Chapter 1

The Current State of Nanopore Sequencing

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Abstract

Nanopore sensing is a disruptive, revolutionary way in which to sequence nucleic acids, including both native DNA and RNA molecules. First commercialized with the MinION™ sequencer from Oxford Nanopore Technologies™ in 2015, this review article looks at the current state of nanopore sequencing as of June 2022. Covering the unique characteristics of the technology and how it functions, we then go on to look at the ability of the platform to deliver sequencing at all scales—from personal to high-throughput devices—before looking at how the scientific community is applying the technology around the world to answer their biological questions.

Key words Nanopore, Sequencing, Applications, MinION, PromethION, GridION, Flongle, Disruptive

1 Introduction

The term "disruptive" is applied to technologies all too often. Without looking far in the biotechnology, consumer electronics, or commercial technology space, you find claims of the next best thing. How then are genuine claims sorted from background noise? The answer lies not in the messaging from any individual company, but in the actions of technology users. Think of the mobile phone—now entirely integrated into society, it was also once viewed as disruptive. There came a tipping point in its adoption when people stepped through their front doors, looked at the mobile in their hand, and collectively decided the landline was a thing of the past. Novel aspects of the technology (hey look, you can pretend to be a snake that eats flowers!) combined well with traditional capabilities, consumers decided the technology was good enough for them, and the disruption was complete.

Nanopore sequencing is a technology demonstrating disruptive capabilities in user's hands right now. In 2022, a group of researchers at Stanford University set a Guinness world record for

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the fastest whole genome sequence [1]—one from a clinical research setting [2]. In 2016, researchers on the International Space Station carried out the first ever sequencing in space—and now are better informed on unknown microbes and their scientific experiments alike [3]. And when the world came to a halt in 2020 due to the global SARS-CoV-2 pandemic, nanopore sequencing aided researchers across the globe to sequence, assess, and track viral genomic information in ways that previously had not been possible within-country [4–6] building on work carried out on Ebola [7] and Zika [8] virus sequencing. It was the scientific community seeing the potential of the sequencing platform provided by Oxford Nanopore Technologies who stepped forward to demonstrate its disruptive capabilities in the real world.

Unsurprisingly, disruptive technologies are often based upon concepts not previously utilized in the space the technology targets. When thinking of whole genome sequencing, individuals commonly jump to short-read optical-based methods: widespread in use, but with limitations in device size and time to answer, among other things. Sequencing in this way has changed little over the decades, as centrally located devices rely upon increased parallelization and chemistry tweaks to demonstrate "progress." Long-read optical-based efforts yield greater biological insight, but are if anything struck with more extreme device limitations than short-read optical methods. Nanopore sequencing enables both long-read and short-read biological insights, while shrugging off optical limitations and instead utilizing digital signals from an electrical output. Put romantically, the analogue wristwatch of optical sequencing, quaint and admired with historical sentiment, is being displaced by the digital smartwatch of nanopore sensing, connecting you to the world and delivering more information than you ever knew a wristwatch could.

2 The Technology

Charming analogies aside, how nanopore sequencing works is beautifully simple yet has proven fiendish to implement. Originally penned in 1989 by Professor David Deamer of UC Davis, the idea then lay dormant until a conversation with Harvard University's Professor Dan Branton in 1991 [9]. Following years of academic development and collaboration, the concept was finally commercialized with the launch of the MinION device in 2015 [9]. The basis of how this system functions is covered in Fig. 1, and the resultant signal is decoded to the sequence of bases on the DNA strand through artificial neural networks akin to those used for speech recognition. Each nanopore does this thousands of times, strand after strand, resulting in an incredibly high-throughput system that can be tuned by altering total nanopore numbers or

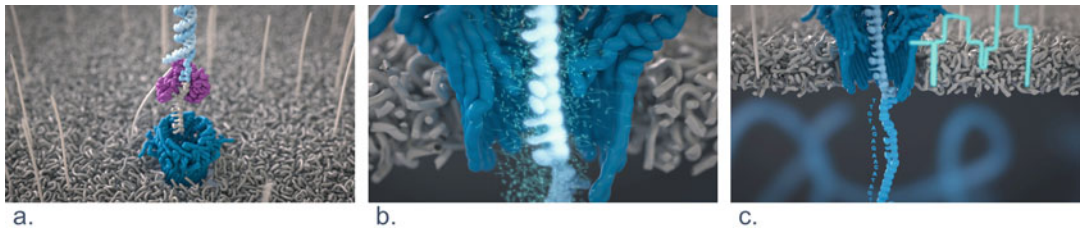

Fig. 1 The principle of nanopore sequencing. (**a**) A protein nanopore (blue) is imbedded into an electronically resistive lipid membrane (grey), before adapted DNA libraries containing a motor protein (purple) are introduced, and the motor feeds DNA progressively through the pore. (**b**) An ionic current (represented by light blue dots) is passed through the nanopore as the DNA translocates through the pore. (**c**) The bases within the nanopore block the current depending on their size and structure. As the strand moves progressively through the pore, a "squiggle" trace is produced, which is decoded into sequence data using artificial neural networks

the speed at which the molecular motor operates, among other variables.

