A world of opportunities with nanopore sequencing

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Abstract

Oxford Nanopore Technologies' MinION sequencer was launched in pre-release form in 2014 and represents an exciting new sequencing paradigm. The device offers multi-kilobase reads and a streamed mode of operation that allows processing of reads as they are generated. Crucially, it is an extremely compact device that is powered from the USB port of a laptop computer, enabling it to be taken out of the lab and facilitating previously impossible in-field sequencing experiments to be contemplated. Many of the initial publications concerning the platform focussed on provision of tools to access and analyse the new sequence formats and then demonstrating the assembly of microbial genomes. More recently, as throughput and accuracy have increased, it has been possible to consider work involving more complex genomes and metagenomes. With the release of the high throughput GridION X5 and PromethION platforms the sequencing of large genomes will become more cost efficient, and enable the leveraging of extremely long (>100kb) reads for resolution of complex genomic structures. This review provides a brief overview of nanopore sequencing technology, describes the growing range of nanopore bioinformatics tools and highlights some of the most influential publications that have emerged over the last two years. Finally, we look to the future and the potential the platform has to disrupt work in human, microbiome and plant genomics.

Introduction

"Great things are done by a series of small things brought together" - Vincent Van Gogh.

In the decade or so since 454 Life Sciences released the first second (or next-) generation sequencing machine, the GS20, few areas of biological science have been left untouched by the potential of genomics technology to deliver insights into the structure and function of living organisms. In the end it was Illumina's technology (having purchased Solexa) of sequencing by synthesis (SBS) combined with bridge amplification of template molecules that won the battle of the next generation sequencing (NGS) platforms and they now dominate the sequencer market worldwide. While these next-gen machines, still account for the largest share of sequencing output, their need to amplify DNA templates limits them to short 100-400 bp read lengths due to phasing (when templates in a polymerase colony lose synchronicity), which makes genome, transcriptome and metagenome assembly more challenging and leaves some areas of even the human genome unresolvable. In contrast the third generation of sequencing platforms uses single molecule sequencing and, with no phasing issues limiting read lengths, the challenge instead is to achieve good signal to noise ratios and base call accuracy. This new wave of technologies is led by the more established player, Pacific Biosciences (PacBio) of Menlo Park, California and by the relative newcomer, Oxford Nanopore Technologies (ONT), of Oxford, UK. Both technologies analyse individual molecules of DNA with no need for artificial amplification, generate longer reads than second generation technologies (typically in the thousands or even tens of thousands of base pairs) but both platforms have a relatively high error rate compared to Illumina's <1% error rates. As the first long read single molecule sequencer, PacBio had to solve problems related to longer

reads (new aligners e.g. BLASR (Chaisson & Tessler 2012)), lower read accuracy (using adapter hairpins to create multipass sequencing of both strands and consensus sequence (Travers et al. 2010)) and genome assembly (read correction, and longread capable assemblers (Chin et al. 203)). Nanopore research has been built on much of this pioneering work.

Beyond these similarities, there are major differences between the technologies. PacBio technology has been established longer and the company's relatively large machines are typically found in centralised sequencing centres where the large sums required to purchase them can be justified. In contrast, Oxford Nanopore's MinION is a four-inch long USB-powered device that is given away free, with the company recouping cost through purchase of reagents. To a certain extent, ONT's approach has already begun to democratise sequencing, enabling almost any research group anywhere in the world to purchase their own sequencer and to use genomics in their research. The portability of the MinION and the fact that it requires only a laptop's USB port in order to power it, has enabled researchers to dream of new experiments that were previously impossible, enabling them to take the sequencer to the sample's natural environ, rather than to bring the sample to the lab. One particularly striking example of this was the recent experiment to deploy a MinION to the International Space Station and carry out sequencing and genome assembly (Castro-Wallace et al. 2016). Finally, and crucially, the MinION operates as a genuine real-time platform, in which individual reads can be analysed as they are sequenced and this information used to determine how long to run the sequencing experiment for - or even, whether to eject specific DNA molecules from individual nanopores.

We have now reached a crucial inflexion point in the progress of Nanopore sequencing. During the first couple of years of the availability of the MinION, researchers were occupied with understanding the technology and its error profile and imagining where the new paradigm might eventually take them. The samples they worked with were typically bacterial, phage or amplicon-based - samples for which the higher error rate and lower yield of the early versions of the chemistry were well suited. However, the last year has seen large increases in the yield of the platform and similarly significant reductions in the error rate. Members of the community are now beginning to work with much more complex organisms including plants and animals and the last few months has seen the release of a number of eukaryotic datasets including two human whole genome nanopore datasets (see https://github.com/nanopore-wgs-consortium/NA12878 and http://www.well.ox.ac.uk/ogc/nanopore-human-genome), a human assembly (Jain, Koren & Quick et al. 2017) and the tomato species *Solanum pennellii* (Schmidt, Vogel, Denton et al. 2017). The announcement of the higher throughput GridION X5 and PromethION platforms should further facilitate this move to more complex organisms.

