

Three decades of nanopore sequencing

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A long-held goal in sequencing has been to use a voltage-biased nanoscale pore in a membrane to measure the passage of a linear, single-stranded (ss) DNA or RNA molecule through that pore. With the development of enzyme-based methods that ratchet polynucleotides through the nanopore, nucleobase-by-nucleobase, measurements of changes in the current through the pore can now be decoded into a DNA sequence using an algorithm. In this Historical Perspective, we describe the key steps in nanopore strand-sequencing, from its earliest conceptualization more than 25 years ago to its recent commercialization and application.

Nanopore sequencing (**Box 1** and **Fig. 1**) has its origins in several laboratories during the 1980s (**Fig. 2**). In 1989, one of us (D.D.) jotted a seemingly implausible idea in his notebook (**Fig. 3**), suggesting that it might be possible to sequence a single strand of DNA being drawn through a membrane's nanoscopic pore by electrophoresis. Around the same time, George Church's interest in scaling up DNA sequencing for the Human Genome Project led him to propose that an electronically monitored phage motor embedded in a bilayer might provide sequence information upon processing of double-stranded (ds) DNA. Hagan Bayley's interest in membrane proteins and the structure and assembly of oligomeric transmembrane protein pores, such as α -hemolysin, also led him and his colleagues to ask if pores could serve as biosensors of molecules and cations¹.

Whereas Church decided to pursue other high-speed sequencing methods, two of us (D.D. and D.B.) had independently noted publications suggesting that α -hemolysin from *Staphylococcus aureus* could form transmembrane channels wide enough (>1.2 nm) to accommodate a single strand of DNA². Furthermore, D.D. had listened to talks by John Kasianowicz at two scientific conferences and knew that he was working with α -hemolysin. In late 1993, D.D. visited Kasianowicz at the US National Institute of Standards and Technology (NIST) to test whether it was possible to electrophorese homopolymers of RNA through an α -hemolysin pore. Kasianowicz had already gained much experience working with α -hemolysin in collaboration with Bayley,

and together with Sergey Bezrukov at the US National Institutes of Health (NIH), had established the conditions that were necessary to avoid spontaneous gating (pore closure) of the α -hemolysin channel³. This was important, because spontaneous gating would have hindered, or confused, observations of nucleic acid translocation through the nanopore. Kasianowicz was also collaborating with Bezrukov to investigate the effect of polyethylene glycol on pore conductance and, consistent with earlier reports², found that a pore radius of ~1.1 nm accounted for their results⁴.

In initial experiments, D.D. and Kasianowicz worked with a single α -hemolysin channel inserted into a lipid bilayer that separated two buffered KCl-filled compartments. RNA homopolymers (polyuridylic or polyadenylic acid) were then added to the *cis* side of the membrane. Immediately after addition of the polymers, the first encouraging results were observed. When a positive voltage bias greater than 80 mV was applied to the *trans* compartment, numerous blockades—transient, millisecond time-scale reductions of the ionic current through the α -hemolysin channel—appeared. No blockades were detected when the *trans* compartment was negatively biased. This was expected because the polyanionic RNA in the *cis* chamber would be inhibited from entering the nanopore when the *trans* side was negative. These preliminary results were consistent with the hypothesis that a voltage applied across the hemolysin pore could electrophorese single molecules of RNA through the α -hemolysin channel.

In February 1994, D.D. and D.B. together visited Kasianowicz to repeat and extend the earlier results. Further experiments showed that the average blockade duration was larger for longer RNA polymers compared with shorter polymers. This led to the hypothesis that the duration of the blockades reflected the length of time required for the polymer to translocate through the nanopore from the *cis* chamber to the *trans* chamber. Shortly after these initial experiments were completed, D.B. and D.D. received a personal communication from Langzhou Song and colleagues⁵, who were investigating the crystal structure of α -hemolysin. The early evidence from X-ray diffraction indicated that the channel diameter in the α -hemolysin pore was between ~1.5 nm and ~2.5 nm. This information facilitated well-controlled translocation experiments. If the narrowest diameter of the channel was only ~1.5 nm, it was reasoned that ssDNA, but not dsDNA, polymers would be able to translocate from *cis* to *trans*.

This hypothesis was tested by Kasianowicz at NIST using a mixture of ssDNA and dsDNA controls designed and synthesized by D.B.'s group at Harvard. The mixture of single- and double-stranded synthetic DNA molecules was sent to Kasianowicz, who placed it on the *cis* side of a membrane that contained a known number of α -hemolysin pores. (In some experiments, more than one pore was inserted in the membrane to more accurately quantify the amounts of DNA translocated to the *trans* side.) The blockades produced in a given time interval were counted at

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Box 1 Nanopore sequencing—the basics

In nanopore sequencing, the nanopore serves as a biosensor and provides the sole passage through which the ionic solution on the *cis* side of the membrane contacts the ionic solution on the *trans* side (Fig. 1a). A constant voltage bias (*trans* side positive) produces an ionic current through the nanopore and drives ssDNA or ssRNA in the *cis* chamber through the pore to the *trans* chamber. An enzyme (e.g., a polymerase or helicase) is tightly bound to the polynucleotide such that its step-wise movement controls and ratchets the nucleotides through the small-diameter nanopore, nucleobase by nucleobase. Because the ionic conductivity through the narrowest region of the nanopore is particularly sensitive to the presence of the nucleobase's mass and its associated electrical field, the ionic current levels through the nanopore (Fig. 1b) reveal the sequence of nucleobases in the translocating strand. Thus, the narrowest aperture region of the nanopore serves as its sensing region. The diagram and current trace shown in Figure 1 are from an experiment carried out in a horizontal bilayer apparatus⁷ using an MspA nanopore and a helicase enzyme that steps along the polynucleotide, thus ratcheting the DNA through the nanopore one nucleobase per step.