Being based upon electrical signals rather than optical outputs, the system provides real-time feedback, permitting experiments to be stopped and started as required. The merits of the nanopore system further extend to its capacity to sequence native DNA and RNA, avoiding amplification bias associated with PCR-reliant technologies [10]. Crucially, this allows native base modifications (e.g., methylation) to be captured in the information-rich nanopore signal with no additional sample preparation. Recent algorithmic developments ensure methylation status is provided alongside canonical base data [11]—this real-time data is permitting innovative research customers to demonstrate potential applications for intraoperative central nervous system (CNS) tumor classification while the patient is still on the operating table [12].

One final, and to some surprising, characteristic of nanopore sequencing is the read length-agnostic nature of the system. Nanopore sequencing has been typecast as a long-read technology, understandable when read lengths of up to 4 million bases in one continuous read have been demonstrated (easily the longest in the world). This sort of capability should not be underestimated, as longer fragments provide comprehensive information not possible with short reads: structural variants implicated in human disease or agricultural traits can reach up to megabases in scale, far beyond the ability of short reads to span; expressed transcripts can be sequenced end to end in single long reads, enabling unambiguous identification of fusion transcripts; and long reads allow you to access more of the genome than short reads [13], unarguably providing insights not possible with other technologies. On the short-read front, following updates to the sequencing software in early 2022, reads as short as 20 bases can now be processed. With

this capability nanopore sequencing can provide insights into applications involving but not limited to cell free DNA, ancient DNA, or liquid biopsy research. The same flow cells, devices, software, and chemistry are used for short and megabase-long reads alike, making nanopore sequencing the only single technology capable of spanning five orders of magnitude. With nanopore sequencing you can observe the true biology present rather than simply sequence a biased proxy of your original sample.

3 The Platform

Putting the performance of the Oxford Nanopore platform on paper always poses a risk: by the time this work (written in June 2022) is published, it is highly likely some of the below information will be outdated. This demonstrates a core merit of Oxford Nanopore, that every aspect of the platform—nanopores, motors, buffers, software, algorithms, and hardware—is consistently iterated and improved upon to deliver ever greater performance (locked-down versions of the platform are available for those requiring revision control). In doing so, however, Oxford Nanopore ensures the community of scientists using its technology has access to the scale and performance of sequencing they need to answer their specific biological question.

Focusing on scale, thanks to a chemistry consistent across all flow cell and device types, a researcher interested in, e.g., fusion genes can use the Flongle™ for cheap, rapid, targeted results in minutes, or can use high-output PromethION™ Flow Cells to identify novel isoforms even if they are in low abundance. This is achieved through a combination of flow cell and device offerings, from the very small to the very high throughput (Fig. 2).

The MinION, the first sequencer launched by Oxford Nanopore, is powered by a USB and runs off a laptop. The size of the MinION (W 105 mm, H 23 mm, D 33 mm, and a featherweight 87 g) makes it simple to ship (just pop it in a jiffy bag!) and lends itself to portability so well that it has been used at some of the farthest-flung regions of the globe [14] and off it [3]. Running MinION Flow Cells with 512 channels for sequencing, it has gone from generating 500 Mbases of data at launch to demonstrated outputs of 43 gigabases (Gb) in-field [15]. Using MinION as a benchmark, it is possible to see how the technology has since been scaled upwards and downwards.

