Pure Appl. Biol., 9(1): 154-161, March, 2020
http://dx.doi.org/10.19045/bspab.2020.90019

Review Article

A review on nanopore sequencing technology, its applications and challenges

Mirza Jawad Ul Hasnain[1], Bakhtawar Afzal[2], Tehreem Anwar[1], Muhammad Tariq Pervez[1] and Tanveer Hussain[3*]

1. Department of Bioinformatics & Computational Biology, Virtual University of Pakistan, Lahore-Pakistan
2. Department of Biosciences, COMSATS University, Islamabad-Pakistan
3. Department of Molecular Biology, Virtual University of Pakistan, Lahore-Pakistan

**Corresponding author's email: tanveer.hussain@vu.edu.pk*

Citation

Mirza Jawad Ul Hasnain, Bakhtawar Afzal, Tehreem Anwar, Muhammad Tariq Pervez and Tanveer Hussain. A review on nanopore sequencing technology, its applications and challenges. Pure and Applied Biology. Vol. 9, Issue 1, pp154-161. http://dx.doi.org/10.19045/bspab.2020.90019

Received: 23/05/2019 Revised: 15/07/2019 Accepted: 14/09/2019 Online First: 04/10/2019

Abstract

In this review, we describe another age of sequencing 'Nanopore Sequencing technology" which is rapidly growing to fulfill the gap in advancements, which hold the capacity for significantly larger perused read lengths, less time consuming and lower by expense. Nanopore-based sequencers, as the fourth-generation DNA sequencing technology, have the potential to quickly and reliably sequence the entire human genome for less than $1000, and possibly for even less than $100.The need has never been more prominent for progressive advances that convey time efficient, reasonable and exact genome data. This quest has led to the advancements in the field of Next Generation Sequencing (NGS). The economical production of huge volumes of sequencing information is the essential advantage over traditional techniques. The preliminary methods of sequencing namely Sanger sequencing and Second Generation Sequencing provide the basis to the propelling and surprising number of logical advances. However, the evolving genomics and specifically the sequences sciences provide prolific basis for extra development in this space of research. We hereby ponder upon the broad range of Nano-pore sequencing techniques along with their applications in the emerging era of genomics and its advancement, also the word goes towards the importance of Nano-pore sequencing advancements in the emerging markets of sequencing like that of Pakistan.

Keywords: Genome; Nano-pore; Next-Generation Sequencing; Sequence analysis; Technology

Introduction

DNA, a molecule that encodes genetic instructions, is the blueprint of life. Accurate and rapid DNA sequencing technology would have profound impacts on human diseases and personalized medicine. So, efforts to sequence human genome was started decades ago. Sequencing word was opted by biologists in order to see the arrangement of the hidden atom/molecules or ions in macromolecules **[1, 2]**. The very first scheme for the DNA sequencing was established in 1970 by Ray Wu using the strategy of Location specific Primer extension **[3]**. In 1973, wandering-spot analysis method was used to report sequence

of 24 base pairs while in 1977. DNA Sequencing with chain-terminating inhibitors methodology was published [4]. First whole genome sequencing of a bacteriophage was done in 1977 while first whole human genome was sequenced completely in April 2003 after an indefatigable effort [5]. Several Sequencing techniques have been introduced and utilized till now including Single-molecule real-time sequencing with an accuracy 87% [6] keeping the read length range from 10,000 bp to 15,000 [7], Ion Torrent sequencing with accuracy of 98% keeping the read length up to 600 bp [8], Pyrosequencing with accuracy 99.9% keeping the read length up to 700bp [1]. Illumina sequencing with accuracy of 99.9% keeping the read length range from 75 bp to 500 bp [1, 9], SOLID sequencing with accuracy of 99.9% keeping the read length range from 85 bp to 100 bp, Sanger sequencing with accuracy of 99.9% keeping the read length range 400 bp to 900 bp [10] and finally the Nanopore sequencing [11] with up to 99.96% consensus where the read length is totally dependent on the library preparation providing the user the of up to 500 kb [9]. The non-nanopore DNA sequencing technologies currently on the market require a great deal of sample preparation and complicated algorithms for data processing. The insight of discussion was to analyze advance sequencing techniques that come under the umbrella of Next generation Sequencing.