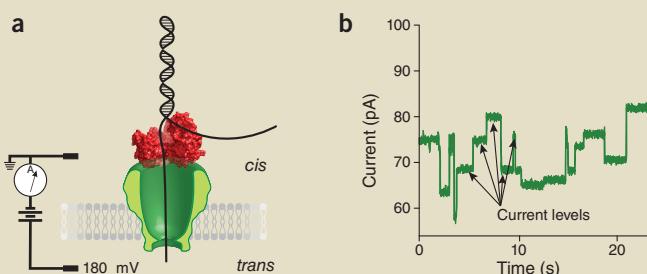


Figure 1 Nanopore sequencing. (a) Two ionic solution-filled chambers are separated by a voltage-biased membrane. A single-stranded polynucleotide (black) is electrophoretically driven through an MspA nanopore (green) that provides the only path through which ions or polynucleotides can move from the *cis* to the *trans* chamber. Translocation of the polynucleotide through the nanopore is controlled by an enzyme (red). (b) Portion of a record showing the ionic current through a nanopore measured by a sensitive ammeter. In nanopore strand-sequencing, the stepping rate is usually 30 bases per second, but this experiment was carried out using exceptionally low concentrations of ATP to slow the helicase activity, thereby increasing the duration of each level to illustrate the resolution that can be achieved.

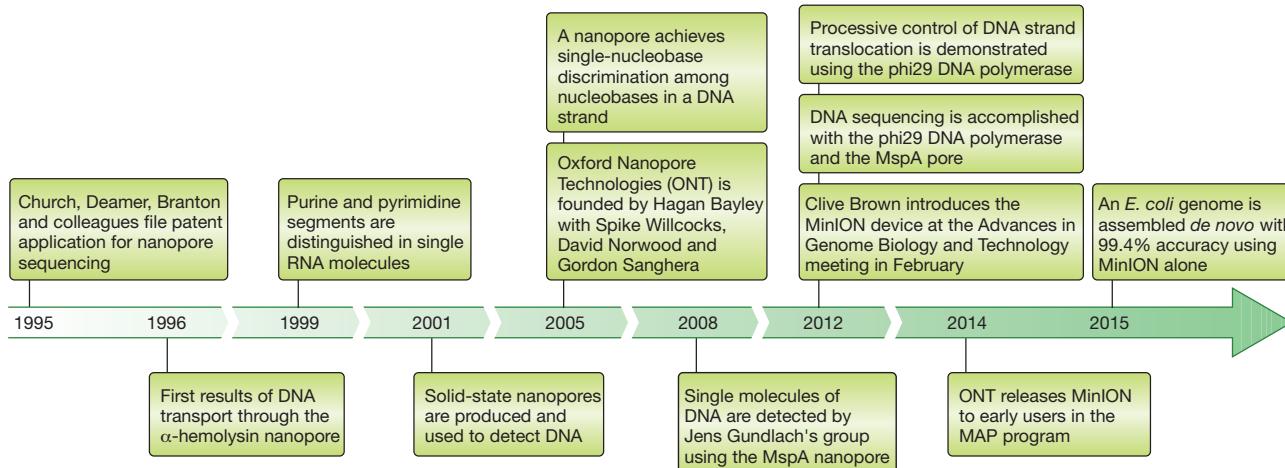
NIST, and quantitative PCR techniques were used at Harvard to quantify the number of DNA molecules that passed to the *trans* side of the pore.

When the results were tallied it was clear that, as predicted, ssDNA but not dsDNA molecules passed through the pore. Furthermore, the number of ionic current blockades matched the number of translocated ssDNA molecules. These results provided the first convincing evidence that single-stranded nucleic acid polymers can be captured by the electrical field around a voltage-biased pore and can be electrophoresed through the pore's nanoscopic aperture.

Publication of the crystal structure of α -hemolysin⁵ shortly after the experiments showing ssDNA translocation through a nanopore⁶ made it possible to interpret the translocation results in detailed molecular terms. The experimental demonstration of oligomer transport and single-molecule detection was the first step suggesting that nanopore sequencing might be feasible, but many other experiments were needed to determine whether nanopore sequencing would actually be possible.

Distinguishing purine and pyrimidine segments

The next important question was whether the α -hemolysin nanopore could discriminate between purine and pyrimidine bases. In 1997, M.A. moved from NIH to the University of California, Santa Cruz, and began to work on this question. The approach was to synthesize an ssRNA oligomer comprising 70 cytosine nucleotides followed by 30 adenine nucleotides. When the 100 mer was added to the *cis* side of the α -hemolysin pore, two distinct current levels were clearly visible in each blockade. It turned out that the variations in current levels were not due to different current-blocking properties of individual nucleobases, but instead to the fact that the cytosine-containing portion of the molecule forms a narrow single-stranded helix that can translocate through α -hemolysin as a helix, whereas the adenine portion forms a larger-diameter helix that must be unwound before the single strand can translocate. Even though individual nucleobases were not resolved, the results showed that an α -hemolysin nanopore can provide information about oligomeric structure or composition⁷.



Marina Corral Spence/Nature Publishing Group

Figure 2 Milestones in nanopore DNA sequencing.

