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Review Article

MinION rapid sequencing: Review of potential applications in neurosurgery

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Abstract

Background: Gene sequencing has played an integral role in the advancement and understanding of disease pathology and treatment. Although historically expensive and time consuming, new sequencing technologies improve our capability to obtain the genetic information in an accurate and timely manner. Within neurosurgery, gene sequencing is routinely used in the diagnosis and treatment of neurosurgical diseases, primarily for brain tumors. This paper reviews nanopore sequencing, an innovation utilized by MinION and outlines its potential use for neurosurgery.

Methods: A literature search was conducted for publications containing the keywords of Oxford MinION, nanopore sequencing, brain tumor, glioma, whole genome sequencing (WGS), epigenomics, molecular neuropathology, and next-generation sequencing (NGS). In total, 64 articles were selected and used for this review.

Results: The Oxford MinION nanopore sequencing technology has had successful applications within clinical microbiology, human genome sequencing, and cancer genotyping across multiple specialties. Technical details, methodology, and current use of MinION sequencing are discussed through the prism of potential applications to solve neurosurgery-related scientific and diagnostic questions. The MinION device has proven to provide rapid and accurate reads with longer read lengths when compared with NGS. For applications within neurosurgery, the MinION device is capable of providing critical diagnostic information for central nervous system (CNS) tumors within a single day.

Conclusions: MinION provides rapid and accurate gene sequencing with better affordability and convenience compared with current NGS methods. Widespread success of the MinION nanopore sequencing technology in providing accurate,

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rapid, and convenient gene sequencing suggests a promising future within research laboratories and to improve care for neurosurgical patients.

Key Words: DNA, MinION, nanopore, neurosurgery, sequencing, tumor

INTRODUCTION

Since its introduction, gene sequencing has revolutionized the understanding of human genetics and the practice of medicine. The advent of sequencing made way for landmark achievements such as the Human Genome Project.[35,43,68] Sequencing has also played an integral role in advancing patient care through genomics and precision medicine. Supporting the practice of personalized medicine, DNA sequencing has informed decisions in pharmacotherapy, infectious disease, genetic counseling, and cancer diagnosis and treatment. Genomic characterization of tumor tissue has become increasingly important over the last decade for creating specific treatment plans and evaluating prognosis. Continued evaluation of the tumor profile is often necessary as frequent mutations can result in initial therapy decisions to be rendered ineffective.

Gene sequencing has become critical to multiple aspects of neurosurgery including the diagnosis, treatment, and evaluation of central nervous system (CNS) tumors.[42,60] Cancers of the CNS are difficult to profile due to the often unique challenges of accessing the tumor tissue. Numerous studies have investigated the use of next-generation sequencing (NGS) on cerebrospinal fluid (CSF) to detect and evaluate CNS tumors. These reports suggest that rapid gene sequencing on CSF can provide improved detection rates, inform genotype-based chemotherapy, and serve as a marker for clinical response.[18,50,69] Recent feasibility studies have trialed the use of NGS within a diagnostic pipeline to rapidly evaluate and categorize patients into biomarker-driven treatment arms.[51] Studies among various specialties have reported that at maximum pace, in-depth molecular analysis with NGS can be completed from biopsy to final report within 4 weeks.[51,71] The benefit of rapid initiation of targeted therapy cannot be overstated, but as such, the molecular analysis pipeline must be improved. This review intends to explore the current state of Oxford MinION–based nanopore sequencing in a clinical context with a particular focus on its applications within scope of neurosurgical specialty.

MATERIALS AND METHODS

This study does not endorse any specific corporate technology and has no marketing or financial relationship with any corporate entity or trademarked technology named in this paper. This study received no outside funding. An electronic literature search was conducted using the National Library of Medicine for publications containing the keywords of Oxford MinION, nanopore sequencing, brain tumor, glioma, whole genome sequencing (WGS), epigenomics, molecular neuropathology, third generation sequencing (TGS), and NGS. Bibliographies of select publications were additionally reviewed to complete the literature search. The articles were screened and selected based on the inclusion criteria for topics that examined the use of nanopore sequencing, particularly the Oxford MinION. Additional articles pertaining to the understanding of DNA sequencing were also included. In total, 64 articles were selected for review.