Nanotechnology in next generation sequencing

Nanotechnology is a rising technology which has luminous applications in the basic and applied sciences. Basically, it is a science of manipulating materials at nanoscale [12]. Nanotechnology is basically a derivative of versatile sources of knowledge including chemistry, engineering, physics and micro fabrication techniques. It can be extensively known as the production, design, system, devices and solicitation of structures via control of size and morphology of material at nanometer scale where the phenomena make the applications possible [13].

A nanometer is a miniscule that is it is too small to see from a human eye. The scale also tends to be specious that would be measuring structures up to 1-100nm, not only this it would also have wide applications and implications that would be indispensable to mankind. In the running era the nanotechnology that makes the highly reactive particles of sizes less than 100nm their base, seem to be central to developing and utilizing new electronics and energy technologies [13, 14].

Nanopore techniques

A pore of nanometer size is called a Nanopore. Nanopores can be created in two ways, either by proteins that form pores or by making holes in artificial molecules. A nanopore can also be depicted as a single molecule identifier when it is present in an electrically insulating membrane mostly coated with iron. Moreover, it can also act as a biological protein network in a lipid bilayer of huge electric confrontation. All types of nanopore technologies are used for the detection of biological and chemical molecules at the molecular level in nanoscale [15].

A nanopore-based device use principle of electrophoresis to drive molecules in solution through nano-scale pore and delivers detection of molecules and analytical competences. Characterization is done in confined spaces within nanopore that allows single nucleic acid polymers to be analyzed. Inexpensive and fast DNA sequencing is made possible with nanopore sequencing technique as it offers sequencing or characterization of single-stranded genomic DNA or RNA or small nucleosides without using labels and amplification [16]. Even though initial experiments regarding nanopore sequencing disappointed the

adolescent expectation but the high sensitivity of nanopores for single molecules, stimulated many types of research in order to study analysis of nucleic acids through nanopore **[17, 18]**. Since the very first experiment that showed that even kbs of single stranded DNA can be drive through nanopores with the help of electric field, this perspective of an inexpensive and fast massive sequencing capability has extensively promoted nanopore research using either plastic materials, lipid bilayer or fabricated nanopores **[19, 20]**.

Biological nanopores

Pore-forming proteins can form Nanopores **[21]**, typically the protein is mushroom shaped and a hollow core pass through it. Phi 29 connector, MspA porin and α-hemolysin are some proteins that make pores. α-Hemolysin (α -HL, also called a-toxin) is the first and most commonly used biological nanopore, holding a tremendous value in the field of DNA sequencing. α-HL is an exotoxin secreted by the bacterium Staphylococcus aureus, a human pathogen. This mushroom-shaped heptamer is a 232.4-kDa transmembrane channel, consisting of a 3.6-nm diameter cap and a 2.6-nm diameter transmembrane β-barrel **[22]**. During the laboratory work, in a lipid bilayer film, a nanopore is inserted to it and subsequently manipulation is done on single channel proteins and measurements are taken **[23]**.

Solid-State nanopores

Solid-State Nanopores are basically created in films of silicon compounds, the frequently used is silicon nitride. Numerous techniques are used for the manufacturing of solid state nanopores involving "Deploying and sculpting with ion beam" and "fabrication by electron beam" **[24]** specifically. The diameter of the solid state nanopores is controllable in a precise range of sub nanometers to hundreds of nanometers, the range varying according to the experimental parameters. Generally, while comparing with lipid membranes, dielectric materials like SiN illustrates superior chemical and thermal stability are used in the solid state nanopores. Though, this stability is dependent on the settings that lead to the formation of these pores **[25]**. For nanopores based on graphene, even with their undemonstrated chemical and thermal stability they hold unique chemical properties that are of great gains over their biological complements **[26]**. Solid state nanopores now have paved the paths to a wide range of research in especially in DNA sequencing **[27]**, protein interaction identifications **[18]**, molecular transport mechanisms and assembly of Nano-fluidic devices **[2]**. An adaptable substitute for biological nanopores has emerged as solid state nanopores because of its unique properties **[26]**. Several electronic or optical measurement techniques are compatibly associated with the solid state nanopore **[27]**. Production of the oxidative film on to a metallic surface **[21]**, ionic beam sculpting **[24]** and fabricated electronic scheme of nanopores in the thin synthetic membrane **[28]**, membrane technology for the ion tracking **[29]** are the recent nanopores fabricated technologies.

