The Potential and Challenges of Nanopore Sequencing

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(Article begins on next page)

Nanopore Sequencing

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A nanopore-based device provides single molecule detection and analytical capabilities that are achieved by electrophoretically driving molecules in solution through a nano-scale pore. The nanopore provides a highly confined space within which single nucleic acid polymers can be analyzed at high through-put by one of a variety of means, and the perfect processivity that can be enforced in a narrow pore ensures that the native order of the nucleobases in a polynucleotide is reflected in the sequence of signals that is detected. Kilo-base length polymers (single-stranded genomic DNA or RNA) or small molecules (e.g. nucleosides) can be identified and characterized without amplification or labeling, a unique analytical capability that makes inexpensive, rapid DNA sequencing a possibility. Further research and development of nanopores offers the prospect of "third generation" instruments that will sequence a mammalian genome for ~$1,000 in about 24 hours.

Introduction

Attaining the Human Genome Project goal of sequencing the human genome while rapidly and publicly disseminating the data was a milestone in human biomedical research that was enabled by scientific, technical and cultural innovation. Central was the development of robust, automated methods and technologies to identify the linear sequence of nucleotides. Recognizing the opportunities to use significantly expanded sequencing technology in the subsequent phase of genomics research, as described in the accompanying Perspective article (NBT ref.), NHGRI initiated a funding program in 2004 aimed at reducing the cost of genome sequencing to about $1,000 in 10 years, with an intermediate goal of reduction to $100,000 by 2009 (http://grants.nih.gov/grants/guide/rfa-files/RFA-HG-04-003.html). Numerous grant awards made in this program (http://www.genome.gov/10000368#6) have stimulated a strong record of publications and patents (http://www.genome.gov/10000368#7) and the successful commercialization of several sequencing platforms now in active use worldwide, with others in the wings. At annual grantee meetings, open discussions of advances and challenges have stimulated collaboration and considerably accelerated research. This perspective on nanopore sequencing and an accompanying one on sequencing by synthesis technologies present current views on these challenges to the broader community of scientists and engineers, with a goal of engaging them to find solutions.

When a small (~100mV) voltage bias is imposed across a nanopore in a membrane separating two chambers containing aqueous electrolyte, the resulting ionic current through the pore can be measured with standard electrophysiological techniques. Bearing in mind that the opening and closing of many biological channels depends on relatively small peptide moieties physically blocking the channel, David Deamer and George Church, independently, proposed that if a strand of DNA or RNA could be electrophoretically driven through a nanopore of suitable diameter, the nucleobases would similarly modulate the ionic current through the nanopore. Subsequently, Kasianowicz *et al.* demonstrated that single-stranded DNA and RNA molecules can be driven through a pore-forming protein and detected by their effect on the ionic current through this nanopore[1] (Fig. 1a). These investigators used the *S. aureus* toxin, α-hemolysin, whose use as a biosensor had been pioneered by Bayley and his colleagues[2]. The α-hemolysin pore is remarkably stable and remains functional at close to the boiling point of water[3]. Because the limiting inside diameter of the α-hemolysin pore is barely as large as the diameter of a single nucleic acid strand, Kasianowicz *et al.*'s results showed that a nanopore can locally unravel a coiled nucleic acid so that its nucleotides are translocated through the pore in strictly single-file, sequential order. Because the current of ions through the nanopore is partially blocked by the translocating molecule, each translocating molecule produces a readily detected reduction of the ionic current relative to that which flows through the open, unblocked pore. Given this fact, Kasianowicz *et al.* hypothesized that if each nucleotide in the polymer produced a characteristic modulation of the ionic current during its passage through the nanopore, the sequence of current modulations would reflect the sequence of bases in the polymer.

To test this hypothesis, the current modulations caused by several different RNA and single stranded DNA (ssDNA) polynucleotides were investigated[4, 5]. The pore current was blocked substantially more by polyC RNA than by polyA RNA, and other experiments with RNA molecules containing 30 A's followed by 70 C's showed that the transition from polyA to polyC segments within a single RNA molecule was readily detectable[4]. These easily measured distinctions between purine and pyrimidine ribonucleotides were unfortunately not as clear with deoxyribonucleotides[5]. In fact, the current level differences that had been observed with RNAs turned out to be a reflection of base stacking and other secondary structural differences between polyA and polyC oligomers[4], and further measurements using various DNA homopolymers revealed only small ion-current differences (~5% or less) between deoxy purine and deoxy pyrimidine oligomers[5]. Single nucleotide discrimination could not be achieved because the ion-current blockades were found to be the consequence of the ~10-15 nucleotides (rather than any single nucleotide) that occupy the membrane-spanning domain of the α-hemolysin pore[6] (Fig. 1a).