HISTORICAL PERSPECTIVE

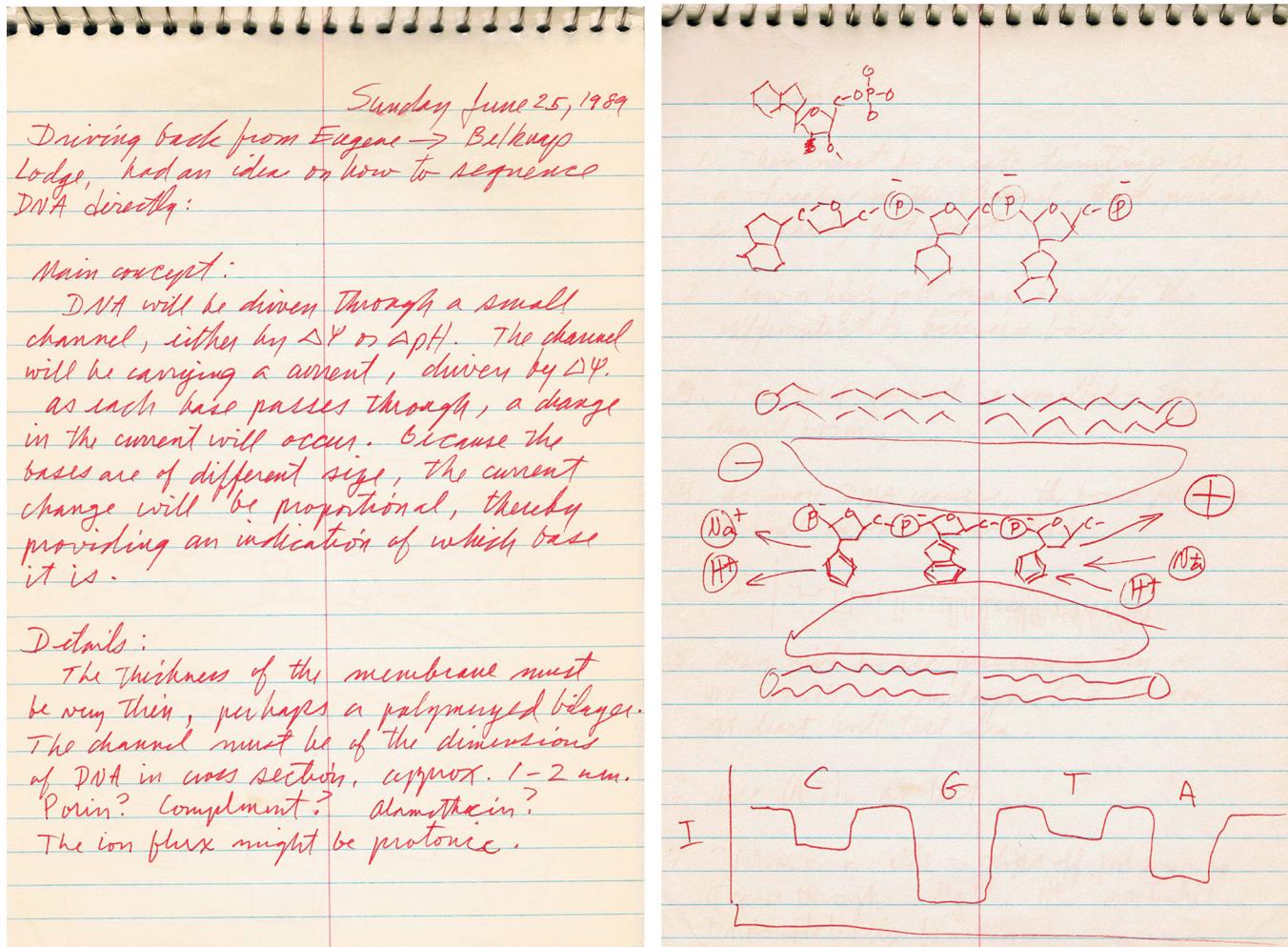


Figure 3 D.D.'s notebook. Two pages of a notebook in which D.D. sketched the original nanopore sequencing concept are shown (dated June 25, 1989). The sketch shows three bases of a DNA strand being drawn through a nanopore by an applied voltage. The voltage also produces an ionic current, and the expected base-specific electrical readout is shown below with each base affecting the current in such a way that the sequence of bases can be determined.

The ability to distinguish between purine and pyrimidine strands in a single RNA molecule generated growing interest in nanopore analysis of nucleic acids in many laboratories. It was soon shown that the blockade signals can distinguish between several identical length ssDNA polynucleotides that differ only in their oligomeric sequence⁸. Further highlighting the sensitivity of the nanopore detection system, later experiments confirmed that the blockade signals can distinguish between polynucleotide strands that traverse the nanopore 3' to 5' from those traversing 5' to 3' (refs. 9–11). This accounted for the earlier puzzling observation that samples containing only one type of polynucleotide consistently produced two types of blockades^{6,8}.

The fundamental question of whether a nanopore is capable of recognizing DNA with single-nucleobase resolution remained unanswered until 2005, when Bayley's and Ghadiri's groups together found that a single adenine nucleotide substitution at a specific location in a strand of poly-d(C) can be distinguished from all the cytosines by its characteristic effect on the ion conductance of α -hemolysin¹². These initial experiments were soon followed by further experiments showing that all four DNA nucleobases can be distinguished from each other, regardless of whether they are located in an otherwise homopolymeric DNA strand or in a heteropolymeric strand¹³. This and subsequent demonstrations of a nanopore's ability

to discriminate among and identify individual nucleobases in a heteropolymeric strand of DNA^{14,15} represented critical steps toward nanopore strand-sequencing. But all of these experiments depended on the DNA strands being immobilized in the α -hemolysin pore using a terminal hairpin or a biotin-streptavidin complex whose width prevented the strand from completing its electrophoretically driven translocation through the small diameter of the pore. Because the DNA strands were stably immobilized, a nucleobase remained at a fixed position in the pore's sensing region. Compared with a freely electrophoresing strand, the immobilized strand greatly improved the signal-to-noise ratio, making it possible to resolve an individual nucleobase. The results pointed to the necessity of ratcheting the strand through the pore so that each step of the ratchet allowed sufficient time to identify the next nucleobase in the strand's sequence. This proved to be a difficult challenge.