Gene sequencing

The first method of sequencing, known as Sanger Sequencing, was introduced in 1977 and remained the gold standard of sequencing until the popularization of second-generation sequencing (SGS), also named NGS, in 2005.[57,61] In contrast to Sanger's sequencing method of chain-termination, SGS improved throughput and reduced costs through a variety of novel methods including pyrosequencing (Roche 454), sequencing-by-synthesis (Genome Analyzer, Illumina), and sequencing-by-ligation (SOLiD).[58] These various sequencing methods continued to dominate for the following decade until the introduction of the next era, TGS. Unlike SGS that functions by breaking long DNA fragments into short segments, TGS introduced sequencing that occurs at the single molecule level. This method not only reduced read time from the scale of days to hours but also significantly increased read lengths. Furthermore, sequencing at the single molecule level also allows for direct detection of DNA methylation and 5'—*C*—*phosphate*—*G*—3' (CpG) sites as well as real-time production of nucleotide reads.[55,63]

Two major technologies exist under the umbrella of TGS: PacBio's single molecular real time (SMRT) sequencing and Oxford's Nanopore Technology (ONT). The latter technology, also called nanopore sequencing, was a concept first suggested in 1995 in a concerted effort by multiple research groups, notably including George M. Church.[14] The research from the following decade accumulated in the foundation of the Oxford startup. In April 2014, the Oxford MinION, a handheld DNA sequencing device, was released for beta-testing through the MinION Access Programme (MAP). Distributed to more than 1,000 labs, the earliest reports characterizing the functionality and applications of the device began appearing.[10,11,29,36,41,54]

Oxford MinION device

Sequencing

Understanding nanopore technology and the molecular science behind the Oxford MinION may present a daunting task for clinicians removed from basic science research. In this paper, we present a simplified version. The MinION is a handheld 90 g device that can plug into any computer with a standard USB 3.0 port. The MinION functions by passing long sequences of DNA (8-20 kbp) through a pore within a small protein, "the nanopore," that is embedded within a membrane. The single stranded DNA molecule (ssDNA) that passes through the nanopore can optionally contain a hairpin adaptor that physically connects the "template" strand and the "complimentary" strand [Figure 1]. If only the template strand is read, the read is considered to be 1D; if both are read, the read is titled 2D. 2D reads provide better accuracy and representation of the true sequence of the strand. However, since May 2017, ONT has discontinued flow cells compatible with 2D technology. This has been replaced by a newer, improved technique named $1D^2$ where both the template and complementary strands are sequentially read but they are not physically connected by an adaptor. As this ssDNA passes through the nanopore, it disrupts an electrical current that exists through the pore. Disruptions in the current are typically recorded as "5-mers" or "6-mers" as each data point represents an electrical disruption caused by a total of five or six adjacent bases. The resultant raw data are represented in a graph referred to as the "squiggle plot" [Figure 2].

Basecalling

Once the device is plugged into a computer, it is operated with the MinKNOW software program. This program is responsible for acquiring and analyzing data, and providing the interface for additional device control. After the raw data, saved as a FAST5 file, are collected, it must undergo an analysis to be converted into a nucleotide sequence; this process is termed basecalling. There are multiple software platforms that are publicly available to complete the basecalling process, namely, Metrichor,[5] Nanocall,[17] Albacore,[1] and Chiron.[66] Metrichor requires internet access, and performs the analysis using a Hidden Markov Model (HMM), whereas Nanocall is an offline alternative that is additionally capable of providing real-time basecalling as the data are being collected. Albacore v2.0.1 is a recently released program that performs "raw basecalling," a method predicted to improve accuracy by bypassing intermediate steps in the recurrent neural network (RNN) analysis. Each program varies in accuracy and should be selected to best serve the needs of the data.[17] More details on analysis and performance comparisons of popular basecalling software can be found in a recent review by Wick *et al*.[70]