Although these early nanopore experiments disappointed naïve expectations of an easy path to inexpensive DNA sequencing, they demonstrated the extraordinary single molecule sensitivity of nanopores and stimulated many very successful applications that have led to a burgeoning literature comprising both theoretical and experimental studies related to the nanopore analysis of nucleic acids[7-10]. Since the first demonstrations that an electric field can drive even kilobase lengths of ssDNA molecules through nanopores, the prospect of an inexpensive direct physical route to massive sequencing capacity has greatly stimulated nanopore research using either protein pores in a lipid bilayer[11] and, more recently, fabricated nanopores in solid-state[12] or plastic materials[13]. Although nanopores are the basis for several important single molecule applications[9, 10], substantial lengths of DNA have yet to be sequenced with a nanopore. In view of the demonstrable progress and cost reductions with sequencing by synthesis (see section XX [NatBioT ref.] of this issue), is continued research toward nanopore sequencing justified?

Justification

The compelling advantage of nanopore sequencing is the prospect of inexpensive sample preparation requiring minimal chemistries or enzyme-dependent amplification. Futhermore, a nanopore sensor eliminates the need for costly nucleotides and polymerases or ligases during readout. Thus, the costs of nanopore sequencing, be it by direct strand sequencing or by one of the other methods discussed here, are projected to be far lower than ensemble sequencing by the Sanger method, or any of the recently commercialized massively parallel approaches (Roche/454, Illumina/Solexa, ABI/Agencourt[14]). Unlike these approaches, the ideal nanopore sequencing approach would not require the use of purified fluorescent reagents and would use unamplified genomic DNA, thus eliminating expensive enzymes, cloning, or amplification steps.

The components of an ideal commercial sequencing system using electrical measurements would then be (1) a disposable detector chip containing an array of nanopores having the required integrated microfluidics and electronic probes; and (2) a bench-top instrument, or portable system, that would control the fluidics and electronic elements of the chip and process the raw sequence data. Assuming one chip will be used to sequence a sample having the complexity of a human genome, the cost of sequencing a complete human genome will be the cost of preparing the genomic DNA from a biological sample (e.g. blood), plus the amortized cost of the instrument, plus the cost of the disposable detector chip that will be used for each genome determination.

Nanopore sequencing could in principle achieve a 6-fold sequence coverage and over-sampling with less than 1 µg of genomic DNA ($<10^6$ copies of the target genome extracted from $<10^6$ cells), but the concentration limited rate at which a nanopore can capture the diffusing DNA molecules from the source volume[15] will probably require $\sim10^8$ copies of the target genome to provide adequate through-put from a practical volume of source material to feed an array of nanopores. This corresponds to about 700 µg of human diploid genomic material, which can be directly obtained without amplification by using commercially available kits for the isolation, purification, and concentration of genomic DNA**.** Such kits can obtain approximately 1,000 µg of purified, high molecular weight (>50,000 base-pair fragments) genomic DNA from ~20 ml of blood at a cost that is likely to be <$40/sample (http://www1.qiagen.com/Products/**)**. The diploid mammalian genome, consisting of 6×10^9 base-pairs, would be fragmented into 50,000 base-pair lengths and dissociated into ssDNA (*e.g.* by high pH) for direct strand-sequencing. The extremely long reads of ~50,000 bases that may be possible with nanopore methods should greatly simplify the genome assembly process. If nanopores indeed enable minimal sample processing and obviate the need for labeling, the cost of such sequencing would be dominated by the cost of the disposable chip and instrument amortization, which is estimated to total less than $1,000 per mammalian genome.

Though the cost and read-length forecasts of nanopore technology are exceptionally promising, a number of key technology challenges must be addressed before nanopore sequencing can be implemented. While specific challenges vary over the several different nanopore sequencing approaches, a discussion of some of the most ubiquitous challenges follows.

Challenges to Nanopore Sequencing

Base recognition and resolution

Several different approaches to using nanopores for sequencing are being considered. Those examined below are not intended to form an exhaustive list of such approaches but instead illustrate the major challenges common to most of these efforts.

a. Measurement of ionic current blockades as ssDNA is driven through a biopore or a solid-state pore. While several experiments have clearly demonstrated that modulations of ionic current during translocation of RNA or DNA strands can be used to discriminate between polynucleotides[4, 5, 16], none of the natural or man-made nanopore structures that has been used has had the appropriate geometry to detect the features of only one nucleotide at a time while the polymer is translocating through the pore. No nanopores that have been investigated have had channels shorter than about 5 nm and, because at least 10-15 nucleotides of ssDNA would extend through a channel of this length, all of these nucleotides together contribute to the ionic current blockade[6] (Fig. 1a). Even an "infinitely short" channel would not achieve the required resolution, as the region of high electric field that determines the electrical "read" region of the channel[17-20] will extend for approximately one channel diameter to each side of the channel. Since the channel diameter needs to be large enough to translocate ssDNA (~ 1.5nm), this puts a fundamental restriction on the spatial resolution of the channel in current blockage readings. Furthermore, when drawn through a nanopore by a ~150mV bias, single-stranded polynucleotides are translocated at average rates that approach ~1 nucleotide/μs. Resolving single bases with small pA currents will require a means of slowing the translocation so that the time that each base occupies the nanopore detector is ≥1 msec, and possibly larger[7].