The Flongle, consisting of the Flongle adapter (with the same footprint as the MinION Flow Cell) and a cheap, disposable Flongle Flow Cell (see Fig. 2), has over fourfold fewer channels (126) than the MinION Flow Cell but can still generate up to 2.8 Gb of data. Perfect for targeted experiments or sequencing of small

Flow cell name	Flongle	MinION Flow Cell	PromethION Flow Cell
Number of channels	126	512	2,675
Theoretical maximum output*	2.8 Gbases	50 Gbases	290 Gbases

Device name	MinION	MinION Mk1C	GridION	PromethION 2 Solo	PromethION 2	PromethION 24	PromethION 48
Flow cell compatibility	Flongle, MinION			PromethION			
Number of flow cells that can be run	1	1	5	2	2	24	48

Fig. 2 The flow cells and devices for nanopore sequencing. The Flongle (**a**) consists of two parts, a reusable adapter, and a single-use flow cell. It has the same footprint as the MinION Flow Cell (**b**) meaning both can be run on the MinION (**d**), MinION Mk1C (**e**), or GridION (**f**) devices. Any combination of Flongle or MinION can be run on the GridION device. The PromethION Flow Cell (**c**) is compatible with all PromethION devices (**g**–**j**). With capacity for different numbers of flow cells, total device yields vary in line with the number of flow cells they can run. Where multiple flow cells can be run, all are individually controllable, meaning no requirement exists to run all flow cells at once and as a result samples can be run on demand. *Theoretical maximum output when flow cell or device is run 72 h (16 h for Flongle) at 420 bases/second. For devices, this is when all flow cells are run at once and the highest yielding flow cell option is chosen. Outputs may vary according to library type, run conditions, etc.

microbial genomes, the Flongle represents an individual flow cell price point that is unmatched and, with its innovative separation of electronics from fluidics, acts as a technological primer for future cost reductions against the higher-output MinION and PromethION Flow Cells.

The PromethION Flow Cell, as with the MinION Flow Cell, comes in a reusable design that can be washed and reused to maximize data output across several experiments (and can be returned to Oxford Nanopore for recycling also). With 2,675 channels for sequencing, as of early 2022, the best-demonstrated yield in-field stands at 245 Gb [16]. As mentioned above, the technology stands to make further improvements and, with adjustments such as passing the DNA through nanopores even faster, holds promise of as much as doubling outputs as they stand today.

Oxford Nanopore sequencing devices (Fig. 2) are designed around the flow cells they run. Anything operating a MinION Flow Cell—the MinION, MinION Mk1C, and GridION™—is also compatible with Flongle Flow Cells. Where multiple flow cell positions are available as with the GridION, any combination of MinION or Flongle can be run alongside one another and stopped and started as experimental requirements dictate. PromethION Flow Cells run exclusively on PromethION devices, with the highest output PromethION 48 running up to 48 flow cells in parallel. In addition to a 24-flow-cell version, two recent additions to the PromethION line (PromethION 2 and PromethION 2 Solo) are capable of running two flow cells each, providing options for those who want PromethION-scale yield but with fewer samples to sequence. Except for the MinION and the PromethION 2 Solo, all devices contain the necessary compute to run sequencing and carry out basecalling—it is perfectly possible for the two remaining devices to be run from commercially available laptops. All devices utilize MinKNOW™, a data acquisition and control software, for run setup, basecalling, and data handling.

Sample preparation is possible through manual or automated methods. Kits are available to prepare native, amplified, or ultralong DNA libraries, along with cDNA and direct RNA. More recently, kits have been adapted for automation on liquid handlers. In addition to this, Oxford Nanopore has created the VolTRAX™, an automated sample preparation device containing magnetic arrays, heating elements, and fluorometers, running from a laptop and working with VolTRAX-enabled forms of Oxford Nanopore library preparation kits. Notable to Oxford Nanopore is the ability to prepare libraries of native DNA samples, which may contain a range of fragment sizes. In conjunction with retained modified base information, the technology is capable of giving a true representation of the biology present.

To complete the "full stack" offering, after basecalling with tools such as Guppy (to be replaced with a new basecaller, Dorado, in late 2022), a number of analysis options are available for those new to data analysis or the very green-fingered alike. The click-and-go, cloud-based analysis platform EPI2ME™ offers predefined workflows for species identification, structural variant calling, single cell analysis, and more. For those wishing to learn to build and manipulate data analysis pipelines, EPI2ME Labs provides step-by-step, didactic guidance for multiple applications including assembly, metagenomic classification, and cDNA isoform detection. Built within the Nextflow framework, EPI2ME Labs leverages the ability to scale to cluster- or cloud-based installations for those who need to deploy and routinely run high-throughput analysis.

