamber above the pore and a *trans*-positive command voltage, a single ssDNA event is detected as a rapid current attenuation (0.2 ms, corresponding to ~ 2 μ s/nt rate, Fig. 1B). To examine the influence of ssDNA and its sequence on the impeded signal amplitude, researchers have used enzymes as a “braking” mechanism, to temporarily stop/slow the otherwise rapid ssDNA passage through the pore. For example, the exonuclease I (ExoI) of *Escherichia coli* binds to and hydrolyzes ssDNA in the 3' to 5' direction. Upon addition of 1 μ M ExoI to the *cis* chamber, ExoI-bound ssDNA events are also observed, detected as a 10 $\times$ increase in the duration of the current shift (Fig. 1B). Here, ExoI binds to ssDNA but hydrolysis is inhibited by adding EDTA to chelate Mg^{2+} , which is required for hydrolysis [25] (template and conditions are identical to those in [14]). In research studies, the measured lifetime distribution of the enzyme-bound events can be used to model the bond energy landscape for this complex, for example, under varying experimental conditions [11]. For sequencing purposes, controlled delivery of DNA through the pore is essential, first to probe the influence of sequence on the measured current, and then to develop and implement the required algorithms for *de novo* sequencing. Recent research shows that DNA polymerases can address the controlled-delivery need [26, 27], and we review this work in Section 2.1.2. As this review is focused on the use of nanopores for DNA sequencing, we refer interested readers to survey papers that cover the use of nanopores for applications other than sequencing [9–11, 28].

The aim of nanopore *sequencing* is to infer the DNA sequence from patterns within the shifted ionic current record as DNA passes through the nanopore. The idea was first envisioned independently by David Deamer of the University of California, Santa Cruz and George Church of Harvard University. In 1996, Deamer, Daniel Branton of Harvard University and colleagues were the first to demonstrate detection of DNA capture and passage through the biological pore α -hemolysin [29]. Despite the simplicity of the nanopore sequencing concept, however, there remained two long-standing obstacles: 1) the need to slow and control the delivery rate of DNA molecules through the nanopore (see Fig. 1B, caption), and 2) to have single-nucleotide sensitivity in the nanopore sensing current.

For bandwidth and noise levels common to nanopore experiments, the specification for rate reduction is that the DNA should be slowed at least three orders of magnitude, from the unimpeded 1–3 μ s/nt [12] to 1 ms/nt or slower [4]. While the ExoI protein bound to ssDNA temporarily halts the ssDNA passage in Fig. 1B, it does not provide controlled delivery. Motivated by the rate control problem, researchers have been seeking molecular motors (enzymes) that are catalytically functional on the nanopore, and robust enough to turn-over processively while overcoming the voltage force that holds the complex in place and generates enough signal for sensing. We review this work, which to date is the leading method for solving the rate control problem. We also review the different biological and solid-state nanopores being pursued to address the sensitivity problem (Fig. 2).

2.1 Biological Nanopores

2.1.1 The sensitivity of biological nanopores—The α -hemolysin nanopore is the most commonly used pore in research [10] (Fig. 2a). It is a protein native to the bacterium *Staphylococcus aureus* and is a primary virulence factor in *S. aureus* pneumonia [30]. α -hemolysin's mechanistic role is to spontaneously insert itself into a foreign cell membrane, disrupting the cell's electrochemical gradient. The protein channel contains a transmembrane α -barrel that will puncture a lipid bilayer [31]. The barrel is ~ 4 nm in length with 2.2 nm inner diameter, which necks at the top to a limiting diameter of ~ 1.5 nm (the necking region is referred to as the lumen). Above the lumen on the *cis* side is a vestibule (2.6 nm maximum diameter). The lumen and barrel (stem) are just wide enough to accommodate ssDNA, while the vestibule can accommodate duplex DNA. Enzymes bound to DNA are too large to pass through the *cis*-side (cap) entry of the pore.

With ssDNA in the length of α -hemolysin channel, several nucleobases contribute to the measured current, with the largest effect at the lumen. The lumen and stem have a length of ~ 5 nm allowing for 10–12 nucleotides of ssDNA to fill the space. Researchers used streptavidin bound to 3' or 5'-biotinylated ssDNA to determine the influence of single nucleotide substitutions in homopolymers [32, 33]. The use of abasic (1,2-dideoxy) residues within DNA that pass through the limiting constriction of the pore causes a larger detectable current shift, making it possible to build an abasic-location map based on the measured current [19]. These studies showed that, while sensitivity to these substitutions is possible, single nucleotide sensitivity is not possible in heteromeric DNA with wild-type (WT) α -hemolysin.