Nanopore sequencing

The MinION was first announced at the Advances in Genome Biology and Technology (AGBT) conference in Florida in February 2012, but it wasn't publically available until an early access programme known as MAP (MinION Access Programme) began in April 2014. However, the idea of using nanopores dates back to the late 1980s and early 1990s and the work of several groups who published key research and patents (see Deamer et al. 2016 for a detailed review). The principle behind the ONT technology is illustrated in Figure 1. A biological nanopore is built into an electrically resistant artificial membrane and a voltage is applied across the membrane. DNA

molecules are prepared according to one of a set of standard library preparation protocols which involve attaching a leader adaptor and motor protein to one strand of DNA. During sequencing, the motor protein unzips double-stranded DNA and passes a single strand through the pore a base at a time. The presence of the DNA molecule in the pore causes a deflection in the current across the pore and this current change can be related to the exact bases present in the pore at that moment - currently, this is based on a 5-mer model. On a MinION flowcell is an array of 512 sensors, each connected to 4 nanopores (one of which is in use at any time), measuring current through the pore thousands of times a second. At any given moment, each of the 512 channels may be in a number of different states depending on whether molecules are being sequenced, captured into the pore or if pores are empty or clogged up. Software called MinKNOW runs on a computer connected to the MinION and records the signal (current) data from all channels, storing it on the local hard drive. This signal data is basecalled to a nucleotide sequence either locally or through a cloud-based service. For the first 2 years of the MinION, there was little option than using the basecalling cloud service provided for free by the ONT subsidiary, Metrichor. This involved uploading the FAST5-format files created by the local control service and downloading basecalled FAST5 reads that needed to be converted to more standard formats such as FASTA and FASTQ. Metrichor characterises basecalled reads as 'pass' or 'fail' according to whether they pass mean quality criteria and, in the case of 2D reads, whether there is both a template and a complement strand read at sufficient quality.

A number of library preparation protocols are available. Initially, the most popular approach was 2D (double stranded) sequencing. This involved preparing molecules that incorporate a hairpin adaptor; the motor protein would process the leader adaptor through the pore, followed by the template strand, then the hairpin adaptor and the complement strand. This approach provided the basecaller with two attempts at reading the bases, and generated a consensus sequence with higher accuracy basecalls than from a single strand alone. However, as overall accuracy has increased on the platform, 1D library preparations involving sequencing only a single strand of DNA have become more popular. The main advantages of 1D sequencing are much reduced library preparation time (as little as 15 minutes for the rapid transposase-based kit) and increased yield due to only sequencing one strand of each molecule and this method is preferred for obtaining the longest reads. The early part of 2017 saw the announcement that 2D libraries would be phased out in preference for what is termed $1D^2$ libraries. Here, the two strands of a DNA molecule are delivered to the pore but are not covalently linked. Once the template strand has been sequenced, this approach relies on the complement strand remaining near the pore and being captured by the pore immediately after the template strand. The exact mechanisms have not been revealed but ONT assert that this happens in 60% of cases (Clive Brown, March 2017 ONT Technology Update) and offers accuracy advantages over the existing 2D approach.

Input DNA requirements for single molecule sequencers tend to be higher than for the second generation of sequencers which use amplification. The standard ONT 1D and 2D ligation kits recommend 1 μg of starting material, but good results are possible with half of that. The new rapid transposase kit has a recommended input of 200 ng. ONT's PCR-based protocol needs only 10 ng input, and it is possible to sequence even lower (picogram) amounts either using increased amplification prior to ONT library creation, or by accepting that pore occupancy, and therefore yields will be significantly lower. There is growing interest in decreasing input requirements using improved library techniques (lower losses) and improved pore loading e.g. using bead bound

library molecules which locate adjacent to pores and load more efficiently. Whilst nanopore (and PacBio) sequencing require large inputs of DNA they remain more susceptible to inhibitors of library construction or sequencing. Most sample types have been successfully sequenced, but you might have a more challenging one. ONT maintain a forum (https://nanoporetech.com/community) with extant protocols and rapid advice from MAP colleagues as well as ONT's staff. Whilst obvious it bears repeating for emphasis, reads can not be longer than the input DNA. Thus the more gentle, careful DNA extraction methods often yield superior data. As many of these methods are based on older protocols it may be worth putting down your kits, reading "Molecular cloning : a laboratory manual" (Green & Sambrook 2012) or finding a green fingered old school molecular biologist and buying them a drink.

Sequencing typically proceeds up to 48 hours, but the first 24 tend to produce much higher yields, as flowcell performance gradually declines. At 8 hour intervals, mux scans are performed by the system in order to chose the highest performing nanopore in each channel's group of 4.