Alternatively, coarser-grained current blockage information could be used to infer sequence by using a nanopore in conjunction with sequencing by hybridization[21]. Although nanopore devices cannot yet resolve the single bases separated by about 0.4 nm in a DNA strand, a pore that is large enough to translocate double stranded DNA (dsDNA) can readily distinguish the passage of ssDNA from the passage of dsDNA[22]. The original concept of sequencing by hybridization contemplates aligning hybridization probes of known sequences to derive the sequence of an unknown ssDNA strand[21], but for *de novo* sequencing both the location and the number of probes bound to the long unknown DNA strand that is to be sequenced must be known. Sequencing by hybridization alone does not provide this information. But since a nanopore is able to discriminate between dsDNA and ssDNA, it may be able to detect and resolve the location of oligonucleotide probes that are hybridized to a long translocating ssDNA. Thus, the standard routines for sequencing by hybridization[21] could be enhanced by nanopore-derived information regarding the number and the location of oligonucleotide probes bound to a long DNA strand. This is the basic concept of Hybridization-Assisted Nanopore Sequencing or HANS[23]. Current research on the HANS method addresses two challenges: Can a nanopore determine the location of a hybridized probe with sufficient accuracy to enhance sequencing by hybridization? What length DNA sequence can be reliably reconstructed, given the practical limitations of detecting bound probes and locating them precisely on the DNA strand that is to be sequenced?

b. Measurement of ionic current blockades from individual nucleotides sequentially cleaved off the end of a DNA strand and driven through a biopore. At the time Keller and colleagues recognized that it might be possible to sequence single molecules of DNA by identifying the deoxynucleoside monophosphates (dNMPs) released by an exonuclease from the end of a DNA or RNA chain[24], there was no obvious way to identify individual unlabelled bases after their release. Recent work indicates that unlabelled bases can be identified by α-hemolysin when fitted with an aminocyclodextrin adapter[25] and methods have now been developed to covalently attach cyclodextrins within the lumen of the α-hemolysin pore[26]. Based on this work, Oxford Nanopore Technologies has recently succeeded in covalently attaching the aminocyclodextrin adapter within the α-hemolysin pore (Fig. 1b). When a dNMP is captured and driven through the α-hemolysin-aminocyclodextrin pore in a lipid bilayer membrane, the ionic current through the pore is reduced to one of four levels, each of which reflects which of the four dNMPs – A, T, G, or C – is translocating. Furthermore, because all four of the ionic current blockage levels are easily distinguished from the current that flows through the open, unblocked pore, the current traces can provide an accurate count of the total number of dNMPs that have translocated through the α-hemolysin-aminocyclodextrin pore. For sequencing, it will now be important to assure that 100% of the exonuclease-released dNMPs are captured in the pore and efficiently expelled on the opposite side of the membrane. Because this approach uses the nanopore to identify the released dNMPs, rather than the bases of an intact DNA strand, the strictly single file, sequential passage of the bases that a nanopore can enforce is lost. It will therefore be especially important to demonstrate that the sequence of independently read dNMPs reflects the order in which the bases are cleaved from the DNA (i.e., no overtaking or double counting). Finally, the choice and attachment of the exonuclease to the nanopore must be considered. A genetic construct in which the nuclease and α-hemolysin genes are spliced together might be used or the nuclease might be chemically attached to assure delivery of the released dNMPs into the nanopore. The enzyme should be processive and, for low noise detection, active in high salt. Preferably, it should digest double-stranded DNA, which is readily produced from genomic DNA and easy to handle.

c. Nanopore sequencing using converted targets and optical readout. A new readout modality in development for nanopore based sequencing converts the sequence information of DNA into a two-color scheme that is then optically read[27, 28]. While attaching fluorescent probes to each and every nucleotide in DNA is difficult, methods are available to systematically encode and substitute each and every nucleotide in the genome with a specific permutation of two different 12-mer oligos (A and B), concatenated in a specific order (AB, BA, AA, BB) that reflects and encodes the nucleotide sequence of the unknown DNA[29] (Fig. 1c). This converts the quaternary DNA code of A, T, G, and C into a binary code in which each base is represented by a pair of 12-mer oligos (A and B). An automated, massively-parallel process developed by Lingvitae Inc. (http://www.lingvitae.com/DPTutorial.php) currently requires ~24 hours to convert a complete human genome into a DNA mixture consisting of fragments, each corresponding to a 24-bp segment of the original genome. Work is currently underway to develop inexpensive error-free conversion of longer segments of the original genome and to greatly reduce the conversion time. The conversion process does introduce an extra biochemical step, which is not ideal, but it side-steps some of the challenges faced by other approaches and thus simplifies the subsequent sequencing readout.