Researchers have used protein engineering of the α -hemolysin nanopore to vary and improve its sensitivity for DNA analysis and sequencing applications [34–37]. In [37], streptavidin-bound to homopolymeric ssDNA with single nucleotide substitutions was used to show improved discrimination with a mutant pore, but heteromeric DNA was not examined. Oxford Nanopore Technologies and researchers at the University of Oxford (led by Hagan Bayley) jointly developed a method that employs a different mutant α -hemolysin, with a covalently attached molecular adapter in the lower stem that binds free nucleoside monophosphates [35]. The mutant pore is capable of discriminating the four nucleoside 5'

monophosphate molecules with high accuracy. Rather than directly sequencing in-tact ssDNA that passes through the pore, ExoI hydrolyzes ssDNA in the bulk-phase *cis* chamber, with excised nucleoside monophosphates translocating in random order. To sequence the DNA would require an ordering mechanism, which the paper suggests could be achieved by covalently tethering the enzyme in close proximity to the pore and optimizing conditions to ensure ordered capture of each hydrolyzed nucleotide. Though no ordering mechanism has yet been demonstrated in the literature, the sensitivity achieved is a milestone in nanopore research.

A group from the University of Washington (led by Jens Gundlach) have engineered an alter- native pore, *Mycobacterium smegmatis* porin A (MspA), which has a funnel-like shape that focuses the sensitivity of the measured current to the bottom (*trans*-side) of the channel [38, 39] (Fig. 2a). By comparison, the current through MspA is influenced by fewer nucleotides than for WT α -hemolysin, making strand sequencing more likely with MspA. The lower limiting aperture has a diameter of ~ 1.2 nm with a height of ~ 0.5 nm. It was determined that current levels for a passing strand do not directly correspond to one nucleobase, but to ~ 4 nucleotides most proximal to the limiting aperture [40]. In addition to fewer nucleotides affecting the current, the signal-to-noise ratio (SNR) of sequence-specific current amplitude shifts is also considerably higher for MspA than for α -hemolysin, due to the funnel-like geometry. At present, MspA is the most promising biological pore for sequencing.

2.1.2 Rate control using molecular motors on biological pores—Polymerase and exonuclease enzymes bound to DNA can be captured and measured on the α -hemolysin nanopore, providing a method to probe and model the kinetics of these complexes [28]. Example enzymes that have been examined on the α -hemolysin pore include the bacteriophage T7, the Klenow fragment (KF) of *Escherichia coli* [16, 17, 41, 42], and the bacteriophage phi29 [18] DNA polymerases. As stated, these molecular motors could also provide the speed reduction and control needed to sequence the DNA strand in the pore. For continuous current sensing during enzyme-catalyzed DNA translocation, the enzyme must meet the following requirements: first, the complex must be stable enough to withstand the constant voltage force during each catalytic cycle, for many cycles in series; second, the force generated by the enzyme to translocate along DNA must be large enough to overcome the competing voltage force on the DNA that holds the complex on the nanopore; third, the force-generating command voltage must also be high enough to generate enough SNR in the measured current to accurately monitor DNA motion progress. The group from the University of California, Santa Cruz (led by Mark Akeson) recently demonstrated that phi29 meets these requirements, while also developing other key procedures that enable sequencing on the nanopore [26]. In their setup, phi29 is bound to each DNA molecule in the bulk-phase *cis* chamber with all ingredients required for catalytic function; however, each bound complex is stalled through the use of a blocking oligomer. Once the complex is captured, voltage unzips the blocking oligomer and pulls the template strand down through the α -hemolysin pore, registering a signal of downward DNA motion progress at ~ 400 ms/nt (2.5 nt/s) that is usable for sequencing. Once the blocking primer is fully unzipped, the complex (and only this complex) is activated for polymerization on the pore. Upon activation, phi29 processes the bound DNA strand, ratcheting it upward through through the pore at ~ 25 ms/nt (or 40 nt/s) registering a second signal that is usable for sequencing in the reverse order. The strategy permits up to 500 DNA molecules to be sequentially controlled through a single nanopore over a period of hours. These results used abasic residues in the templating strand to signal DNA motion progress through α -hemolysin, and although this achieved single nucleotide (5 Å) resolution, strand sequencing was not demonstrated due to the lack of sensitivity of α -hemolysin.