4 Applications

While perhaps obvious due to the breadth of offerings covered thus far, it is worth reenforcing that Oxford Nanopore is *not* a company making a single device do a single thing for a single group of people. Oxford Nanopore is creating the next generation of tools for use by tomorrow's scientists, thriving and succeeding off the incredible work by the scientific community: from clinical research on NICU cases [2] to tracking the endangered Kakapo [17], from sequencing on Icelandic glaciers [18] to identifying unwanted yeasts in breweries [19], research into the genomes of cancer patients in multi-sample studies [20], or checking the sequence of plasmid constructs [21]. Through the hard work of this community, the boundaries of what is possible are consistently redrawn, and in return Oxford Nanopore continues to drive their products to generate faster, more accurate, and lower-cost results. On MinION alone, yields have increased over a hundredfold since the device's introduction, developing hand in hand with accuracy improvements. As of June 2022 (taking note of the cautionary words at the start of The Platform section), single-pass, single-molecule accuracy sits at 99.6% modal. Duplex accuracy, where both strands of the DNA molecule are sequenced, is at 99.92% modal accuracy, greater than Q30—and there is still room for improvement. For years nanopore sequencing has provided insights well beyond the reach of alternative technologies, and with the mission to enable the analysis of anything, by anyone, anywhere, these insights will continue to develop not only in the DNA sequencing space, but in the transcriptomic, proteomic and, epigenomic space also. Here is a collection of just some of the ways nanopore technology is currently being used around the world.

4.1 The World's Fastest Genomes

Alluded to already, Oxford Nanopore worked with a team led by Stanford University School of Medicine to develop a rapid, whole genome sequencing approach, setting a world record for the fastest human genome ever sequenced in the process: 5 h and 2 min [1]. Headed by Dr Euan Ashley with collaborators from Oxford Nanopore; University of California, Santa Cruz; Baylor College of Medicine; NVIDIA; and Google, this stunning new benchmark was possible in part thanks to the PromethION 48, its utilization maximized by splitting one genome across all 48 flow cells at once [2, 22]. In one iteration of their experimental setup, the PromethION generated 204 Gb in 2 h and 42 min [2], just over 60-fold coverage of the human genome with one complete pass sequenced every ~2.5 min. This unprecedented speed of sequencing enabled the team to identify key variants in experimental data within 7 h and 18 min [22], almost half of that previously achieved with short-read sequencing [23].

4.2 The World's Largest Genomes

PromethION Flow Cells are not only powerful in parallel, but when run individually provide hundreds of Gb of sequence data, including long sequencing reads. This capability enabled the successful assembly of the Australian lungfish genome, which, at 43 Gb, is ~30% larger than the previous record assembly belonging to the axolotl [24, 25] and currently the largest known animal genome assembly in the world. Using data batched into buckets of read N50 9 kb, 27 kb, or 46 kb, the resultant assembly possessed a contig N50 over 6000 times greater than the alternative long-read axolotl assembly, with over 17,000-fold fewer contigs [26].

4.3 The World's Most Complete Human Genome

It probably has not escaped the attention of readers that in 2021, the Telomere-to-Telomere Consortium announced the full completion of the human genome, addressing the remaining gaps and removing "a 20-year-old barrier that has hidden 8% of the genome from sequence-based analysis, including all centromeric regions and the entire short arms of five human chromosomes" [27]. Specifically, reads greater than 100 kb in length generated with nanopore sequencing enabled the complete assemblies of the Y centromere and the entirety of chromosome X, excelling at highly repetitive regions of the genome that could not be adequately resolved by other technologies [27]. This work enables comprehensive study of genomic variation across the entire human genome, poised to drive future discovery in human health and disease [27].

4.4 Viral Genomes the World Over

So much has been written about the global SARS-CoV-2 pandemic and its impact on our lives, there is no succinct way to introduce this topic. Oxford Nanopore was humbled to be identified by researchers the world over as able to play a key part in the tracking of this ever-evolving situation [28], where rapid sequencing workflows are coupled with data streaming and analysis in real time. Thanks to the ARTIC Network and their comprehensive library preparation [29] and data analysis [30] workflows, modular, scalable devices such as GridION could conduct on-demand experiments offering rapid access to data for public health scientists. With devices easily deployed around the world within days and requiring little to no up-front cost, the decentralized network of public health scientists supported by nanopore sequencing has been able to generate over one million SARS-CoV-2 genomes as of Spring 2022, ensuring the evolution and spread of the virus can be accurately measured as required. In the growing field of genomic epidemiology, Oxford Nanopore is proud to offer a technology platform for supporting public health scientists now and in the future.