For readout, this converted DNA mixture is then hybridized with a mixture containing two different "Molecular Beacons" (http://www.molecular-beacons.org/Introduction.html), each of which is a 12-mer oligo designed to complement either A or B. When free in solution, the molecular beacons produce only a very low background fluorescence due to self-quenching (see Fig. 1c). Similarly, when hybridized to the converted DNA, the molecular beacons produce only low background fluorescence because the universal quencher at one end of each beacon is in close proximity to the fluorophore of its nearest neighbor (Fig. 1c). Because the beacons do fluoresce briefly as they are stripped off the complementary converted DNA strand, readout is performed by sequentially stripping off the fluorescent 12-mer oligos one at a time by driving the converted DNA strand through a sub-2 nm diameter nanopore (i.e., a pore diameter that strips off the complementary fluorescently labelled 12-mer oligos[30]). The original DNA sequence is obtained by determining the color sequence of the photon bursts, where each pair of two successive bursts corresponds to a specific base. With high-density nanopore arrays[31], optical readout can facilitate massive parallelism, and a high resolution electron-multiplying CCD camera could be used to probe thousands of nanopores simultaneously. Because the nanopores require no on-chip electrical contacts, surface modification, or mechanisms to regulate the translocation process, improved nanofabrication methods may make it possible to develop such nanopores in very high density arrays. Nevertheless, at this time, fabricating high-density arrays of 1.7 – 2 nm diameter nanopores remains a significant challenge.

d. Measurement of transverse tunneling currents or capacitance as ssDNA is driven through a solid-state nanopore with embedded probes. It has been proposed that tunneling currents through nucleobases that are driven through a nanopore articulated with tunneling probes may be able to distinguish among the four nucleobases of ssDNA (Fig. 1d)[32-36]. Single bases should be resolved because it is the transverse tunneling current from an emitter probe tip of ≤1 nm diameter that generates the nucleobase-identifying signal rather than the nucleotide occupancy through the entire length of the nanopore channel. Although simulations of attainable base contrast when using tunneling measurements for nucleobase identification have presented encouraging but differing insights into the challenges this approach must address[32, 35, 37-40], the ability of a scanning tunneling microscope (STM) to reveal the atomic scale features of matter is well established[41].

As in a STM, electron tunneling currents can be in the nano-ampere range with appropriate probes[34, 42, 43]. The nanoamp electron currents would make it possible to read the nucleotides at a greater speed than is possible with the pico-ampere ionic currents that flow through a <3 nm-diameter nanopore. Although this approach using only robust solid-state components and electrical measurements may ultimately be the least expensive and fastest way of sequencing a genome, the major challenges that must be addressed include[40]: (a) The voltage bias and solution conditions that optimize contrast between the bases must be determined and maintained to provide unambiguous nucleobase identification; it is difficult to predict beforehand exactly what the electronic response of the detector will be to the different DNA bases, particularly in a fluid system such as is envisioned here. (b) The device must provide a mechanism to assure that each base will assume a reproducible orientation and position on the collector probe while it is being interrogated; tunneling currents are exponentially sensitive to atomic scale changes of orientations and distances. (c) Unidirectional translocation of the DNA must be controlled so that each nucleobase remains between the tunneling probes at least 0.10 msec to sample over inevitable noise and molecular motion; this translocation rate will assure that each nucleotide is sampled over a time period that is two orders of magnitude longer than required for a state of the art preamplifier[44] to sense nanoamp currents. (d) It remains to be shown whether the transverse current measurements can provide sufficient contrast to not only discriminate between the bases, but also provide a signal characteristic of the gaps between bases that could be used to distinguish each base from the next base in the unknown DNA sequence.

It has been proposed that the use of single walled carbon nanotubes (SWNT) may address challenges (b) and (c), and even challenge (a) if the carbon nanotube is appropriately functionalized[34]. Nanotubes bind and orient nucleobases in a specific manner[45] and the binding activation enthalpies per base lie in a range that can be modulated by temperature, ionic strength, or a voltage bias so as to control the DNA as it slides on the nanotube (Albertorio, F., Hughes, M.E., Golovchenko, J.A., & Branton, D. Base dependent DNA-carbon nanotube interactions: Assembly and disassembly of the hybrid. *J. Amer. Chem. Soc.* submitted (2008))..

Another inventive solution to the challenge of identifying each base using transverse tunneling currents is to form base specific hydrogen bonds between chemically modified metal electrodes and the nucleobases in the molecule that is to be sequenced. Ohshiro and Umezawa[42] showed that in a STM whose metallic probe had been modified with thiol derivatives of adenine, guanine, cytosine, or uracil, tunneling was significantly enhanced between a sample nucleobase and its complementary nucleobase molecular tip. Using a cytosine modified probe, they demonstrated base identification and electrical signals able to distinguish between TTTTTTTTGTTTTTTTTT and TTTTTTTGGTTTTTTTTT. Their work has led Lindsay to propose a nanopore reader bearing pairs of two chemically functionalized probes, one probe of each pair able to couple to the nucleotide's phosphate moiety while the other probe base-pairs with the nucleobase (Fig. 2)[43]. The nucleobases would be identified by the current-distance responses as the DNA moves through the nanopore and past the reader, rather than the tunneling current in a static configuration. The functional groups on each of four such readers -- A, C, G, or T -- would be designed to form a hydrogen bonded path when the cognate base translocates through the nanopore between the pair of probes[43]. Four such readers would be needed to generate a complete sequence, each one reading a duplicate strand. Synchronizing the translocation of four duplicate strands through four readers will pose a major challenge for this approach.