For biological nanopore sequencing, the most compelling results to date combined the phi29 motor and blocking oligomer strategy in [26] with the MspA nanopore (Fig. 2c), as a result of collaboration between the Akesson and Gundlach groups [27]. The work combines the most viable method of rate control with the most sensitive nanopore conducive to enzyme function. Still, as the results are very new, there are several challenges that must be addressed before the method can provide direct strand sequencing. First, distinguishing each base of a translocating DNA strand involves significant computational effort to determine what combination of ~ 4 bases are in the sensing region. Second, a significant subset of current records display sustained amplitudes for ~ 1 s, observed during unzipping and synthesis, as compared to mean unzipping time of 100 ms and synthesis time of 40 ms. The pauses in the current will make it challenging to differentiate homopolymeric regions from stalled complexes. Additionally, because the dwell time between nucleotide additions is exponentially distributed, the motor will invariably (and nondeterministically) exhibit these longer-than-normal dwell times between nucleotide additions, making this an issue for other methods that rely on enzymatic function for sequencing to progress. Third, errors due to motor steps faster than the recording bandwidth, and due to backtracking of the motor to re-register sequences, confound the accuracy of the sensing and ordering (these errors were quantified in [26]). This is not an exhaustive list, but these challenges highlight the need for new algorithms to identify the sequence estimate and assign confidence intervals. The opportunity to re-read the same molecule will undoubtedly play a role in reducing errors.

2.1.3 Commercial biological nanopore sequencers—In the commercial sector, Oxford Nanopore Technologies has achieved single-nucleotide reading with biological nanopores. In February of this year, the company introduced new nanopore-based sequencing devices: the GridION and MinION. Both claim high-throughput DNA sequencing with significantly longer reads than modern sequencing platforms. The GridION platform has an array of 2000 individual nanopores at present. On each nanopore the claimed read length is 5-100kb at a rate of 150 Mb per hour for up to 6 hours, and with 20 GridIONs working in parallel, the company claims that the human genome could be sequenced in 15 minutes [43]. The MinION is a smaller USB-sized sequencer with 512 nanopores with similar specifications. Though the company has committed the majority of their effort to develop commercialized biological nanopore devices, licensing relevant IP from UC Santa Cruz, Harvard and Oxford Universities, the company is reserving a portion of their research capacity for solid-state-based nanopore sequencing according to their webpage press release announcing their licensing solid-state nanopore IP from Harvard University.

2.2 Solid-state nanopores

This section first provides a brief overview of solid-state nanopore research to define the field, then covers recent developments that have promise for sequencing technology. We note that solid-state nanopores are likely to find commercial applications outside of sequencing, and refer interested readers to other surveys on this topic [9, 10].

Solid-state nanopores are nano-sized holes formed by drilling or etching the opening in a solid-state substrate, with the substrate material typically being fabricated by conventional semi-conductor processes [9, 44] (Fig. 2b). In comparison to biological pores, solid-state pores have superior mechanical, chemical and thermal stability [10, 45–49]. The most common solid-state membranes are silicon-based, including silicon mononitride (SiN), disilicon nitride (Si₂N), silicon sesquinitride (Si₃N₄) and silicon dioxide (SiO₂). In 2001, researchers at Harvard (led by Gene Golovchev) were the first to demonstrate nanometer precision pores drilled in silicon substrates using ion beam milling, and detection of DNA capture and passage through such pores [50]. Pores are now routinely fabricated using

methods that involve chemical etching, ion beam sculpting, e-beam drilling, atomic layer deposition, feedback controlled sputtering, or combinations of these [9].