MinION performance

Since the MAP programme started in 2014, the MinION platform has seen regular updates to the cradle, the flowcell design, the sequencing pore, the sequencing kits and the signal capture and basecalling software. Thus any discussion about MinION performance is a reflection of a particular time point and is quickly out-of-date. The first sequencing pore used by members of MAP was termed R6 ('R' for 'Reader'), then came R7, R7.3, R9 and finally R9.4, which is the CsgG-derived pore currently used in flowcells. An international consortium of MAP members, the MinION Analysis and Reference Consortium (MARC) set out to produce an assessment of MinION quality and consistency by conducting an experiment in which 5 different labs across the world sequenced DNA from the same *Escherichia coli* K-12 sample using the R7.3 pore and flowcell (Ip et al. 2015). They found a high variation in yield amongst the 20 flowcells used (ranging approximately 20,000-140,000 per flowcell), but a much more consistent error rate with median accuracy of 89% for 2D pass reads. Recently, the consortium published a Phase 2 analysis which updated results to R9 (Jain, Tyson, Loose et al. 2017), demonstrating an increase of median accuracy to 92% and much increased yield (127,000 - 217,000 per flow cell, 4 flow cells sequenced). MARC have yet to publish an update for R9.4, but the general consensus within the nanopore community is that accuracy is now up to around 95% for 2D pass reads and yields of 3-5Gb are typical (Figure 2). As with the PacBio platform, insertions and deletions are the predominant error type - R7.3 MARC data found roughly three times the number of indels as substitutions across 2D pass and fail data. This is in contrast to Illumina technology where errors, while rare, tend to be substitutions. The MinION has had particular difficulties with homopolymer sequences, as a stream of identical bases will result in the same current level and variability in the speed of procession through the pore makes it difficult to discern the exact number of bases from temporal data alone. A new basecaller termed 'scrappie' has been developed by ONT to address this problem and early results seem to indicate substantially improved homopolymer resolution (Jain, Koren & Quick et al. 2017).

ONT have long asserted that nanopore sequencing quality is the same at the beginning and end of the DNA molecule, thus read length is dependent on the DNA extraction and preparation. This means that if you can prepare long DNA molecules for sequencing, the device will produce long sequence data. The standard protocols for 2D library preparation involve a recommended shearing

step which tends to produce a mean length of around 7-8kb. However, even with this approach, long reads are possible and the MARC Phase 2 data includes mapped reads up to 50kb. Recently, Nick Loman and Josh Quick garnered much attention within the nanopore community for their attempts to sequence extremely long molecules on the MinION. Loman's blog (http://lab.loman.net) details recent success in sequencing accurate E.coli and human DNA molecules over 900 kb long using older phenol-chloroform extraction and minimal use of pipetting in order to avoid unwanted shearing. The same protocols were also used in a recent human sequencing preprint publication which described generation of reads of up to 882 kb (Jain, Koren & Quick et al. 2017).

Basecalling

At launch, the only basecalling option for the MinION was using the EPI2ME platform provided by ONT subsidiary Metrichor (http://www.metrichor.com/). The client software ran on the same computer as the MinION and uploaded raw signal FAST5 files to a remote server. After a slight delay, basecalled FAST5 files are downloaded back onto the client machine. Initially, ONT adopted an approach to basecalling in which the raw signal was first converted to events (with each event ideally corresponding to a single base movement through the pore - Figure 2c) and the event space modelled as a Hidden Markov Model, utilising the Viterbi algorithm to find the most likely sequence of states consistent with the event data (see David et al. 2017, Schreiber & Karplus 2015 for more detail). Subsequently, ONT found increased accuracy could be obtained using Recurrent Neural Networks (RNN) to model the raw signal, moving away from event-based analysis.

MinKNOW 1.0.2, released in mid-2016, introduced 1D local basecalling by RNN and removed the reliance on the Metrichor service, though still providing it as an option. Using local basecalling, MinKNOW will attempt to capture signal data and basecall it on the same laptop; depending on the processing power available, the basecalling may lag behind. ONT subsequently released the standalone Albacore basecaller, which can be run locally or on a High Performance Compute (HPC) cluster. This added the ability to perform 2D basecalling and with MinKNOW 1.5.5 (March 2017), MinKNOW was brought into parity with Albacore. This coincided with ONT switching off cloud basecalling, though it is unclear at this stage if it will subsequently be offered as a paid for service, something ONT have previously indicated. The period in which there was no local basecalling option provided by ONT saw the release of two third-party basecallers: Nanocall was the first to be published, providing an HMM-based implementation of 1D basecalling and producing comparable results to the Metrichor HMM basecalling of R7.3 data (David et al. 2017), DeepNano emerged at a similar time, adopting an RNN approach and claiming to offer slightly higher accuracy than Metrichor then did (Boza et al. 2016). For a period, both tools provided a viable basecalling approach for those needing to move away from an internet service but have subsequently been left behind by updates to the core platform and ONT's own move to local RNN basecallers. Nevertheless, they represent useful examples for those looking to understand and work with nanopore signal data.