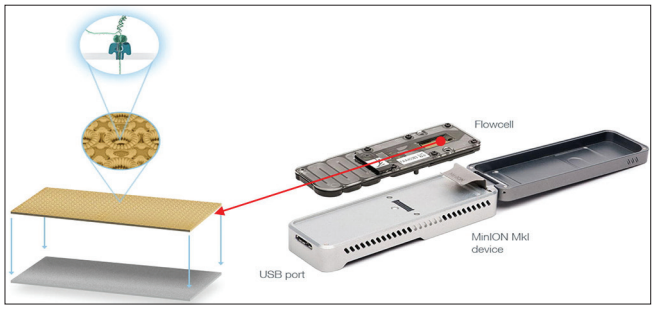

Figure 1: Miniature nanopore sequencing device with microscopic view of flowcell containing embedded nanopore[43]

Further analytics

Once the initial data are collected, further analysis can provide additional information regarding the presence of mutations, characterization of base repeats, and specific methylation patterns. Many of these specific analytical tasks require supplemental software to accomplish. To detect mutations within the targeted sequences, the template reads must be compared with a reference human genome. This can be accomplished using Galaxy,[4] a web-based platform that uses a BWA-MEM algorithm for alignment analysis or directly with the Burrows-Wheeler Aligner (BWA) software.[2] Many other alignment methods exist including basic local alignment search tool (BLAST), local alignment search tool (LAST), basic local alignment with successive refinement (BLASR), and GraphMap. Among these, GraphMap was specifically developed to analyze data produced by nanopore sequencing.[64]

The detection of copy number variation is a complicated process that is prone to error. To maximize accuracy, multiple steps are typically involved in analyzing the DNA sequence. The sequence is first "normalized" using specific software such as QDNAseq[7] to evaluate for multiple characteristics, including G-C percentage in exons and structural aberrations.[59] The sequence can then be analyzed by a separate program, particularly R,[8] to measure copy number variations.[20,50]

Direct detection of methylation patterns of DNA is one of the unique strengths of nanopore sequencing. Specific studies have shown that the 5-methylcytosine (5-mC) modified base can be directly detected based on unique electrical signals detected by the MinION device without the need for additional chemical treatment. HMM models can be trained to detect specific methylation patterns and distinguish them from other base pairs. However, this technology is still under development and has shortcomings. Not all methylated bases can be detected, and when both methylated and unmethylated bases are present within the same k-mer, the detection system is inaccurate. Improvement in basecalling software can be expected to resolve these obstacles.[63]

Sample preparation

A DNA-containing sample needs to be treated with a variety of steps before it is ready to be analyzed with the MinION device. This process, named library