Processive control of DNA translocation

The pace of progress increased substantially in 2003 when the National Human Genome Research Institute announced the Revolutionary Genome Sequencing Technologies project. This program, directed by Jeffery Schloss, eventually made over \$50 million available in grants to explore methods for sequencing the three billion base pairs of the

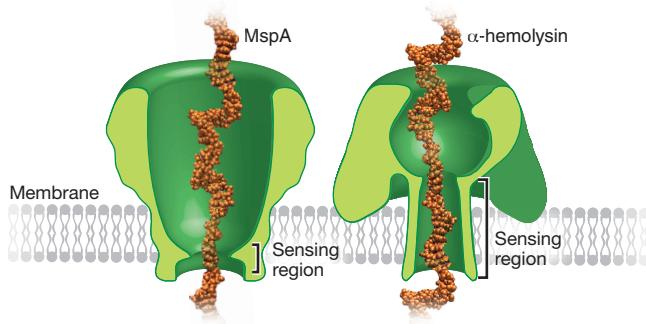


Figure 4 Sensing regions in MspA and α -hemolysin. The sensing region in MspA is much shorter than in α -hemolysin.

human genome for \$1,000 or less. Nanopore sequencing was an obvious candidate, and multiple research groups were soon supported.

Early on, it was clear that even the smallest voltage bias that drives a DNA strand through the pore moves each base through the sensing region in less than \sim 1–10 μ s. Nucleobase identification using the small picoampere current modulations measured in a nanopore requires a higher signal-to-noise ratio than can be achieved in <10 μ s. Achieving the higher signal-to-noise ratio required that each base reside in the nanopore's sensing region for >100 μ s. Attaining such slow ratchet-like ssDNA transport through the pore was one of the key feats that made nanopore strand-sequencing a reality.

The successful strategy, first proposed in 1998, was to use processive enzymes to control DNA movement through the nanopore¹⁶. Initial experiments demonstrated that, absent catalysis, binding either the Klenow fragment¹⁷ or the *Escherichia coli* exonuclease I¹⁸ to the DNA strands slowed their translocation through the α -hemolysin pore by orders of magnitude. Still, these and other methods to slow translocation through the pore without true nucleobase-by-nucleobase ratcheting proved to be insufficient.

Subsequent experiments by Ghadiri and colleagues¹⁹ progressed further step toward ratcheting. Using an A family DNA polymerase, Ghadiri's group showed that primer strand elongation could be detected with single-nucleotide resolution by alternating the voltage applied to the *trans* side of the membrane between -30 mV (an elongation mode wherein the 3'-OH of the primer is accessible and binds to DNA polymerase in bulk phase) and $+30$ mV (a monitoring mode wherein the polymerase usually dissociates from the DNA, thereby placing the extended primer/template junction at the detecting aperture in α -hemolysin). Attempts to sequentially ratchet DNA through the nanopore sensor driven by A family polymerases were limited by the fact that these enzymes dissociate under the load imposed by the applied voltage required to drive DNA through the nanopore. At best, only two consecutive nucleotide additions were observed^{20,21}.

Experiments using phi29 DNA polymerase (phi29 DNAP) were more successful. Under conditions where catalysis was prevented, binary phi29 DNAP–DNA complexes were retained on the α -hemolysin pore $>10,000$ times longer than were binary Klenow fragment–DNA complexes²². Subsequent experiments used ‘blocking oligomers’ that favored nanopore-controlled activation of phi29 DNAP–DNA complexes while preventing DNA polymerization in the bulk phase of the *cis* compartment²³. Up to 500 synthetic DNA strands could be processed by a single nanopore. In most of those cases, 25 nucleotides of the captured template strand were examined one by one in both the 3'-to-5' and 5'-to-3' direction. This level of translocation control was an essential component in five publications that demonstrated practical nanopore DNA strand sequencing^{24–28}.

Engineering protein nanopores to discriminate nucleobases

It was apparent early in nanopore sequencing development that the wild-type α -hemolysin pore was not optimal as a DNA sequencing sensor. The crystal structure of α -hemolysin revealed it to be a mushroom-shaped heptamer with a 5-nm stem whose inside channel diameter varies from \sim 1.4 nm to \sim 2.4 nm, depending on the volume of the amino acid side chains protruding into the channel cylinder⁵. Although there are three particularly good sensing spots within the 5-nm-long stem^{14,15}, experiments with differing length oligomers showed that all of the \sim 12 nucleobases that occupy the 5-nm-long stem during strand translocation are sensed and reflected in the current level of a blockade²⁹. Having \sim 12 nucleobases contributing to the measured current levels obscures identification of individual bases. One solution, proposed by Bayley and colleagues^{13–15}, was to engineer α -hemolysin to create a nanopore with a sensing region shorter than 5 nm. They tested a series of modified channels for this purpose. Using synthetic DNA strands immobilized in the modified pores, they demonstrated that the four canonical nucleotides could be individually resolved in DNA strands^{13–15}.

The second solution, initially suggested in the first nanopore sequencing patent¹⁶, was to find a nanopore with a relatively short sensing aperture. Early work in Roland Benz's laboratory had characterized the biophysical properties of MspA^{30,31}, a porin from *Mycobacterium smegmatis*, whose crystal structure was later shown to have a funnel-like geometry that narrowed to a \sim 1.2 nm diameter, \leq 0.6-nm-long aperture³². This sensing region was about the same diameter as in α -hemolysin but nearly an order of magnitude shorter (Fig. 4). Jens Gundlach at the University of Washington predicted, correctly, that MspA would therefore be a better detector of individual nucleobases in a DNA strand than α -hemolysin (personal communication).