Data analysis

The FAST5 format used on the MinION platform is an implementation of the HDF5 standard for hierarchical data storage (https://support.hdfgroup.org/HDF5/). Each read has its own FAST5 file and each file is substantially larger than the size of the sequence data alone, containing signal data

and large amounts of other metadata. Standard bioinformatics tools typically require FASTA or FASTQ format files, so it is necessary to convert reads to one of these formats for downstream analysis. ONT do not provide toolsets to enable this, but a number have been developed by the community. Poretools (Loman & Quinlan 2014) was the first to be released, offering a command line interface to extract FASTA/Q data and to report and plot basic statistics such as yield over time or read length distributions. Released slightly later, poRe offers similar functionality for users of the R statistical environment (Watson et al. 2015). NanoOK also provides FASTA/Q extraction, and will align reads against references and generate a comprehensive report analysing accuracy and error profile of the data (Leggett et al. 2016). minoTour implements a web front-end to interact with nanopore sequencing data or to monitor and control runs on remote machines in real-time (http://minotour.nottingham.ac.uk).

Once converted to FASTA/Q format, it is important to use alignment tools that can handle the particular error profile of nanopore reads. Early data was error prone enough to be challenging to analyse using standard tools, but as MinION accuracies have increased this has become easier. LAST was initially popular within the nanopore community, offering BLAST-like searches but with an adaptive seed approach in which seeds are chosen for their rareness instead of fixed length matches (Kielbasa et al. 2011). After a nanopore specific option was added by the authors of BWA-MEM (Li & Durbin 2009), it soon attained ascendancy. Good results can also be obtained from BLAST, especially with tuned penalty parameters (Goodwin et al. 2015). marginAlign is a nanopore-specific alignment tool that uses expectation maximisation to train an HMM and estimate Maximum Likelihood Estimation parameters in order to find higher confidence alignments (Jain et al. 2015). Using marginAlign, the MARC Phase 1 researchers were able to decrease their estimate of overall error rate by 1.1% for 2D pass reads, compared to alignments with BWA or LAST (Ip et al. 2015). GraphMap is an alignment tool for nanopore reads which adopts a process of refinement that progressively reduces the candidate alignment sites until a good quality alignment is obtained (Sović, Šikić M & Wilm 2016).

Table 1 provides a summary of key third-party software tools for working with nanopore data.

A portable lab

One of the most exciting attributes of the MinION is its compact size and the ability to power it and operate it from a standard laptop computer. This has enabled researchers to design experiments that would previously have been impossible or that are made difficult by restrictions (physical or legal) when transporting biological materials. Instead of collecting samples and couriering them frozen to the sequencing centre, it is now possible to take the sequencer to the samples in the field for faster results and potentially highlighting sites with special genetic diversity. One high profile example of this was the project to carry out surveillance of the West African Ebola outbreak of 2014-2015 using the MinION (Quick et al. 2016). All instruments and consumables necessary to set up a genome surveillance lab were packed into less than 50kg of aircraft baggage and flown to Guinea. Here, hundreds of samples were sequenced, basecalled and analysed. This took place before local basecalling was available and there were some challenges in obtaining sufficient mobile internet data bandwidth. These were overcome and the researchers were able to carry out detailed evolutionary analysis of the outbreak. Some of the same team responsible for the Ebola work then became part of the ZiBRA collaboration which is using MinIONs to carry out surveillance

of the Zika virus outbreak in Brazil (Faria et al. 2016, Faria et al. 2017). Another group evaluated the use of nanopore sequencing as part of a rapidly deployable laboratory during a 2016 NATO exercise which simulated an outbreak due to a bioterrorism attack (Walter et al. 2016). Using a MinION operated inside an inflatable tent, they were able to prepare samples, sequence and perform metagenomic analysis to determine the causative agent. Some challenges remain - such as the amount of input DNA required - but improved yields and accuracy, combined with emerging simplified and automated sample preparation will make outbreak surveillance activities even simpler in the future.

The utility of the MinION in a range of clinical settings has also been demonstrated. Applications have included real-time sequencing of a hospital outbreak of *Salmonella* (Quick et al. 2015), identification of antibiotic resistance profiles (Ashton et al. 2015, Bradley et al. 2015, Cao et al. 2016, van der Helm et al. 2017), same day diagnostics and surveillance for tuberculosis (Votintseva et al. 2016), prenatal testing (Cheng et al. 2015, Wei & Williams 2016), detection of viral pathogens in blood samples (Greninger et al. 2015), identification of bacterial pathogens in urine samples (Schmidt, Mwaigwisya, Crossman et al. 2017), structural variant analysis in cancer (Norris et al. 2016).

Researchers have attempted to test the MinION in what might be termed 'extreme' environments. Edwards et al. 2016 took sequencers to a European High Arctic glacier in order to sequence microbial communities, finding profiles coherent with those obtained by Illumina sequencing. Another group sequenced in the McMurdo Dry Valleys, Antarctica (Johnson et al. 2017). But perhaps the most extreme environment is represented by a recent NASA experiment aboard the International Space Station (Castro-Wallace et al. 2016) in which MinIONs were used to sequence mouse, *E.coli* and lambda libraries. Zero gravity was no barrier to sequencing and researchers found no discernable decrease in MinION quality and throughput. However, the successful sequencing runs were carried out using pre-prepared libraries and preparing these in space may be more of a challenge.