Electrostatic DNA detection and sequencing based on a metal-oxide-silicon (MOS) capacitor incorporated into the nanopore has also been proposed[46-48]. Using the electron beam of a transmission electron microscope[46, 49], a nanopore is fabricated in a membrane consisting of two layers of doped silicon, separated by a 5 nm thick insulating SiO_2 layer. As DNA translocates through the pore, variation of the electrostatic potential in the pore polarizes the capacitor, and voltage fluctuations on the two silicon layers are measured. Simulation results have demonstrated that A, C, G, and T give distinct capacitance signals and that the instrument could, in principle, resolve single-base substitutions in a DNA strand[50]. In an early trial of this approach, a voltage signal associated with DNA translocation was detected with one such device, but the time resolution was inadequate to distinguish between nucleotides[46]. The control of DNA velocity and orientation during translocation would also be a major challenge in this approach.

Achieving the promise of long reads

One of the compelling potential advantages of nanopores for sequencing is the promise of long reads. Because the nanopore sensor reads molecules sequentially, base by base, as they thread through the pore, its fundamental strength is that the accuracy of a base call at one instance in time does not depend on the prior history of the system. In principle, the length of DNA that could be read with a nanopore is limited only by the practicalities of avoiding shearing during sample preparation and of limitations yet to be explored with respect to capture and threading of exceptionally long molecules through individual pores. To date, it has been demonstrated that lengths of ssDNA on the order of 25 kb have been threaded through biopores (A. Meller and D.Branton – personal communication) and up to 5.4 kb lengths of ssDNA have been threaded through solid-state nanopores[22, 51]. A unique feature and promise of nanopore technologies is therefore that if a detection scheme is developed that allows reading of a few bases on the fly during unidirectional translocation of the DNA strand through a pore, then the extension of the technology from reading a few bases to reading thousands of bases should be straightforward. While the expected accuracy of the read is yet unknown, insertions, deletions, and other sequence errors will not compromise the read length as de-phasing is not an issue in independent single molecule reads. Sufficient averaging or depth of coverage could then reach any desired level of accuracy, as long as sequence errors are random rather than systematically sequence- or position-dependent.

Furthermore, given the high throughput available and anticipated in short reads from current and next generation sequencing instruments, it may be that nanopores will play a role in providing an assembly scaffold of very long reads at low accuracy to facilitate assembly of short read sequences. A hybrid combination of low accuracy long reads and high coverage, high accuracy short-reads may be one path to inexpensive and rapid *de novo* sequencing. Ultimately, both of these two classes of data could be collected from nanopores.

Considering the central importance of long reads to the future of sequencing methods, additional work needs to be undertaken to determine the limitations of nanopores in capturing and sequentially translocating very long fragments. Very high throughput detection of short single-stranded oligomers (<50 nucleotides) can be achieved[5, 52], and for these the measured concentration-normalized capture rate constant in α-hemolysin[15] is ~5.8 oligomers $(sec\ \mu M)^{-1}$. Since the capture rates depend on the solution molarity and since the molar concentration (or concentration of fragment ends) must be limited to reasonably low *w/v* concentrations of long fragments to avoid excessive viscosities, it remains to be seen whether ~50 kb fragments can be captured and threaded through small nanopores at reasonable rates. Although several publications using 3 – 6 nm diameter pores show a reasonable number of capture-translocations per minute can be achieved with native 3 - 10 kb or kbp fragments of native ssDNA or dsDNA when the source chamber concentrations are in the range of 10 – 20 nM[22, 53, 54], the precise capture rate constants were not determined. Although full length lambda DNA (48 kbp) has also been captured and translocated through nanopores[49, 55, 56], achieving high coverage of such long reads might be most efficiently achieved by using the recently demonstrated trapping and recapture ability of a nanopore[54]. The discovery and accompanying theory that show how the same molecule that has translocated all the way through a nanopore can be recaptured and interrogated multiple times are particularly relevant to implementing accurate sequencing. If the initial passage of an individual molecule provides an incomplete or poor quality read-out, real-time software could drive that molecule back to be re-sequenced multiple times without having to re-sample the entire genome.

Controlling DNA motion and translocation in a nanopore

The high speed at which DNA translocates through nanopores[4, 5, 53] holds the promise of ultra-fast sequencing; but the rate at which unconstrained DNA moves through these pores is also the Achilles' heel of many approaches because it implies unattainable measurements of very small currents. At 120mV, DNA typically translocates through an α-hemolysin pore at a rate of ~1 - 20 μs per nucleotide[57]. This pushes the detector bandwidth requirements to the Mhz region which precludes the measurement of pico-ampere steps in ion current.