2.2.1 The sensitivity of solid-state nanopores—While biological nanopores have fixed and atomic-precision geometries, solid-state nanopores can have a range of diameters depending on various semiconductor fabrication techniques. Diameters range from 50 nm (or larger) to as small as 1 nm in diameter—smaller than α -hemolysin's (1.5 nm) and MspA's (1.2 nm) most limiting diameters. Although solid-state pores can reach the dimensions of biological nanopores in diameter, the thickness of the membranes is generally greater (>10 nm) than the length of biopores, and they exhibit much higher noise than biological pores. Both of these issues prevent solid-state pores from demonstrating the necessary sensitivity. Another disadvantage is that no two fabricated pores are the same, even if they undergo the same process with the same specifications. Moreover, two pores that look the same by electron imaging can behave radically differently (in terms of noise) when applying voltage and measuring current [24]. Still, alternative materials and surface treatment methods are improving the performance of these pores [51]. Recently, researchers from University of Illinois, Urbana-Champaign (led by Rashid Bashir) have employed aluminum oxide (Al₂O₃) for solid-state DNA analysis [45]. Compared to its silicon counterparts, aluminum oxide has increased mechanical durability and improved electrical properties, exhibiting higher SNR [45–47]. In any nanopore sequencing approach, SNR must be high enough to achieve single-nucleotide sensitivity; thus, noise reduction techniques are essential for solid-state nanopores to progress as a potential sequencing approach.

Another material used as a substrate for solid-state pores is graphene. A single sheet of graphene is only one atom thick but the material is exceptionally strong. The membrane thickness (0.34 nm) is comparable to the height of a nucleotide and it has been proposed that nucleotide discrimination is possible with graphene due to this property. In initial experiments [49, 52], graphene was shown to act as a nanopore-bearing and insulating membrane, offering an ionic-current based sensing method. Alternatively, graphene can also act as a conductor for transverse current that passes through the material, and with current that tunnels across a nanopore drilled in the membrane. In numerical simulations, Postma showed that nucleobase detection is achievable by transverse conductance shifts due to an ssDNA strand passing through a graphene nanogap [53]. Experimentally, however, high noise is a challenge [49, 54] as graphene exhibits larger noise than Si-based pores. Graphene monolayers coated with TiO₂, which insulates the graphene pores once drilled, were shown to exhibit significantly lower noise [54]. In another work, a nanopore formed in stacked alternating layers comprised of graphene monolayers and AlO₂ insulating layers showed improved noise characteristics, and made it possible to distinguish between various DNA structures and DNA-protein complexes [46]. To date, achieving the required sensitivity for sequencing with graphene has been demonstrated only in numerical experiments [53].

Embedding electrodes in nanopores to create a tunneling current across a nanopore has also been pursued (a review of relevant work prior to 2008 is in [4]). A recent example of commercial relevance for the prospect of sequencing DNA is the tunneling work at Osaka University (led by Tomoji Kawai, with involvement by Toshiba) [55,56]. The transverse (tunneling current) signal can be correlated with the trans-membrane (ionic current) signal, and the transverse voltage can create friction to reduce DNA translocation rates that are otherwise driven solely by the trans-membrane voltage [55]. Another approach that has significant technological promise is the use of surface modifications to functionalize the pore-embedded electrodes for nucleobase recognition. Notably, researchers at Arizona State University (led by Stuart Lindsay) have designed a benzamide-based molecule (4(5)-

Substituted 1-H-Imidazole-2-carboxamide) that will hydrogen-bond to DNA bases T, A, C and G in different orientations [57] (Fig. 2d). By functionalizing the tip of an electrode pair with this molecule, and integrating the electrodes within a solid-state nanopore, translocating nucleobases interact with the molecule and form a transient electron-tunneling bridge across the electrodes. A transient signal is present for each nucleobase due to the specific bond orientation [58], and the detection SNR is considerably higher than appears to be possible with ionic-current based sensing through biological nanopores. In conjunction with a rate control mechanism, the functionalized-electrode approach by Lindsay's group appears to be a leading solid-state nanopore platform for sequencing.

2.2.2 Rate control for solid-state pores—We first consider candidate rate control methods, as well as alternative approaches that propose to obviate the need for rate control. Controlled translocation rates for DNA sequencing have yet to be demonstrated on any solid-state platform, with the exception of methods that involve attachment of beads to the DNA that are manipulated by an auxiliary source [59]. While such methods have proven useful as scientific instruments [60–63], they are unlikely to be incorporated into commercial sequencing devices due to instrument complexity. In a commercial-based effort, Roche and IBM have jointly licensed the IP on the functionalized-electrode approach by Lindsay's group, with the aim of using it in conjunction with their DNA-transistor method of rate control. No experimental demonstrations of the DNA-transistor method have been published, though idealized simulation results are in [64].