Genome assembly

Due to the similarities in read length and error profiles between PacBio and ONT technologies, it has been natural for researchers working with the MinION to adopt many of the assembly approaches and tools used for PacBio data. This has meant a return to overlap, layout, consensus assembly methods (originally used with Sanger reads), as well as exploration of hybrid assembly approaches that utilise long reads to scaffold Illumina contigs or Illumina reads to correct the long reads.

The first de novo assembly of a complete bacterium from only MinION data was Loman, Quick and Simpson's assembly of *Escherichia coli* K-12 MG1655 from R7.3 data (Loman, Quick & Simpson 2015). Their three-stage pipeline consisted of a custom read correction step (nanocorrect), assembly with Celera (Myers et al. 2000), followed by an assembly polishing step with Simpson's nanopolish tool. This final step involves using the raw pore signal data in order to compute an improved consensus sequence based on iterative evaluation of possible changes with a Hidden Markov Model. The resulting assembly is a single contig with 99.5% nucleotide identity to the already available reference sequence. The polishing step played a crucial part in the final quality of

the assembly, decreasing the substitution and indel rate from 80 and 921 per 100kb respectively to 26 and 321 per 100kb. The drawback of the polishing process is that it is relatively processor intensive, though it is easily parallelised for users with a HPC cluster.

Currently, Canu appears to be the most popular assembly tool for nanopore data. Based on Celera, but with new overlap and assembly algorithms, it has been designed to provide improved support for the error profile of third generation sequencers and improved runtimes (Koren et al. 2017). Canu provides its own efficient read correction step which negates the need for nanocorrect. However, the resulting assembly will still be improved by finishing with nanopolish. Miniasm is an assembler for PacBio and Nanopore data that sacrifices accuracy for speed (Li 2016). By omitting read correction or consensus sequence stage, it is able to assemble small genomes in minutes (for example *C. elegans* in only 9 minutes on 16 cores). However, the resulting contigs reflect the underlying errors in the input reads and are significantly less accurate than Canu. Running nanopolish on miniasm assemblies can significantly improve accuracy, but the speed advantage is then diminished. An alternative to nanopolish is provided by Racon (for Rapid Consensus) which provides a stand alone assembler-neutral consensus module that can be paired with miniasm (Vaser et al. 2017).

A number of assemblies utilising hybrid approaches have been published which combine nanopore data with highly accurate short read Illumina data. Madoui et al. describe what they term Nanopore Synthetic-long, or NaS, reads which are formed by using nanopore reads as a template for local assemblies of Illumina short-reads (Madoui et al. 2015). LINKS uses nanopore reads to scaffold contigs assembled from Illumina MiSeq data (Warren et al. 2015). Karlsson et al. 2015 also used MinION reads to scaffold Illumina data. Another tool, npScarf, takes advantage of the fact that nanopore reads can be used immediately they are generated in order to scaffold Illumina data in real-time (Cao et al. 2017). Although it is still necessary to wait for an Illumina run to complete before running npScarf, the authors argue it favours a reduction in Nanopore resources (flowcells, consumables) as sequencing can be stopped when the assembly has reached an acceptable quality. The assembler SPAdes has an option to utilise Nanopore or PacBio data for gap closure and repeat resolution in hybrid assemblies with Illumina data (Bankevich et al. 2012). Nanocorr uses alignments of Illumina reads against nanopore reads to correct the longer reads (Goodwin et al. 2015). MiSeq reads are aligned against the nanopore reads and an algorithm selects an optimal set of alignments spanning the read. A consensus basecall for the read is then determined using the PacBio tool PBDAG-Con (Chin et al. 2013). In general, as the yield and read accuracy of the MinION have increased, there has been less focus on hybrid approaches for assembly of small genomes and a realisation that nanopore data alone is sufficient to produce accurate results. An evaluation of some of the approaches discussed can be found in Sović et al 2016, Deschamps et al. 2016, Judge et al. 2016, Lu et al. 2016.

MinION reads have contributed in part or full to assemblies of a wide range of bacteria (Millard et al. 2015, Ashton et al. 2015, Deschamps et al. 2016, Turton et al. 2016, Greninger et al. 2016, Greninger et al. 2017), yeast (Goodwin et al. 2015, Istace et al. 2017), viruses (Wang et al. 2015, Karamitros et al. 2016). In general, nanopore assemblies of bacteria typically produce more contiguous results than Illumina-only assemblies and the platform is starting to be seen as a competitor to the PacBio RS2 which has become the de facto gold standard for microbial assemblies. Thanks to increases in yield and accuracy, researchers are now starting to assemble

larger genomes with MinION data. Preprints have recently become available outlining assemblies of the 54 Mb fungal genome *Rhizoctonia solani* (Datema et al. 2016), the 100 Mb C. elegans genome (Tyson et al. 2017), the 860 Mb endangered European eel (Jansen et al. 2017) and the gigabase genome of the tomato species *Solanum pennellii* (Schmidt, Vogel, Denton et al. 2017). The human assembly (Jain, Koren & Quick et al. 2017) is particularly impressive as it features ultra-long reads (N50 of 99.7kb) a data type which is still only possible on MinIONs. Adding only 5x genome coverage of this doubles the contiguity of the assembly and their models suggest that 30x coverage of such data would have led to a 10-fold increase in contiguity (NG50 >30Mbp).