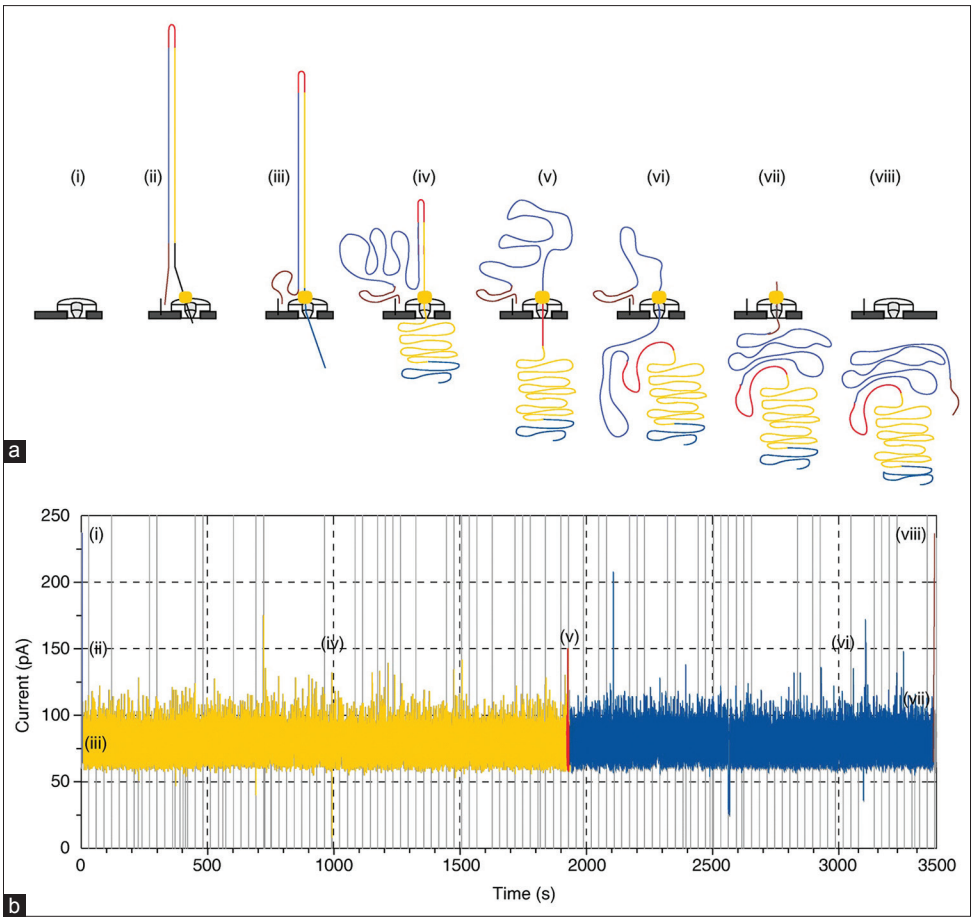

Figure 2: Principle of nanopore sequencing. (a) Visualization of 2 ssDNA strands connected by adaptor passing through the nanopore. (b) Graphical representation of raw data produced by MinION, also known as "squiggle plot"[29]

preparation, has recently been simplified and is available in predesigned kits. The components and protocol included in each kit are designed to achieve a particular purpose desired by the investigator. Kits available for purchase online include Ligation Sequencing Kit 1D (SQK-LSK108), Rapid Barcoding Kit (SQK-RBK004), Rapid Sequencing Kit (SQK-RAD004), and 1D^2 Sequencing Kit (SQK-LSK308). Kits that focus on preparation of a cDNA or RNA sample are also available. ONT continuously updates these kits, improving on the protocol and components to provide the best outcome and experience in library preparation. Intersection of computer programming and the electrical manipulation of fluids resulted in the creation of VolTRAX, a handheld automated library preparation device with consumable cartridges. The device allows the user to load samples into the cartridge and run a preprogrammed code that manipulates, moves, and mixes the liquid samples as desired. VolTRAX is designed to provide reproducible, portable, and simplified library preparation.[6] Its potential applications exceed far beyond DNA sequencing.

Improvements in technology and throughput

The final accuracy of the nucleotide sequence is largely influenced by both the basecalling process and the internal chemistry of the nanopore. Basecalling software includes technology that uses deep and machine learning models to convert the raw electrical signal into a nucleotide sequence. Basecalling accuracy has rapidly improved between 2014 and 2017 with new software releases by ONT, such as Albacore v2.0.1, and third-party researchers publishing open-source software. Both Chiron and Albacore v2.0.1 are recently released programs (September 2017) that bypass error-prone steps to provide an improved accuracy in basecalling.[1,66] Each program utilizes one or multiple machine learning models such as convolutional neural network (CNN) and RNN. Chiron and Albacore v. 2.0.1 have been reported to have the highest accuracy, also called identity rate, when compared with other basecalling software. Both outperformed other softwares when using samples from virus, *E. coli*, M. tuberculosis, and human DNA.[66] Other existing basecalling software include Metrichor, Nanonet, DeepNano, BasecRAWller, and Scrappie, each with unique capabilities and accuracy ratings.