Although initial tests indicated that ssDNA could not be detected by, and would not translocate through, wild-type MspA, engineered mutants of MspA in which negatively charged amino acids lining the wild-type pore were replaced by neutral or positively charged amino acids were fully capable of electronically detecting and characterizing single molecules of ssDNA³³. Using these mutant MspA constructs, Gundlach's group then established that the mutant nanopores

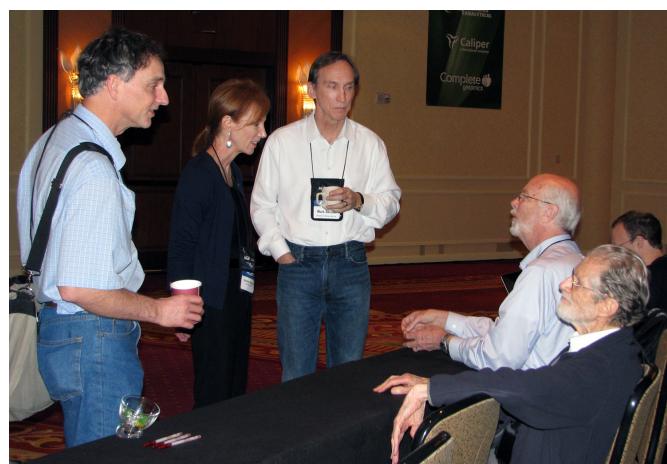


Figure 5 Nanopore researchers at the Advances in Genome Biology and Technology Meeting, Marco Island, Florida, 2012. Left to right: (standing) Jens Gundlach, Kristen Stoops (ONT) and M.A.; (seated) D.D. and D.B. The photograph was taken shortly after Clive Brown's plenary seminar introducing an early prototype of the MinION sequencer developed by ONT.



Figure 6 Hagan Bayley (center) conversing with Jene Golovchenko (Harvard, left), Muthukumar Murugappan (University of Massachusetts, Amherst, back to camera) and Reza Ghadiri (Scripps Research Institute) at the April 2011 meeting of NHGRI Principal Investigators.

discriminated between all four different nucleobases and achieved significantly larger ionic current differences between nucleobases than did α -hemolysin nanopores³⁴.

Reading DNA using phi29 DNAP coupled to the MspA nanopore

The two essential components of a functioning nanopore sequencing device—processive control at single-nucleotide precision and good discrimination among nucleobases—were now in place, and it was logical to put the two together. In October 2011, Max Cherf traveled from UC Santa Cruz to Seattle with phi29 DNAP, blocking oligomers and a synthetic DNA substrate that he had successfully used to monitor DNA displacement through the α -hemolysin pore. He and Liz Manrao in the Gundlach laboratory at the University of Washington used phi29 DNAP translocation control in combination with an engineered MspA nanopore. They were soon rewarded with their first convincing sequential reads of DNA template strands. The ionic currents they recorded revealed progressive ionic current levels that reflected a ‘C-A-T’ trimer repeat within the template strand. These results were reported by the two groups in a poster at the Advances in Genome Biology and Technology (AGBT; Marco Island, FL, USA) meeting in February 2012 (Fig. 5), followed shortly thereafter by publication of the conceptual and research details^{23,24}. Since 2012, nucleobase calls using the phi29 DNAP–MspA combination have been documented for both synthetic strands^{23–26,28} and native bacteriophage DNA²⁷, thus establishing the feasibility of DNA nanopore sequencing.

The academic experiments described in the preceding text were accomplished using single nanopores in lipid bilayer membranes. Development of a high-throughput, widely accessible nanopore sequencing device would require sustained effort from the private sector.

Delivering nanopore sequencing to the genomics community

In 2003, Bayley returned to his native England to become professor of chemical biology at the University of Oxford. Together with a team experienced in the development and commercialization of intellectual property, biotech and point-of-care diagnostics, Bayley joined Gordon Sanghera, Spike Willcocks and David Norwood to found ONT in 2005 (Fig. 2). ONT licensed DNA strand sequencing patents in 2008, and embarked on a focused internal strand sequencing effort in 2010. This culminated in the ONT MinION device first described by ONT’s Chief

Technology Officer Clive Brown during a plenary talk at the AGBT Meeting in 2012. Two years later, in June, 2014, ONT released individual MinION devices in a large-scale collaborative MinION Access Program (MAP).

The MinION is compact and portable, weighing about 100 g and controlled by a laptop computer with software developed by the company. A flow cell in the device has 2,048 individual protein nanopores, each embedded in a separate stable membrane, of which 512 are selected for monitoring by the software. Samples are prepared by adding a hairpin loop to one end of double-stranded genomic DNA or cDNA fragments. During analysis of the sample, DNA molecules in the *cis* compartment of each nanopore are continuously captured as a helicase enzyme unwinds the double-stranded DNA into a continuous single strand that is drawn through the pore to produce an electrical signal containing sequence information (Fig. 1). Because the DNA molecules are hairpins, both strands of the dsDNA molecule can be analyzed—a so-called 2D read—to substantially improve base-calling accuracy. The electrical signals yield long continuous DNA sequence reads with lengths being a function of the fragmentation method. In the MAP user group, common fragment lengths are in the range of 6,000 to 48,000 bases³⁵.

If commercial nanopore sequencing is to succeed, it must compete with other high-speed sequencing methods both in terms of accuracy and cost. Reading accuracy will depend in part on the chemistry and enzyme used to ratchet the DNA through the nanopore in a controlled fashion. There have now been several MinION chemistry versions and numerous base-calling algorithm updates that have improved device performance. For example, in the Nanopore Research Group at the University of California, Santa Cruz, the average identity observed (the proportion of bases in a single-molecule read aligned to matching bases in a reference sequence) ranged from 66% in June 2014 (release R6.0) to 85% in November 2014 (release R7.3)³⁵ and to >90% in unpublished results from some MAP researchers.