4.5 Targeted Sequencing on the Other Side of the World

Continuing the world theme, the Garvan Institute for Medical Research in Sydney, Australia, is situated on the opposite side of the world from the headquarters of Oxford Nanopore in Oxford, UK. In 2022, scientists from The Garvan, lead by Dr Ira Deveson, published impressive work on targeting short tandem repeat (STR) expansion disorders with programmable nanopore sequencing [31]. For those unfamiliar with the term "programmable" in this context, it refers to a feature unique to nanopore sequencing: adaptive sampling (see Fig. 3 and accompanying legend). By simply inputting a reference file and genomic coordinates, the group were able to target all known neuropathogenic STRs in a single experiment with a single MinION flow cell [31]. With STR expansions responsible for heritable disorders including Huntington's disease, fragile X syndrome, cerebellar ataxias, epilepsies, dementia, and ALS [31], the real-world implications and future potential of this work need little elaboration. This elegant and streamlined solution has unquestionable impact based on the evidence presented by Ira and his team—as a group they anticipate that adaptive sampling "will be a powerful approach to STR gene discovery" in addition to resolving "many previously unsolved cases in the future" [31].

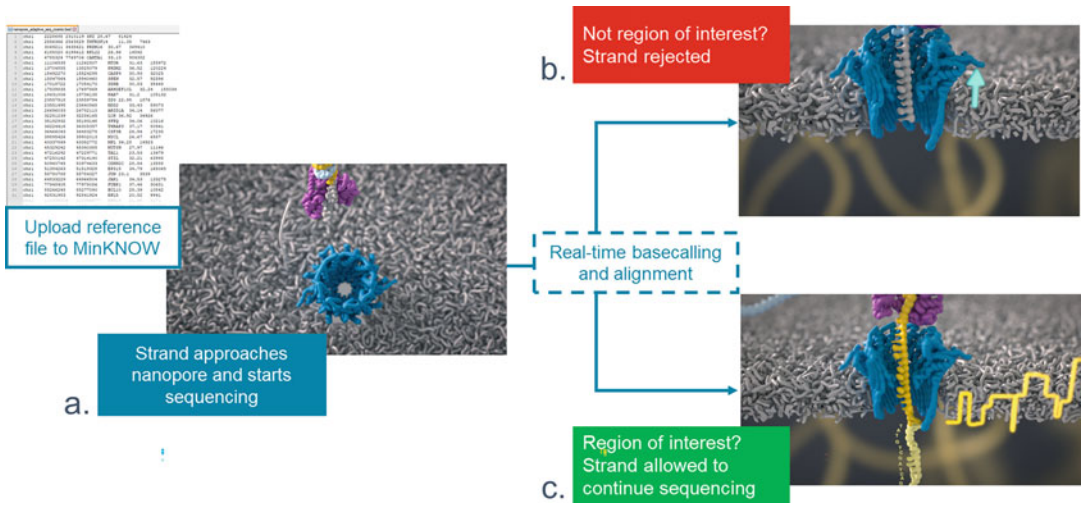

Fig. 3 Adaptive sampling. Following upload of a file containing a genome reference and genomic coordinates to the operating software MinKNOW, the sequencing run starts and a strand of DNA moves to and through the nanopore (**a**). As the strand moves through the pore, basecalling begins and alignment happens in real time. Within ~0.5 s, a decision can be made. If the sequence does not match the regions of interest within the reference files, the strand is ejected from the nanopore (**b**). If the sequence does match, the strand is permitted to continue sequencing through its entirety (**c**). Once the strand is through the pore, the process begins with the next strand and so on

4.6 A World of Potential

This is not an exhaustive list of applications, merely a snapshot into areas where nanopore sequencing is already making a difference. While these focus upon research-based examples, Oxford Nanopore has taken position in the foothills of applied markets including but not limited to agriculture, food safety, veterinary science, public health, and more. Additionally, with the establishment of subsidiary company Oxford Nanopore Diagnostics, the aim is to specifically enable actionable decisions in healthcare with end-to-end solutions. By collaborating with talented and visionary scientists the world over, nanopore sequencing will enter a forum where human lives are positively impacted every single day.

5 Summary

To close this introduction, the contents of this book demonstrate exactly why nanopore sensing is a technology for the future. Oxford Nanopore provides a toolkit for innovators, tinkerers, and the most inquisitive to assess the living world around them, encompassing a much broader scope than Oxford Nanopore can research themselves. Along this path of broadening biological understanding, there will be difficulties to work through and obstacles to overcome, but ultimately the outcome from doing so will lead to everyday breakthroughs that, collectively, combine into something much greater than the sum of their parts. The authors of the chapters within this book have begun to remove these obstacles for you, demonstrating brand-new capabilities and sharing with you the nuances they encountered. With their help, the community can continue to develop their understanding of the living world around us and generate the most important of answers—the ones for their most burning biological questions.

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