Another aspect of the MinION pipeline that can significantly affect the throughput and accuracy of the sequencing is the flow cell. The flow cell is the consumable part of the MinION that contains

the nanopore proteins and the Application-Specific Integrated Circuit (ASIC) sensor. The flow cell is also the site where the prepared DNA sample is directly added. Currently, there are two flow cells manufactured by ONT, the R9.4 and R9.5. Previous flow cells that have since been improved include the R7.3 and R9. The R9.4 flow cell is graded for all 1D experiments, while the R9.5 is compatible with both 1D and $1D^2$.[6] Few investigators have quantified the improved chemistry reporting a 91% accuracy for the R7.3 flow cell and a 94% accuracy for the R9. Similar studies comparing the R9.4 and R9.5 have yet to be published as both are recently released products.[63]

Current applications

After only 4 years since its release, ONT's MinION has been utilized in a wide variety of applications, including WGS,[28] single nucleotide polymorphism (SNP) identification, genotypic analysis of cancer,[20]forensics,[15] and microbiology characterization.[47] Its immediate relevance to neurosurgery is the use of the MinION in the context of rapid genetic profiling of normal or pathological tissue samples for improvement in diagnostics and implementation of personalized treatment approaches.

Tumors

Recent (2016) World Health Organization (WHO) classifications for CNS tumors, for the first time, require molecular and genetic analysis for definitive diagnosis.[42] Currently, only a single investigation by Euskirchen *et al*. published in June 2017 has employed the MinION in the use of genomic diagnosis of brain tumors. Using the MinION to create a same-day diagnostic pipeline, the investigation examined single nucleotide variations, copy number, and methylation patterns from tumor tissue from 28 patients, although not all 28 samples yielded sufficient read depth. Samples underwent ultra-low pass WGS and amplicon sequencing to identify mutations in many cancer-related genes, including TP53, IDH1, TERT, and 1p/19q. 1p/19q was correctly detected in three fourth of the samples. Using machine-learning-based molecular classification based on copy number and methylation data, 7/7glioma and 2/2 medulloblastoma samples were correctly classified based on WHO classification. Remaining samples were either determined to be not classifiable or the sample quality was determined too poor to accurately attempt classification. Overall, this proof-of-principle study reported on the feasibility of accurate rapid diagnosis, and significantly low costs of equipment.[20]

The MinION device has also been used in other cancers, including leukemia,[45,46]lung cancer,[65] and pancreatic cancer.[49] Each study has cited excellent accuracy, detection, and reliability in the identification of mutations, even when compared with current gold-standard methods. The MinION has demonstrated rapid detection of single nucleotide variants (SNVs), insertions, deletions, and translocations in many cancer and therapy relevant genes, including TP53, EGFR, KRAS, NRAS, NF1, CDKN2A/P16, SMAD4/DPC4, and BCR-ABL.[45,46,49,65] Certain studies have suggested that the MinION, in its current state, is ready to be deployed to hospital pathology laboratories to aid in genetic profiling.[45]

Microbiology

In addition to its application for gene profiling in cancer, the MinION has had considerable success in the identification of microbiology, WGS, SNP identification, and forensics. Multiple studies have utilized the MinION to identify and characterize specific pathogen species within known microbiological communities and clinical samples.[23,31,32,47,52] Significantly, MinION technology was used by Quick *et al*. that subjected 142 Ebola virus samples to real-time genome sequencing during the recent West African epidemic.[53] The MinION was shown to be a transportable and affordable alternative to the cumbersome and delayed process of transporting samples to distant laboratories. This study reported that, even in remote settings, the complete workflow from data collection to sequencing could be completed within a single working day, with sufficient reads (5,000 to 10,000) generated within an hour. The versatility of nanopore sequencing technology may become practically important for application in rapid diagnosis of perioperative neurosurgical infection pathogens.