With the recent success of phi29 [26, 27], one could imagine coupling a processive enzyme to a solid-state pore. Though static DNA-protein complexes have been captured on solid-state pores in force spectroscopy studies [22], preserving enzymatic function on such pores is not trivial because the precise surface structure of a solid-state pore opening is in general unknown and may not be conducive to function. In any case, a method that could position phi29 in proximity to functionalized electrodes is attractive, as it would provide a direct strand-sequencing platform that overcomes the lack of single-nucleotide sensitivity associated with ionic currents through biological pores. A hybrid biopore/solid-state pore [65] on which the enzyme could sit, in series with a pore with embedded functionalized electrodes, could achieve the desired setup.

Other recent research aims to reduce high-frequency noise, through optimizing the electrical engineering of the amplifier and setup, which in turn allows higher bandwidth recording. Specifically, by reducing the capacitive elements of the circuitry and wiring, Rosenstein *et al.* demonstrated significant reduction of high-frequency noise, permitting detection of DNA passage through solid-state pores at 1 MHz bandwidth (200 times the standard 5 kHz bandwidth used) [66]. We report this work in this section for the following reason: the setup could potentially relax the requirements on DNA rate reduction for sequencing with solid-state nanopores. That is, DNA can go faster for sequencing, but only if the necessary SNR for sequencing is achievable at higher bandwidth. For example, if there is enough SNR at 1 MHz bandwidth for sequencing, then DNA can go $\sim 5\text{--}10\text{ }\mu\text{s/nt}$, which is only slightly slower than the unimpeded rates of $1\text{--}3\text{ }\mu\text{s/nt}$ [12] and could be achieved with slightly lower voltage than 120 mV. Presently, though, there is no verified solid-state platform exhibiting single nucleotide sensitivity, so we do not yet know what the necessary SNR is for sequencing. It is also unlikely (and perhaps theoretically impossible) that noise levels will ever be low enough at 1 MHz for direct sequencing. We can establish this by considering MspA performance as the criteria, where noise levels are $\sim 1\text{ pA RMS}$ at 5 kHz bandwidth, and lower after subsequent filtering used for step-detection analysis [27]. Regardless, provided a solid-state or biological nanopore uses some form of control to achieve the necessary rate reduction, there is value in using the setup in [66]. The reason is that additional filtering can be done offline to further reduce noise, as so the same SNR achieved

with a commercial amplifier at a given bandwidth should be achievable at a *higher* bandwidth using the integrated setup in [66], provided filtering is done offline. We note that the setup appears to have higher low-frequency noise than the commonly used commercial amplifier (Molecular Devices Axopatch 200B), which means SNR is not as good if both setups use the standard 5 kHz bandwidth online. Another consideration is that if the driving voltage and ionic current of a single pore are used only for the detection of DNA in the pore, and an auxiliary method is used for sensing nucleotides (e.g., tunneling electrodes), achieving sufficient SNR for detection at MHz bandwidth is very useful. The results in [66] are significant, and speak to the value and need for further integration and performance optimization of the amplifier circuitry.

Since lowering the filtering bandwidth always improves SNR, and the noise specification for sequencing are to be determined, there is value in continuing to develop DNA rate reduction methods for solid-state nanopores. Bashir and coworkers reported the potential for Al_2O_3 pores to significantly reduce translocation rates due to their unique ability to form positively charged crystal domains from sputtering [45, 47] —the negatively charged phosphate backbone of DNA, while traversing an Al_2O_3 pore, interacts with the positive surface, slowing its movement. Recent work by Dekker and coworkers showed that LiCl slowed DNA rates by 10 $\times$, compared to the commonly used KCl ionic solution [67], provided a method that any nanopore setup could benefit from.

In the commercial sector, NABsys has taken an alternative approach that aims to eliminate the need for DNA motion control by using hybridization-assisted nanopore sequencing [68]. For the method to work, accurate mapping of double-stranded segments within otherwise ssDNA must be achieved. While accurate differentiating individual ssDNA molecules from dsDNA molecules has been shown [13], the mapping problem within a single DNA molecule bearing ssDNA and dsDNA sections has not been resolved. In a comparable approach, the mapping problem is solved by incorporating an optical reader with the nanopore setup [69]. The method involves a DNA conversion scheme, in which each nucleotide is mapped to a larger sequence, and hybridization of complementary 12 mer strands to each of the four sequences would be detected in DNA that passes freely through a pore by electrophoresis, without added rate control. The same conversion scheme can also be applied using the MspA nanopore [38]. However, conversion is very costly in time and reagents, typically being accomplished by cyclic application of DNA restriction and ligation enzymes [4]. The commercial potential of the method is enticing, since sensitivity is achieved and rate reduction is no longer necessary, but conversion costs must first be sufficiently reduced.