In a recent study published online³⁶, an international consortium of five laboratories associated with MAP compared results from the same strain of *E. coli* K-12, using identical protocols for extraction, shearing and library preparations. They reported consistent results from the five laboratories, with 2D read accuracies of 92–93%. Recently developed algorithms for nanopore sequencing^{35,37–39} are able to analyze and combine multiple reads of the same DNA sample and achieve higher accuracies, as expected. As a result, nanopore sequencing has already been used to generate scaffolds that resolve complex and often unfinished regions of a genome, such as the position of repetitive insertion sequences^{35,40}.

As with routine shotgun sequencing, high-coverage read depth yields more accurate MinION sequencing data. For example, single-nucleotide variant calls approaching 99% accuracy were achieved using a maximum likelihood algorithm to align M13mp18 genome segments³⁵. This advance allowed Jain and colleagues³⁵ to establish copy number of the CT47 (cancer/testis) gene family in a previously unresolved segment of human chromosome X cloned into a bacterial artificial chromosome. Another early test of the MinION sequencing accuracy used shotgun analytical methods to generate a whole-genome data set from *E. coli* K-12 (ref. 41).

In many of the early studies testing the MinION on viral and microbial genomes, a reference sequence was required for genome assembly and read accuracy. More recently, improved algorithms have been developed, making it possible to use only MinION data for full *de novo* assembly. One laboratory has now assembled the *E. coli* genome into a single 4.6-Mb contig that achieved 99.4% identity at 30 \times coverage relative to a reference *E. coli* genome³⁷. Again using only MinION data and a different improved sequencing algorithm, a second laboratory has

sequenced M13 bacteriophage DNA to 99% accuracy at moderate coverage, and assembled *de novo* the sequence of *E. coli* and lambda DNA at a range of coverages. They also demonstrated the algorithm's ability to accurately classify sequence variants at far lower coverage than with existing methods³⁹.

New publications demonstrating the utility of nanopore sequencing are appearing monthly, suggesting that the MinION and its successor nanopore sequencing instruments will soon be routine next-generation sequencing instruments. For instance, in many genes, alternatively spliced transcription occurs at multiple places that are often located farther apart than the read lengths of most current high-throughput sequencing platforms. The recent identification of ~8,000 of the possible isoforms of the *Drosophila melanogaster Dscam1* gene by nanopore sequencing⁴² shows that transcriptome characterization can now be accessible to a much broader range of investigators than had previously been the case. Using short read length second-generation sequencing poses similar problems for the detection of tumor-associated alterations and is further complicated by low tumor cellularity (the relative proportion of tumor to normal cells in a sample) in most biopsy samples. The success of nanopore sequencing to detect deletions, translocations and inversion in mutant pancreatic cancer tumor suppressor genes diluted 1:100 with wild-type sequences shows that "...nanopore sequencing could become the ideal tool for the low-level detection of cancer-associated... [sequence variants] needed for molecular relapse, early detection, or therapeutic monitoring"⁴³.

Conclusions and outlook

The commercial development of nanopore strand-sequencing as a portable device is a major technological achievement. Nevertheless, two challenges persist, whose resolution would increase nanopore sequencing accuracy to the level needed for single-nucleotide variant calling in the clinic⁴⁴. The first problem is that enzyme turnover is stochastic, so that an enzyme provides an imperfect ratchet in which the intervals between each advance of the DNA are variable. It is during these time intervals that the nanopore current identifies each successive base in the sequence. Some of the intervals may be so short that they are lost or overlooked in system noise and some of the long intervals may be indistinguishable from a repetitive sequence of identical bases. In fact, the most common errors are deletions (~0.075/aligned read base) with about two- to threefold fewer insertion errors³⁵. An improved ratcheting mechanism will be an important step toward more accurate nanopore sequencing.

A second, perhaps more important, problem is that several nucleobases in a strand of DNA contribute to each of the measured current levels. Even though MspA's limiting aperture has been estimated to be only ≤ 0.6 nm long³², molecular dynamic simulations suggest that several nucleobases extending over a length of ~1.2 nm contribute to the current levels⁴⁵. Given an effective sensing length of ~1.2 nm for the sensing region, it is not surprising that up to five neighboring bases simultaneously influence the current levels as a single DNA strand traverses through MspA's limiting aperture^{24,46}. The base multiplicity affecting each current level substantially diminishes the signal-to-noise ratio for base determination, so that only the most likely sequence can be determined, usually by applying dynamic programming, such as the Viterbi algorithm^{47,48} used by ONT's base-caller hosted by Metrichor (Oxford, UK). Reducing the number of nucleobases affecting each current level to improve nanopore sequencing accuracy will probably require testing different biological nanopores or pores through a single layer of graphene⁴⁹ or other atomically thin material that can serve as a stable ionic insulator. Because a single layer of graphene is only ~0.35 nm thick, experiments and modeling suggest that a nanopore pierced

through a graphene layer may be short enough to yield ionic current blockade levels to which only 1–2 nucleobases contribute⁵⁰.

Although nanopore sequencing technology was developed for DNA, it is sufficiently flexible to provide information about a variety of polymers such as RNA and proteins, so it is likely that other applications will emerge. Some of these may be groundbreaking. For instance, because nanopore sequencing does not alter the DNA strand it reads, the accuracy usually achieved by high coverage of PCR-amplified material can be realized by repeated resequencing of the same native genomic fragment multiple times. Although this has not yet been achieved, recapturing a segment of DNA multiple times in the same nanopore through which it has already translocated has been demonstrated⁵¹. When single molecules of native DNA can be read multiple times to achieve high accuracy reads, users will be able to directly analyze epigenetic modifications, base adducts, strand breaks and depurination within genomic DNA obtained from only a single cell.