Human genome sequencing

Current commercial and hospital laboratory-based methods of WGS are limited to NGS techniques. As research continues to implicate genetic mutations, specifically SNPs, in context of countless diseases, the need for genetic screening and WGS steadily rises. Advancement in precision medicine and personalized treatment plans are facilitated by research demonstrating actionable results and progress in the speed, cost, and accuracy of WGS. Within neurosurgery, many disease processes have been found to have a major genetic component. Recent reports on intervertebral disc disease suggest that up to 75% of the underlying etiology is attributed to genes.[44] Other diseases of neurosurgical importance found to have a component of genetic etiology include gliomas,[9,48] schwannomas,[24] meningiomas,[12] intracranial aneurysms,[26,38,72,73] arteriovenous malformations,[19] cavernous malformations,[21] subarachnoid hemorrhage,[25] idiopathic scoliosis,[22] moya-moya disease,[27] hemorrhagic, and ischemic strokes. The results generated from these genome wide association (GWA) studies, revealing specific SNPs and their association with disease, are the stepping stones for better understanding of disease risk, prediction, treatment, and prognosis.[16,62] A major limitation to the widespread conduction of GWA studies

and advancement of this frontier is the steep initial investment required for equipment and high run cost per sample.[16] Nanopore sequencing has the potential to remedy these obstacles of WGS in the future.

Nanopore sequencing has demonstrated the capability to accurately and rapidly sequence the whole genome of bacteria, eukaryotes, and viruses reliably.[30,41,52,67] Successful completion of genome sequencing requires additional steps in analysis within the pipeline, namely genome assembly and genome polishing. Whole genome assembly (WGA) can be accomplished through a variety of available software including PacBio Corrected Reads (PBcR) Assembler, Canu, Falcon, and Miniasm [Table 1]. Each assembly software has unique strengths and weaknesses based on its algorithm and associated error correction methods. The assembly software takes into account overlapping and repetitive regions and is responsible for synthesizing the individual reads into a contiguous, consensus sequence with the greatest accuracy. This assembly process requires the most computational power when compared with other steps within the analytic pipeline. Once assembled, the sequence can undergo polishing with software such as Nanopolish [Table 1] to improve nucleotide consensus. The software uses the original raw data produced by the MinION and extrapolates additional information to improve the accuracy of the sequence.

Jain *et al*. reported the use of the MinION in assembling a high consensus human genome.[28] The capability of the MinION device for ultra-long reads enables it to serve as a superior method of WGS when compared with established NGS methods. This is due to the improved representation of tandem repeats, closure of gaps, and identification of nucleotide sequences previously unknown. This study shows practicality for the continued use of nanopore sequencing for human genome assembly and represents advancement in personalized medicine.

Table 1: Software for genome assembly and preparation used in conjunction with Oxford Nanopore Technologies MinION

Software name	Link	Ref.
PBcR	http://wgs-assembler.sourceforge.net/wiki/index.php/PBcR	[33]
Canu	https://gitub.com/marbl/canu	[34]
Falcon	https://github.com/PacificBiosciences/falcon	[13]
Miniasm	https://github.com/lh3/miniasm	[37]
Nanopolish	https://github.com/jts/nanopolish	[41]

DISCUSSION

MinION in the context of neurosurgery

Based on the current applications of the MinION, its potential use in neurosurgery is diverse. Particularly for CNS tumors, integration of the MinION into the diagnostic pipeline promises to reduce costs and turn-around time for actionable results. The 2016 WHO classifications of CNS tumors require molecular profiling for final diagnosis. Common genes that delineate this classification include IDH, 1p/19q, SHH, WNT, TP53, and RELA.[42] To date, only one study has been published using the MinION to support molecular diagnosis of CNS tumor tissue. Despite a small sample size, this study demonstrated that the MinION can provide critical diagnostic information regarding SNPs, copy number variations, and methylation patterns within a single work day.[20] This accuracy and rapid turn-around time suggest integration of the MinION into the diagnostic pipeline for CNS tumors can improve patient care and accelerate treatment decisions. Although studies reporting the use of the nanopore sequencing for neurosurgical applications are currently limited, examining the role of NGS in neurosurgery reveals many areas of future effective application for the MinION. Recent studies have demonstrated that genetic profiling of CSF provides superior detection and surveillance for CNS tumors when compared with plasma.[18,50] Application of nanopore sequencing in this context could improve diagnostic time and return of actionable results.