Signal level analysis

One of the unique attributes of the MinION platform is that it provides access not only to the DNA sequence, but also to the raw signal used for basecalling. As we have discussed previously, this has enabled researchers to implement their own basecallers and to create algorithms for genome polishing going back to the raw signal in a method reminiscent of examining Sanger trace files when reads disagree. Two other exciting uses of signal data are in the analysis of DNA modification and in the direct control of individual nanopores.

Before the release of the MinION, researchers had already demonstrated that nanopores can discriminate between 5-methylcytosine, 5-hydroxymethylcytosine and cytosine bases (Laszlo et al. 2013, Schreiber et al. 2013) through changes in the ionic current levels. Unlike with second generation sequencing approaches, no special treatment of the DNA is required in order to detect the modifications. Two groups have since demonstrated this practically on the MinION, both producing tools that train HMMs to discriminate modified bases. Simpson et al. implemented their model within their existing nanopolish package and demonstrate 95% accuracy in detecting 5-mC (Simpson et al. 2017). Rand et al. have produced SignalAlign, a tool for alignment of signal data to a reference with mapping of methylation data and demonstrated mapping of three cytosine variants and two adenine variants (Rand et al. 2017). Both groups anticipate the further development of these models for the detection of other classes of DNA modification. ONT's recent release of a direct RNA sequencing kit also offers the unique possibility to discriminate RNA modification signals, though this research is currently immature. PacBio can also call DNA modifications from untreated DNA, due to changes in the sequencing polymerase kinetics as it interacts with the base, but the depths needed to accurately call a specific modification vary depending on the modification and can be as very high e.g. 250-fold for the commonest eukaryotic modification 5-methylcytosine (Davis, Chao & Waldor 2013).

ReadUntil is the name of an Application Programming Interface (API) from Oxford Nanopore that gives software control over individual nanopores. Specifically, it provides a function to reverse the voltage across a pore causing the ejection of the molecule and offering the possibility of rejecting DNA molecules which the user is not interested in sequencing. This unique capability of nanopore sequencing could enable a researcher to target sequencing to species of interest, or to specific regions of interest in a genome. For the approach to be efficient, a decision must be made as quickly as possible and from the earliest bases. However, standard basecallers do not call the sequence until the whole molecule has been sequenced. Thus, in order to decide if a molecule is required or not, software must make a decision based on analysis of the raw signal data or basecall using partial data. Loose, Malla and Stout demonstrated the first practical application of

ReadUntil, using an established signal processing algorithm called Dynamic Time Warping to match nanopore event data to pre-calculated signals (Loose, Malla & Stout 2016). They used 11 amplicons covering 22kb of the lambda genome and showed they were able to selectively sequence amplicons of choice - generating more even coverage of all amplicons, or high coverage of specific amplicons and low coverage of others. Possible future uses for ReadUntil might include targeting reads from a large insert library to address specific issues e.g. a duplicated gene cluster, gaps in genome assemblies or sequencing specific species within a metagenomic community. However, there are significant technical challenges in performing the analysis rapidly enough to be efficient, especially as the number of targeted sequences increases and the speed of nanopore sequencing also increases.

The future for nanopore sequencing

Early 2017 saw the first labs take delivery of the PromethION, ONT's high throughput sequencing platform. While the MinION takes a single 512-channel flowcell, the PromethION provides for up to forty eight 3000-channel flowcells, with each flowcell able to be run independently. ONT suggests that the PromethION will exceed the best platform Illumina has, with a theoretical maximum of 11 Tb per PromethION run. At the time of writing, PromethION flow cell manufacturing is not fully underway and we are still waiting to see what the throughput is in the hands of typical users. In the first quarter of 2017 ONT also announced the GridION X5 which is effectively a unit consisting of 5 MinIONs and some built-in computing for base calling. Both of these platforms enable enormous quantities of long read data to be generated at a price point close to or matching Illumina short reads and will be crucial in the continued expansion of nanopore sequencing into complex plants and animals. At the other extreme, ONT has also announced that they are developing the SmidgION, a tiny 128-channel nanopore sequencer that attaches to, and is powered by a mobile phone, this is planned for a late 2017 release. Such a device would offer incredible possibilities for ecological, environmental and clinical applications.