2.3 Summary

Single nanopores have now developed to the extent that they are incorporated into a commercial NGS platform. Biological nanopores are presently leading the charge, considering jointly the impacts of MspA and phi29 academic research, and Oxford Nanopore's commercial claims. Solid-state nanopores could make a similar impact, likely with the use of tunneling electrodes and chemical modification, provided a rate control mechanism can be coupled with these single-nucleotide sensors, and that they can be used in parallel on the same DNA strand. While the long-touted promises of nanopore sequencing are beginning to be realized, it is not yet clear how many of the promises will ultimately materialize. Oxford Nanopore's claim of sequencing a full phage genome suggests that the long read-length promise is viable. But can the promised time-to-sequence be achieved? How do the total and per-uses costs compare to Ion Torrent's device? What error rates will register in the hands of the user? While we cannot yet answer these questions, we can consider an issue that will affect the outcome: the scalability of nanopores for multichannel array implementations.

3. Multichannel nanopore sensing

If a nanopore can sequence genome-length DNA, using an enzyme perched on a sufficiently sensitive biological pore, then why develop a multichannel nanopore array, particularly if the same DNA can be repeatedly sequenced by cyclically re-unzipping and re-synthesizing at one pore? In the ideal setting where there are no time limits on the measurements or device operation, there is technically no advantage to using an array of independent nanopore sensors. Practically, of course, the need for a multichannel implementation is motivated by cost and time. A nanopore array would enable measurements in parallel, which reduces the time necessary to glean the estimated sequence *and* the statistical errors associated with the estimates. It also makes it possible to use a heterogeneous concentration, in which a distinct sequence for each captured DNA would be measured. To preserve the single-molecule-at-a-time feature, each nanopore in the array would need to function independently and asynchronously, with no requirement that molecules be progressing at the same rate, or in the same strand location. In turn, this requires advances in microfluidics and integrated circuitry. The fluidic chip should support a set of pores in parallel, with one common fluidic chamber in which DNA samples are added, for example, and isolated chambers on the other side of each pore. The circuitry should be fully integrated to maximize the number of channels, and optimized for low-noise sensing.

3.1 Recent research

Building arrays of nanopores has become an active area of research [70], and fabrication methods are improving [71]. In initial work on nanopore arrays, each pore (in a common membrane) shared both volumes in common with all the other pores. This is the simplest array setup, requiring no advancements in terms of fluidics or circuitry. A single voltage source is used such that all channel currents are superimposed on the recorded signal, which complicates the subsequent analysis for detecting distinct DNA capture events. This setup is comparable to measuring multiple gating channels within a single membrane patch in cellular recording [72]. While such a setup alone would not be viable for sequencing, using it in concert with other methods has proven useful. For example, researchers have used optics in conjunction with the nanopore array to provide hundreds- to-thousands of distinct signals that can be correlated with each location in the optical field of view [69, 73].

For the development of microfluidics and circuitry to support functionalization of an array of nanopores, consideration should be given to the precedent already set in the field of cellular channel recording [72]. Devices that incorporate multi-array amplifiers for simultaneous patch-clamping of cells (each by a giga-ohm seal formed with a micropipette exposed to a channel flow) are commercially available, and much research has set the stage for this. Using expertise from this field [74], researchers at the University of Freiburg, Germany (led by Jan Behrends) and collaborators have recently demonstrated a device with an array of 16 functionalized α -hemolysin nanopores [75]. An earlier work showed an 8 channel platform [76], and both works utilized commercially available multichannel patch-clamp amplifier technology (Tecella).

3.2 The costs and scale of a multichannel nanopore array

In the remained of the paper, we consider a simple Gedankenexperiment (thought experiment) that considers the scalability of the circuitry for a hand-held nanopore array. That is, we assess how many channels can be functionalized on a device based on existing fabrication methods and relevant patch-clamp amplifier technology; we do not consider the scalability of the fluidics. A multichannel nanopore array on the small-footprint device requires a multichannel patch-clamp amplifier integrated on a single chip, and each amplifier should be connected with an individual nanopore in order to prevent signal

interference between nanopores. Thus, the number of patch-clamp amplifiers should be the same as the number of nanopores. There is also analog signal acquisition from each nanopore through the patch-clamp amplifiers, and each analog signal must be digitized using an analog-to-digital (ADC) converter.