NGS panels directed at detecting critical mutations in CNS tumor tissue are routinely used in the hospital setting.[56] However, current NGS protocols are associated with several disadvantages including long turnaround time, limitations in copy number analysis, expensive

Table 2: Comparison of portable NGS approaches to existing technologies

Parameter	Sanger Sequencing[39]	Next Generation Sequencing (NGS)[39]	MinION Device[6]
Method	Dideoxynucleotide chain termination	Pyrosequencing, Sequencing-by-synthesis, Sequencing by Ligation	Nanopore sequencing
Accuracy of Single-Read	99.9%	98%-99.9%	Up to 99%
Read length	400-900 bp	35-700 bp	Up to 1Mb (1,000,000bp)[40]
Time per Run	20 min - 3 h	24 h - 2 weeks	Data can be streamed real-time. Available within 1 min. Run time: 1 min - 48 h
Cost per equipment	$65,000-95,000	$500,000+	$1000
Cost per Million bases	$2400	$.05-10	$500-999 per flow cell
Advantages	High accuracy	High throughput	Longest read length, portable, affordable

initial investment cost, and logistical limitations and unavailability in the remote areas. Each of these factors is adequately addressed by nanopore sequencing which boasts greater accuracy in copy number analysis, inexpensive initial investment ($1,000 for Oxford MinION), and turnaround times reported on the scale of hours. These advantages create promise for the nanopore sequencing to become a future standard in gene sequencing. Reduced cost allows even smaller neurosurgical centers to equip themselves with the latest gene sequencing technologies. This can extend even beyond well-equipped neurosurgical centers and laboratories to allow gene sequences to become a worldwide standard of care. However, more studies regarding MinION's accuracy, cost, and speed in genomic assessment of diseased and healthy human tissue are required before making a definitive conclusion regarding the clinical and economic benefits and recommending incorporation of new technology for patient care in neurosurgery.

Limitations

An identifiable challenge that will hinder the rapid and widespread use of portable nanopore sequencers is its competition with genomic services that provide "all-inclusive" (sample preparation, sequencing, computational analysis, and interpretation) sequencing in a timely manner, with medical center samples submitted by mail. These companies, providing WGS services, offer a price per sample that is roughly comparable with the entire cost of a portable sequencer.[3] Despite the financial incentive, scientists and hospital laboratories may be reluctant to relinquish current hardware or train in new technologies. Scientists without the direct availability of expensive bulky sequencers, including those working in remote areas, will certainly benefit from the use of portable nanopore sequencers. However, even in remote areas, basecalling and data analytics require robust electricity supply, robust computer processing, and dependable internet connection.

Comparison of the NGS MinION with the previous generation and more common sequencing methods is summarized in Table 2.

CONCLUSION

Low cost, portability, speed, and versatility make MinION a promising tool for low-resolution, rapid nucleic acid sequencing. As the accuracy of MinION increases with improved chemistry and basecalling software, we predict that nanopore sequencing may become a widely used tool for rapid gene profiling. Based on the first reports available so far, nanopore sequencing technology has a potential to improve the molecular diagnostic pipeline for CNS tumors, infectious diseases, and other clinical or laboratory research purposes within neurosurgery. Further studies comparing cost, speed, and accuracy of MinION with current NGS solutions are required to establish its clinical value.

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Conflicts of interest

There are no conflicts of interest.

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