The situation is worsened by diffusion as the DNA is electrophoretically driven through the pore. Stochastic DNA motion, which is reflected in the broad distribution of transit times in both experimental[1, 4-6, 22, 49, 51, 53, 55, 58], and theoretical studies[19, 59-65], can, as indicated above, generate uncertainty in the number of bases that have passed through the nanopore. Furthermore, non-specific interactions between the translocating DNA and the nanopore's surface may be dominated by discontinuous stick-slip phenomena[66]. Variability in the nature and frequency of interactions can give rise to non-Poisson distributions of escape times[67-69, 70], such that the translocation time for two identical molecules can differ by orders of magnitude[1, 4-6, 49, 53, 58, 69]. If some of the nucleotides in a DNA strand slip between the probing elements of a nanopore in time periods that are significantly less than the average, these fast-translocating nucleotides may be missed.

Thus, a key challenge to DNA sequencing with nanopores is to find methods to slow down and control DNA translocation and reduce the fluctuations in translocation kinetics due to pore-surface interactions. DNA translocation speeds can be reduced somewhat by decreasing temperature[5, 71], or increasing solvent viscosity[72], but these methods do not reduce the variations in the translocation dynamics due to DNA-pore interactions[67-70, 73]. Substantial reductions of the translocation rate can be achieved with processive DNA enzymes[74-76], which limit the translocation rate by binding to the DNA strand and preventing it from moving into the narrow confines of the pore faster than the enzyme processing rate; or by successive unzipping of DNA oligos, which then becomes the rate limiting step for the translocation process[27, 28]. These processing rates are typically on the time scale of a few milliseconds per base and can be controlled through ion concentrations[75-77], temperature, and the voltage bias through the nanopore.

Ultimately, eliciting a distinct electrical signal from the space between bases to provide a clear count of the number of bases that translocate would be ideal. Such signals would greatly facilitate further analysis of translocation kinetics and base dwell time distributions so that the detection system developers can determine the required bandwidth and performance specifications of their systems. But until such signals are available, a detailed understanding of, and methods to control, the kinetics of DNA strand translocation through a narrow pore need to be obtained. Fabricating nanopores provides the opportunity for generating nanopores with tailored surface properties that could both regulate DNA-pore surface interaction[78] and reduce noise[79, 80]. Ultimately, a combination of methods to control translocation rate and DNA-pore interaction will need to be coupled to high-bandwidth, low-noise detection to achieve the fast sequence analysis that is the promise of many nanopore approaches.

Biopore stability and fabrication of solid-state pores

The hemolysin heptamer, which until now has been the usual protein that is used to form nanopores in lipid bilayers, is remarkably stable[3]. The primary instability therefore arises from the support, typically a fluid lipid bilayer, which is difficult and time consuming to set up.

A bilayer encapsulated between two thin layers of agarose with a single inserted α-hemolysin pore is sufficiently stable to be sealed in teflon film and stored for weeks before use[81]. A single α-hemolysin pore could be introduced in each element of an array of such bilayers using agarose-tipped plastic or glass probes[82, 83]. Another approach to stabilizing bilayers is to use nano-scale, rather than micron scale, apertures. Bilayers across 100 - 1,000 nm diameter apertures at the end of glass capillaries coated with a specially formulated silanizing agent have been shown to be stable for over two weeks[84].

Very stable, functionally useful solid-state nanopores can be fabricated in silicon nitride, silicon oxide, or metal oxides, using ion beam sculpting[53], e-beam drilling[85] and atomic layer deposition[79], but generating arrays of a large number of uniform solid-state nanopores with diameters in the 1.5-2.0 nm range remains a daunting task, particularly for a research laboratory. Articulated nanopores with buried nanotube probes for tunneling measurements have been realized, but the current fabrication methods are so tedious, slow, and manpower expensive they often cannot be used to provide even the limited number of such nanopores required for research scale development. There is little doubt that the accelerating rate of discovery in the field of nano-scale electronics and the proven ability of the electronics community to develop mass-production strategies for high value components will be able to master the nano-scale science required to fabricate massive nanopore arrays. But until such time as nanopore sequencing in any form is shown to be feasible and valuable, nanopore sequencing researchers face the challenge of using only research scale facilities rather than those that are to be found, or could be developed, in a specialized, mass production plant.

For some nanopore applications, the ultimate stable pore is likely to be a hybrid between a solid-state pore and α-hemolysin. This might involve producing a ~5 nm pore in a synthetic membrane such as silicon nitride, then capturing an α-hemolysin heptamer in the pore in the absence of a lipid bilayer. Should this prove possible, the resulting nanopore is likely to be both highly reproducible and indefinitely stable.