Library preparation is already a relatively simple process for MinION, but ONT are keen to make it simpler and more consistent. VolTrax is a compact microfluidic device designed to automate nanopore library preparation. It consists of a USB-powered base into which a consumable cartridge is placed with an array of fluid containing pixels on its surface. Software running on a connected laptop controls the movement of drops of fluid around the cartridge surface, from pixel to pixel, including through defined heating zones. ONT provide a protocol for their 1D rapid library prep, but are in the process of developing other protocols and will enable users to develop their own workflows. Original designs for VolTrax showed it attached to the top of a MinION, offering the tantalising prospect of putting cell solutions into the device and receiving raw sequencing data out of the bottom. Though this isn't realised in the current first release, it remains a target for ONT, possibly via another straw like device, currently codenamed Zumbador, in which cells or tissues are inputted at the top, and material flows between partitions filled with chemicals and lyophilised enzymes until ready to sequence libraries emerge from the bottom.

New library preparation protocols continue to be developed by ONT and one of the most eagerly awaited has been the direct RNA sequencing protocol which was recently made available. This offers the prospect of amplification free (unbiased), full-length transcripts, as well as access to RNA base modification data. Early versions of the RNA basecaller are not yet as accurate as for

DNA, but we are likely to see rapid progress as more members of the nanopore community start to exploit the potential of direct RNA sequencing.

ONT are not the only company working on nanopore technology. Hitachi have recently presented work on solid state nanopores (Goto et al. 2016) and Genia (owned by Roche) have a technology based on biological nanopores and highly modified DNA copies. However, no other company has yet reached market with a product. ONT's main competitors then are PacBio's long-read technology and Illumina's short-read, high throughput technologies. Both companies have tended to downplay the impact of ONT's technology publically, but it is clear that ONT's continuing improvements in throughput and accuracy have moved nanopore sequencing to a point where it has become a contender to both of the longer standing platforms. As ONT's business model includes options to buy platforms for little or no cost (paying only for consumables) many labs may be attracted to nanopore sequencers without writing an equipment grant. Assuming the continuation of improvements in nanopore sequencing (by no means guaranteed), PacBio are probably the most threatened followed by Illumina's assembly and then resequencing markets. Illumina SBS and cluster chemistry is strongest in the "tag-counting" space of functional genomics e.g. RNA-seq, ChIP-seq etc. but one can envisage how a long read SAGE type method (Velculescu et al. 1995) might threaten even this. As users of all three technologies we hope that competition drives innovation, increased performance and lower prices that enable new biological discoveries.

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Conflicts of interest

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Figure 1: Nanopore sequencing: (a) A biological nanopore is inserted into an electrically resistant synthetic membrane. A potential is applied across the membrane, resulting in ion flow. Library DNA molecules have adapters with aliphatic tethers (not shown) which preferentially locate to the membrane for a localised library concentration. (b) The motor protein bound to the other adapter, docks with the pore, and passes the DNA molecule through it. (c) Bases in the nanopore cause disruptions in the current which are characteristic of their sequence (blue line). In some basecallers, the signal is further refined to events (red line) which correspond to distinct pore kmers.
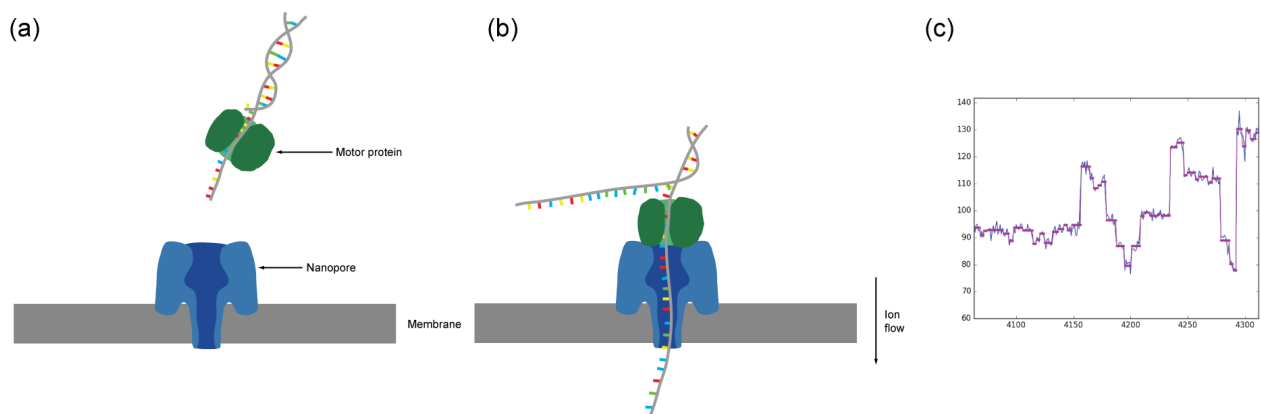
Figure 2: Improvements in MinION accuracy and yield: (a) Indicative read identity vs length plots for standard 2D library prep on R6, R7.3 and R9.4, based on flowcells run at Earlham Institute using ONT suggested 2D protocol (with ~8kb shear). (b) Distribution of read identities for the R9.4 run, showing difference between template, complement and 2D consensus read accuracy. (c) Indicative yields. In all cases, results are affected sample and DNA quality, but the general trend has been upwards. Read length dependent on DNA preparation. Plots produced using NanoOK (Leggett et al. 2016).