The multichannel patch-clamp amplifier should have two main features: miniaturization and high channel density. For the purpose of establishing a geometric size constraint, within which the circuitry should be housed, we consider the iPhone as our ideal housing dimension ($5.86 \times 11.52 \times 0.93 \text{ cm}^3$). Ideally, then, several hundreds or thousands of patch-clamp amplifiers have to fit in an iPhone. A patch-clamp amplifier from Tecella supports up to 384 channels (according to their company webpage), but its size ($19.8 \times 23.6 \times 35.6 \text{ cm}^3$) is too large for our thought exercise here.

CMOS process technology enables space minimization and thus helps to increase channel density on a limited area. Many research groups have developed a low-noise CMOS patch-clamp amplifier in order to detect the ionic current variations through a cellular membrane channel or a nanopore. For instance, Dr. Culurciello's group has designed a low-noise patch-clamp amplifier [77, 78]. Using a silicon-on-sapphire CMOS technology, they reduced electronic coupling noise present in sensitive current-recording equipment such as patch-clamp amplifiers. Dr. Shepard's group developed the low-noise preamplifier that was part of the reduced high-frequency noise setup referenced in the previous section [66]. They formed a solid-state nanopore on the CMOS amplifier chip using a post-fabrication process, resulting in a reduction of input parasitic capacitance and nanopore capacitance, which in turn reduced the high-frequency noise and permitted higher bandwidth detection of DNA events. Our own lab has also designed a low-noise CMOS patch-clamp amplifier and demonstrated it using solid-state and α -hemolysin nanopores [79, 80]. The amplifier in [79] presents a novel method of capacitive compensation that minimizes the hardware complexity, compared to conventional techniques [72,78,81], and thereby increases the patch-clamp amplifier density that is possible on a chip (i.e., more channels).

Based on the single patch-clamp amplifier area, we can estimate how many amplifiers can be integrated on a silicon chip while also considering cost and yield. First, we assume that the maximum die size we can use in a $0.35 \mu\text{m}$ CMOS process is about 600 mm^2 . This maximum die area varies with IC foundry and process technology. For a realistic single patch-clamp amplifier size, we have to consider contact pads, electrostatic discharge circuits, and ADC circuitry. Including all of those components, we assume a single patch-clamp amplifier occupies an area of 0.4 mm^2 , which is more than our chip occupies [79, 80]. Using mass production, the cost to fabricate a single wafer with a diameter of 300 mm would be approximately \$2000, though the actual cost varies with IC foundries. Using a gross die per wafer (GDW) formula from [82], we calculate the available number of die per wafer as

$$\text{GDW} = \pi R^2 / A - (\pi \times 1.16 \times R) / \sqrt{A} \quad (1)$$

where R is the radius of the wafer ($=150 \text{ mm}$) and A is the total amplifier area, which is defined as the number of channels times the single channel amplifier area (0.4 mm^2). Because we already set the maximum available die size at 600 mm^2 , the number of channels varies from 1 up to $600 \text{ mm}^2 / 0.4 \text{ mm}^2 \approx 1500$ channels. Employing a yield formula from [83], we can estimate the die yield (%) which varies with the number of channels as

$$\text{Die Yield} = WY \times (1 + DR \times A/\alpha)^\alpha \quad (2)$$

Here, WY is wafer yield, DR is defect rate per unit area, and γ is a yield coefficient (we set $WY = 1$, $DR = 0.75$, and $\gamma = 4$ from [83]). A single die cost (\$) can be calculated as

$$\text{Die Cost} = \text{Wafer Cost} \times (\text{GDW} \times \text{Die Yield}) \quad (3)$$

Fig. 3 illustrates a trade-off graph in die cost and die yield varying the number of patch-clamp amplifiers on a die. Although we could integrate the maximum of 1500 patch-clamp amplifiers on a die area of 600 mm^2 , the yield would be poor, resulting in increased cost. Considering the tradeoff in cost and yield, the curve suggests an optimal choice of integrating 820 patch-clamp amplifiers on one die, corresponding to a cost of $\sim \$70$. If other costs related to circuitry are considered, such as testing verification procedures and chip packaging processes, the chip cost would be increased up to a few hundred dollars. Including packaging, the maximum area corresponds to roughly $4 \times 4 \text{ cm}^2$, which would leave more than 50% of the iPhone for the fluidics. Using 820 channels uses about half the maximum die area, and so reserves more of the iPhone for fluidics, which are likely to take considerably more space per channel.