Conclusion

A number of advantages are offered by nanopore sequencing if it can be achieved. The most important are minimal sample preparation, sequence readout that does not require nucleotides, polymerases, or ligases, and the potential of very long read lengths (>10 – 50,000 nt). It follows that a successful nanopore sequencing device will provide a tremendous reduction in costs and should achieve the $1,000 per mammalian genome goal set by NIH. The instrument itself will be relatively inexpensive, and the time required for 6-fold coverage could be as little as one day if 100 nanopores having the required integrated microfluidics and electronic probes can be fabricated into each sequencing chip. But significant challenges remain. An important short-term challenge is to slow DNA translocation from microseconds per base to milliseconds, and several recent studies indicate that this can be achieved by using DNA-processing enzymes. If a future instrument incorporates the hemolysin heptamer, it will also be necessary to establish a stable support of some kind. Again, there is recent progress toward this end, though in the longer term it seems likely that synthetic solid-state nanopores will be preferred for a commercial instrument. Electronic sensing based on either tunneling probes or a capacitor is being tested for its ability to detect a DNA strand during translocation, but whether this is possible remains to be demonstrated. A continuing concern is that stochastic motion of the DNA molecule in transit will increase signal noise in such a sensor, thereby reducing the potential for single-base resolution. All that said, the advantages of nanopore sequencing are so attractive that work will continue unless a fundamental limitation is discovered. So far, no such limitation has emerged, and the progress toward the goal of fast, inexpensive nanopore sequencing has been both impressive and encouraging.

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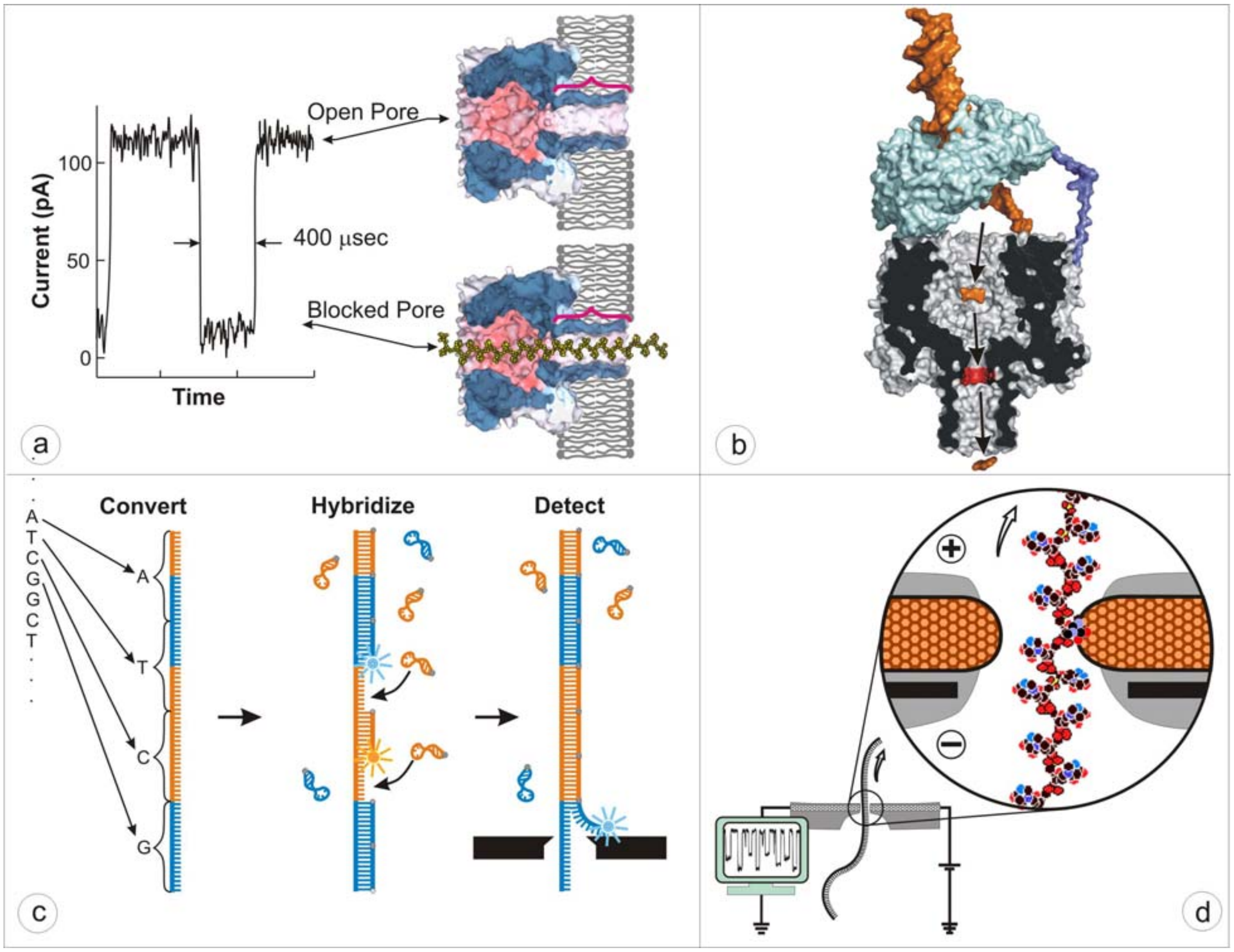