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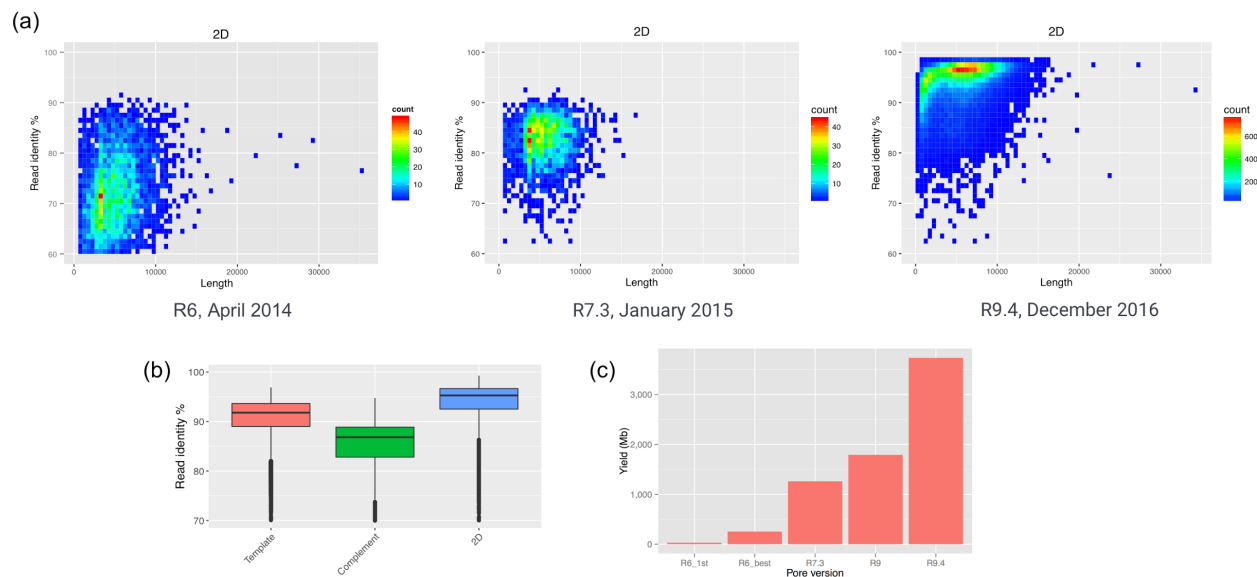
Table 1: Commonly used third-party nanopore software tools.

Tool	Brief description	Website	Reference
poretools	Read extraction and run metrics	http://poretools.readthedocs.io/en/latest/	Loman & Quinlan 2014
poRe	Read extraction and run metrics via R	https://github.com/mw55309/poRe_docs	Watson et al. 2015
NanoOK	Read extraction and alignment-based analysis	https://documentation.tgac.ac.uk/display/NANOOK/NanoOK	Leggett et al. 2016
minoTour	Web-based monitoring and remote control of runs	http://minotour.nottingham.ac.uk	
BWA-mem	Read alignment	http://bio-bwa.sourceforge.net	Li & Durbin 2009
LAST	Read alignment	http://last.cbrc.jp	Kielbasa et al. 2011
marginAlign	Tuning of nanopore read alignments	https://github.com/benedictpaten/marginAlign	Jain et al. 2015
GraphMap	Read alignment	https://github.com/isovic/graphmap	Sović, Šikić & Wilm 2016
Canu	Correction and assembly of long read data	http://canu.readthedocs.io/en/latest/	Koren et al. 2017
minimap and miniasm	Rapid mapping and assembly	https://github.com/lh3/miniasm	Li 2016
SPAdes	Hybrid assembly	http://bioinf.spbau.ru/spades	Bankevich et al. 2012
nanopolish	Signal level analysis - including assembly polishing, event alignment, methylation detection, variant calling	https://github.com/jts/nanopolish	Loman, Quick & Simpson 2015
SignalAlign	Signal mapping and methylation detection	https://github.com/ArtRand/signalAlign	Rand et al. 2017
Racon	Assembly consensus	https://github.com/isovic/racon	Vaser et al. 2017

NanoCorr	Error correction using Illumina data	https://github.com/jgurtowski/nanocorr	Goodwin et al. 2015
LINKS	Scaffolding with long reads	http://www.bcgsc.ca/platform/bioinfo/software/links	Warren et al. 2015
npScarf	Scaffolding with long reads	https://github.com/mdcao/npScarf	Cao et al. 2017
Nanocall	Basecalling	https://github.com/mateidavid/nanocall	David et al. 2017
DeepNano	Basecalling	https://bitbucket.org/vboza/deepnano	Boza et al. 2016
PoreSeq	Variant calling	https://github.com/tszalay/poreseq	Szalay & Golovchenko 2015