Notes added in proof: Until recently, ONT has not disseminated information about which nanopores are incorporated in the several different flow cell versions shipped to MAP participants. In an 8 March 2016 presentation for MAP participants (www.youtube.com/watch?v=nizGyutn6v4&feature=youtu.be), Clive Brown, ONT's Chief Technology Officer, indicated that ONT's future products would utilize mutants of the CsgG⁵² nanopore. During final production of this Historical Perspective, two papers were published that detailed use of MinION nanopore sequencers for Ebola outbreak surveillance on site in Liberia⁵³ and Guinea⁵⁴ West Africa. These papers underscore the future importance of inexpensive, portable DNA sequencers for monitoring and combating infectious disease outbreaks at the point of care.

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AUTHOR CONTRIBUTIONS

The three authors contributed equally to writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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- Walker, B., Kasianowicz, J., Krishnasamy, M. & Bayley, H. A pore-forming protein with a metal-actuated switch. *Protein Eng.* **7**, 655–662 (1994).
- Menestrina, G. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: voltage-dependent inhibition by divalent and trivalent cations. *J. Membr. Biol.* **90**, 177–190 (1986).
- Bezrukov, S.M. & Kasianowicz, J.J. Current noise reveals protonation kinetics and number of ionizable sites in an open protein ion channel. *Phys. Rev. Lett.* **70**, 2352–2355 (1993).
- Bezrukov, S.M., Vodyanoy, I., Brutyan, R.A. & Kasianowicz, J.J. Dynamics and free energy of polymers partitioning into a nanoscale pore. *Macromolecules* **29**, 8517–8522 (1996).
- Song, L. *et al.* Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* **274**, 1859–1866 (1996).
- Kasianowicz, J.J., Brandin, E., Branton, D. & Deamer, D.W. Characterization of individual polynucleotide molecules using a membrane channel. *Proc. Natl. Acad. Sci. USA* **93**, 13770–13773 (1996).
- Akeson, M., Branton, D., Kasianowicz, J.J., Brandin, E. & Deamer, D.W. Microsecond time-scale discrimination among polycytidylic acid, polyadenylic acid, and polyuridylic acid as homopolymers or as segments within single RNA molecules. *Biophys. J.* **77**, 3227–3233 (1999).
- Meller, A., Nivon, L., Brandin, E., Golovchenko, J. & Branton, D. Rapid nanopore discrimination between single polynucleotide molecules. *Proc. Natl. Acad. Sci. USA* **97**, 1079–1084 (2000).
- Wang, H., Dunning, J.E., Huang, A.P.-H., Nyamwanda, J.A. & Branton, D. DNA heterogeneity and phosphorylation unveiled by single-molecule electrophoresis. *Proc. Natl. Acad. Sci. USA* **101**, 13472–13477 (2004).
- Mathé, J., Aksimentiev, A., Nelson, D.R., Schulten, K. & Meller, A. Orientation discrimination of single-stranded DNA inside the alpha-hemolysin membrane channel. *Proc. Natl. Acad. Sci. USA* **102**, 12377–12382 (2005).
- Butler, T.Z., Gundlach, J.H. & Troll, M.A. Determination of RNA orientation during translocation through a biological nanopore. *Biophys. J.* **90**, 190–199 (2006).