Figure 1. Approaches to Nanopore Sequencing. **(a)** Strand-sequencing using ionic current blockage. A typical trace of the ionic current amplitude *(left)* through an α-hemolysin pore clearly differentiates between an open pore *(top right)* and one blocked by a strand of DNA *(bottom right)*, but cannot distinguish between the ~12 nucleotides that simultaneously block the narrow transmembrane channel domain *(red bracket)*. **(b)** Exonuclease-sequencing by modulation of the ionic current. An exonuclease (pale blue) attached to the top of an α-hemolysin pore through a genetically encoded (deep blue), or chemical, linker sequentially cleaves dNMPs (gold) off the end of a DNA strand (in this case one strand of a double-stranded DNA). A dNMP's identity (A, T, G, or C) is determined by the level of the current blockade it causes when driven into an aminocyclodextrin adapter (red) lodged within the pore. After a few milliseconds, the dNMP is released and exits on the opposite side of the bilayer. **(c)** Nanopore sequencing using synthetic DNA and optical readout. Each nucleotide in the target DNA that is to be sequenced is first converted into a longer DNA strand composed of pairs of two different code-units (colored orange and blue for illustration); each code-unit is a 12-base long oligomer. After hybridizing the converted DNA with "Molecular Beacons" that are complementary to the code units, these "Beacons" are stripped off using a nanopore. The sequence of the original DNA is read by detecting the discrete short-lived photon-bursts as each oligo is stripped. **(d)** Strand-sequencing using transverse electron currents. DNA is driven through a nanopore articulated with embedded emitter and collector tunneling probes *(orange)* and a backgate *(black)*. The amplitude of the tunneling currents that traverse through the nucleotides is expected

to differentiate each nucleobase as the DNA is electrophoretically driven through the pore (arrow).

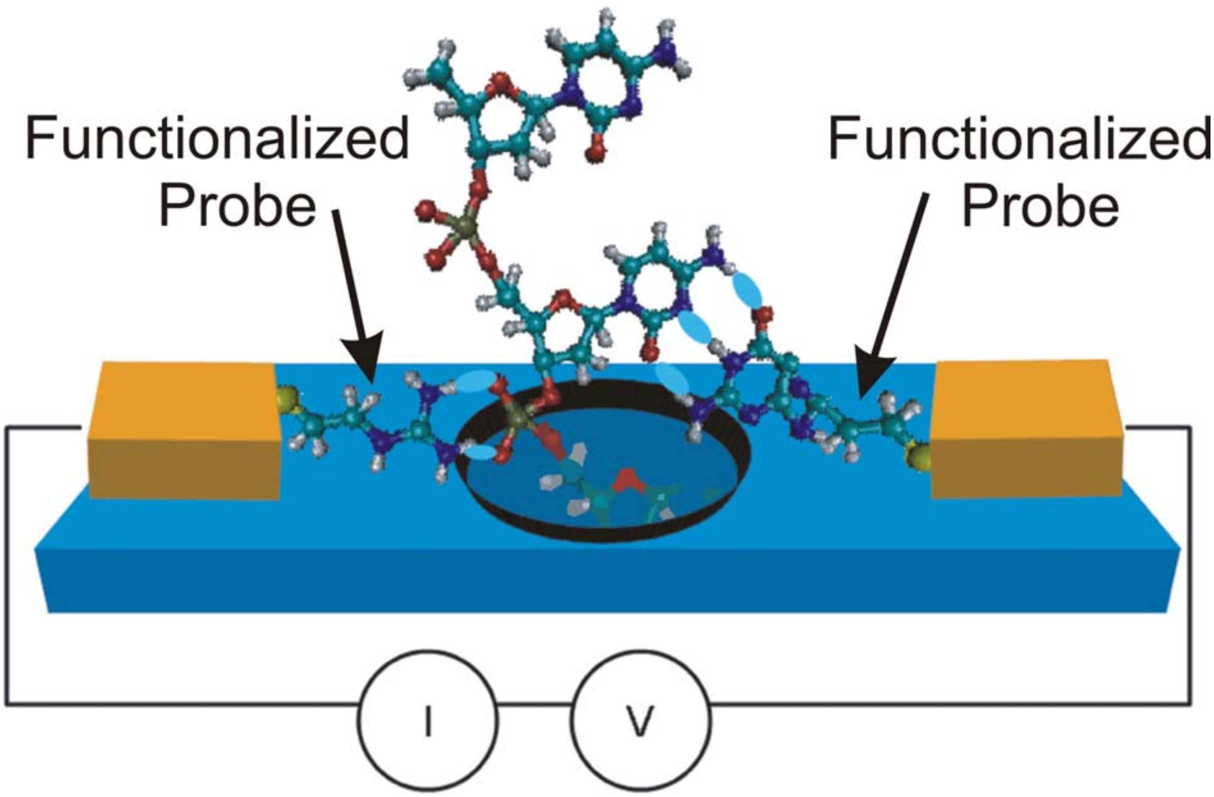

Figure 2. A nanopore reader with chemically functionalized probes. As a strand of DNA emerges from a nanopore, a "phosphate grabber" on one functionalized electrode and a "base reader" on the other electrode form hydrogen bonds (light blue ovals) to complete a transverse electrical circuit through each nucleotide as it is translocated through the nanopore.