This section considered the scalability of the circuitry to support a multichannel nanopore array, housed in a device the size of an iPhone. By comparison, and ignoring the iPhone size constraint, Ion Torrent's device can accommodate 1.2 Million measurements in the array [84], because the technology is based on voltage sensing using an ion-sensitive field-effect transistor, and each sensor requires little space on an integrated chip. Additionally, the chip contains both the circuitry and the sensors. A nanopore array, on the other hand, has to combine two arrayed chip, one bearing the circuitry and one bearing the fluidics for nanopore sensing. Moreover, measuring currents through nanopores requires an amplifier for each channel, and this requires more space on the chip. Based on our thought experiment, we reason that 800-1000 channels is reasonable for a hand-held device, with costs (excluding usage and microfluidics fabrication) well below \$1k per instrument.

4. Concluding remarks

In this paper, we reviewed recent achievements in nanopore sequencing research and technology. Sustained and progressive research efforts at universities that has focused on improving the sensitivity of biological pores, and on using DNA polymerases for DNA rate control through the pore, are the principle reasons that nanopore sequencing is now staking a claim in the NGS technology landscape. The recent press on Oxford Nanopore Technologies suggests that the commercial potential of nanopores is at last being realized, including the achievement of kbp read lengths. We also considered solid-state nanopore methods that could emerge as NGS platforms. In any commercial platform, multichannel arrays of nanopores will be implemented, and we reviewed some of the recent research on this topic, and presented an analysis on the scale of circuitry to support the array. There remain open challenges for the research community, including the need for algorithm that can glean the sequence from the data, the need for optimization of integrated circuitry to functionalize each pore in the array, and the need for microfluidics to house the array.

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Abbreviations

NGS	next-generation sequencing
SNR	signal-to-noise ratio

Figure 1. Schematic of the biological nanopore instrument with representative ssDNA and protein-bound DNA events

A) The nanopore instrument uses an amplifier to apply a command voltage V_c and measure ionic current I_p through the nanopore channel. B) At 120 mV in 1 M buffered KCl solution, 120 pA of open channel current is attenuated to ~15 pA for ~0.2 ms upon capture of ssDNA into the channel from the *cis*-chamber, until the DNA passes through the pore. With ExoI and ssDNA in the *cis* chamber, bound events are also observed in which the duration of the current shift is extended (~2 ms), while the DNA is stalled in the pore channel, until ExoI is forced to dissociate from the DNA and the DNA translocates into the *trans* chamber (hydrolysis is here inhibited by adding EDTA to chelate Mg^{2+}).

Figure 2.

Varying types and geometries of nanopores (a,b) and leading configurations for sequencing (c,d). (a) Relative dimensions (approximate) of biological pores α -hemolysin and MspA. (b) Dimensions of graphene with single-atom thickness, and range of pore diameters in varying solid-state substrates. (c) The mechanism of phi29 polymerase mediated DNA translocation developed in [26] and implemented on the MspA nanopore in [27]. The motor's ability to both polymerize (at ~40 nt/s) and unzip (at ~2.5 nt/s) the strand is utilized to register DNA motion progress and position sensing, with the unzipping direction shown in this illustration. Though dwell times at each position are not constant, but exponentially distributed, the rates meet the requirements for DNA speed reduction [4]. (d) Functionalized electrode readers of nucleobases via 4(5)-Substituted 1-H-Imidazole-2-carboxamide. Different 180° rotations occur over the specified bonds on the carboxamide molecules to allow hydrogen bonding with different nucleobases, causing detectable variations in electron tunneling signals between two electrodes attached to the two carboxamides.

Figure 3.

Tradeoff curve between die yield and cost, varying the number of patch-clamp amplifiers on the die. The optimal value is ~820 patch-clamp amplifiers on one die, resulting in a cost of ~\$70. The actual cost is driven by the wafer cost (assumed to be \$2000 here), and other factors described in the text.