12. Ashkenasy, N., Sánchez-Quesada, J., Bayley, H. & Ghadiri, M.R. Recognizing a single base in an individual DNA strand: a step toward DNA sequencing in nanopores. *Angew. Chem. Int. Ed.* **44**, 1401–1404 (2005).
13. Stoddart, D., Heron, A.J., Mikhalova, E., Maglia, G. & Bayley, H. Single-nucleotide discrimination in immobilized DNA oligonucleotides with a biological nanopore. *Proc. Natl. Acad. Sci. USA* **106**, 7702–7707 (2009).
14. Stoddart, D. *et al.* Nucleobase recognition in ssDNA at the central constriction of the alpha-hemolysin pore. *Nano Lett.* **10**, 3633–3637 (2010).
15. Stoddart, D., Maglia, G., Mikhalova, E., Heron, A.J. & Bayley, H. Multiple base-recognition sites in a biological nanopore: two heads are better than one. *Angew. Chem. Int. Ed.* **49**, 556–559 (2010).
16. Church, G., Deamer, D., Branton, D., Baldarelli, R. & Kasianowicz, J. Characterization of individual polymer molecules based on monomer-interface interactions. US patent 5,795,782 (1998).
17. Benner, S. *et al.* Sequence-specific detection of individual DNA polymerase complexes in real time using a nanopore. *Nat. Nanotechnol.* **2**, 718–724 (2007).
18. Hornblower, B. *et al.* Single-molecule analysis of DNA-protein complexes using nanopores. *Nat. Methods* **4**, 315–317 (2007).
19. Cockroft, S.L., Chu, J., Amorin, M. & Ghadiri, M.R. A single-molecule nanopore device detects DNA polymerase activity with single-nucleotide resolution. *J. Am. Chem. Soc.* **130**, 818–820 (2008).
20. Olasagasti, F. *et al.* Replication of individual DNA molecules under electronic control using a protein nanopore. *Nat. Nanotechnol.* **5**, 798–806 (2010).
21. Chu, J., González-López, M., Cockroft, S.L., Amorin, M. & Ghadiri, M.R. Real-time monitoring of DNA polymerase function and stepwise single-nucleotide DNA strand translocation through a protein nanopore. *Angew. Chem.* **49**, 10106–10109 (2010).
22. Lieberman, K.R. *et al.* Processive replication of single DNA molecules in a nanopore catalyzed by phi29 DNA polymerase. *J. Am. Chem. Soc.* **132**, 17961–17972 (2010).
23. Cherf, G.M. *et al.* Automated forward and reverse ratcheting of DNA in a nanopore at 5-Å precision. *Nat. Biotechnol.* **30**, 344–348 (2012).
24. Manrao, E.A. *et al.* Reading DNA at single-nucleotide resolution with a mutant MspA nanopore and phi29 DNA polymerase. *Nat. Biotechnol.* **30**, 349–353 (2012).
25. Laszlo, A.H. *et al.* Detection and mapping of 5-methylcytosine and 5-hydroxymethylcytosine with nanopore MspA. *Proc. Natl. Acad. Sci. USA* **110**, 18904–18909 (2013).
26. Schreiber, J. *et al.* Error rates for nanopore discrimination among cytosine, methylcytosine, and hydroxymethylcytosine along individual DNA strands. *Proc. Natl. Acad. Sci. USA* **110**, 18910–18915 (2013).
27. Laszlo, A.H. *et al.* Decoding long nanopore sequencing reads of natural DNA. *Nat. Biotechnol.* **32**, 829–833 (2014).
28. Wescoe, Z.L., Schreiber, J. & Akeson, M. Nanopores discriminate among five C5-cytosine variants in DNA. *J. Am. Chem. Soc.* **136**, 16582–16587 (2014).
29. Meller, A., Nivon, L. & Branton, D. Voltage-driven DNA translocations through a nanopore. *Phys. Rev. Lett.* **86**, 3435–3438 (2001).
30. Niederweis, M. *et al.* Cloning of the mspA gene encoding a porin from *Mycobacterium smegmatis*. *Mol. Microbiol.* **33**, 933–945 (1999).
31. Trias, J. & Benz, R. Permeability of the cell wall of *Mycobacterium smegmatis*. *Mol. Microbiol.* **14**, 283–290 (1994).
32. Faller, M., Niederweis, M. & Schulz, G.E. The structure of a mycobacterial outer-membrane channel. *Science* **303**, 1189–1192 (2004).
33. Butler, T.Z., Pavlenok, M., Derrington, I.M., Niederweis, M. & Gundlach, J.H. Single-molecule DNA detection with an engineered MspA protein nanopore. *Proc. Natl. Acad. Sci. USA* **105**, 20647–20652 (2008).
34. Derrington, I.M. *et al.* Nanopore DNA sequencing with MspA. *Proc. Natl. Acad. Sci. USA* **107**, 16060–16065 (2010).
35. Jain, M. *et al.* Improved data analysis for the MinION nanopore sequencer. *Nat. Methods* **12**, 351–356 (2015).
36. Ip, C.L.C. *et al.* MinION Analysis and Reference Consortium: phase 1 data release and analysis. *F1000Res.* **4**, 1075 (2015).
37. Loman, N.J., Quick, J. & Simpson, J.T. A complete bacterial genome assembled *de novo* using only nanopore sequencing data. *Nat. Methods* **12**, 733–735 (2015).
38. Madou, M.-A. *et al.* Genome assembly using Nanopore-guided long and error-free DNA reads. *BMC Genomics* **16**, 327 (2015).
39. Szalay, T. & Golovchenko, J.A. *De novo* sequencing and variant calling with nanopores using PoreSeq. *Nat. Biotechnol.* **33**, 1087–1091 (2015).
40. Ashton, P.M. *et al.* MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. *Nat. Biotechnol.* **33**, 296–300 (2015).
41. Quick, J., Quinlan, A.R. & Loman, N.J. A reference bacterial genome dataset generated on the MinION™ portable single-molecule nanopore sequencer. *Gigascience* **3**, 22 (2014).
42. Bolisetty, M.T., Rajadinakaran, G. & Graveley, B.R. Determining exon connectivity in complex mRNAs by nanopore sequencing. *Genome Biol.* **16**, 204 (2015).
43. Norris, A.L., Workman, R.E., Fan, Y., Eshleman, J.R. & Timp, W. Nanopore sequencing detects structural variants in cancer. *Cancer Biol. Ther.* doi:10.1080/15384047.2016.1139236 (19 January 2016).
44. Peters, B.A. *et al.* Accurate whole-genome sequencing and haplotyping from 10 to 20 human cells. *Nature* **487**, 190–195 (2012).
45. Bhattacharya, S. *et al.* Molecular dynamics study of MspA arginine mutants predicts slow DNA translocations and ion current blockades indicative of DNA sequence. *ACS Nano* **6**, 6960–6968 (2012).
46. Manrao, E.A., Derrington, I.M., Pavlenok, M., Niederweis, M. & Gundlach, J.H. Nucleotide discrimination with DNA immobilized in the MspA nanopore. *PLoS One* **6**, e25723 (2011).
47. Viterbi, A.J. Error bounds for convolutional codes and an asymptotically optimum decoding algorithm. *IEEE Trans. Inf. Theory* **13**, 260–269 (1967).
48. Timp, W., Comer, J. & Aksimentiev, A. DNA base-calling from a nanopore using a Viterbi algorithm. *Biophys. J.* **102**, L37–L39 (2012).
49. Kuan, A.T., Lu, B., Xie, P., Szalay, T. & Golovchenko, J.A. Electrical pulse fabrication of graphene nanopores in electrolyte solution. *Appl. Phys. Lett.* **106**, 203109 (2015).
50. Garaj, S., Liu, S., Golovchenko, J.A. & Branton, D. Molecule-hugging graphene nanopores. *Proc. Natl. Acad. Sci. USA* **110**, 12192–12196 (2013).
51. Gershaw, M. & Golovchenko, J.A. Recapturing and trapping single molecules with a solid-state nanopore. *Nat. Nanotechnol.* **2**, 775–779 (2007).
52. Goyal, P. *et al.* Structural and mechanistic insights into the bacterial amyloid secretion channel CsgG. *Nature* **516**, 250–253 (2014).
53. Hoenen, T. *et al.* Nanopore sequencing as a rapidly deployable Ebola outbreak tool. *Emerg. Infect. Dis.* **22**, 331–334 (2016).
54. Quick, J. *et al.* Real-time, portable genome sequencing for Ebola surveillance. *Nature* doi:10.1038/nature16996 (3 February 2016).