Anodic oxidation method on the metal aluminum

Fabrication of several self-ordering Nano porous oxides of metals has been resulted by the observation of electrochemistry and electrophysiology during anodic oxidation of metals. These Nano porous oxides of metals include anodized aluminum oxide, porous silicon (psi) and Nano tubular titania oxide (TNT) **[30]**. A key instance of such highly ordered processes is of Nano porous anodic alumina oxide that is produced by anodization of aluminum. Notable characteristics such as thermal and chemical stability, hardness and high surface area make ordered anodic alumina oxide to stand out **[31, 32]**. Ordering behavior during anodic oxidation is studied for selective metals that

include aluminum, Zr, Nb, Ti etc. most commonly stated as valve elements and also alloys of these metals, under suitable investigational situations. Several factors that emphasize and enhance this process are the type of electrolyte, pH and concentration of electrolyte, its applied current and voltage, temperature and finally the surface pre-texturing [33, 34].

Ion track-etching technology

Ion track-etching technology is a very well stabled technology for pore generation in insulating materials. Filtration films are normally produced from several polymers. The basic principle is that on a straight ion path, permanent material changes are induced due to the penetration of heavy ions having high energy, into a solid. By etching with suitable reagent (the one that quickly and specially attacks damaged zone), ion tracks can be enlarged to pores. Between a few nanometers and several micrometers, cylindrical pores of huge aspect ratio can be formed [35, 36].

To obtain more uniform etching and for improved wetting, surfactants are added during ion track-etching [37]. Following facts must be considered to understand the ion track etching process in the presence of surfactants:

- Susceptibility to chemical attack is changed when surfactant molecules adsorb on the surface.
- Surfactant molecules are very small, a few nanometers in size, analogous to the radius of the specially etched track core [38].

Ion-beam sculpting

Deploying and ‘‘sculpting’’ materials [24] under 10 nm becomes currently a crucial point for nano-fabrication [28], and for its several applications in biology, chemistry and electronics. Ion beam sculpting is tremendously rising interest among many other research tools developed for meeting nanopore challenges. In comparison to other methods, due to its less shattering rate of ions, it proposes very good firmness. and offers direct substrate patterning which becomes crucial to meet the nanopore challenges [15, 24]. The high resolution focused ion beam offers nanometer based sculpting of matter. Using focused ion beam sculpting to carve membranes unlocks new emergent directions for nanoscience tooling [39].

Ion current rectification

A specific transportation effect has been experienced in unevenly shaped Nano-capillaries and nanopores, which is because of their small Nano sized openings. It has been observed that ion current is rectified in these nanopores, however, electrolyte pH and concentration is the same that make contact with both openings of pores. Current–voltage curves are used for observation of rectification, with the currents observed for one voltage polarity higher than the currents observed of opposite polarity but for the same absolute voltage [40, 41, 42]. Many Nano porous systems show ion current rectification behavior. Biological pores encompass the first section of such systems and they are present in lipid cell membranes [43, 44]. Another Nano porous system that shows rectifying behavior consists of lipid bilayer in which artificial nanopores are inserted [45]. It has been shown that totally artificial systems can also rectify ion current. Nanopores have been prepared in polymer films and in gold nanotubes [33, 46].

Electron-beam fabrication

Fabrication of small diameter solid-state nanopore is difficult. For example, by the use of FIB systems, it is nearly impossible, the fabrication of a nanopore that is below 30nm dependably in relation to shape and size. Nanopores can be etched by the use of FIB with high energy, but there is a limitation on film thickness due to a low etch rate [47]. By the use of an ion beam that is unfocused [24, 48] or an electron beam with high energy [49], the diameter of nanopore can be condensed very significantly, from

approximately 50 to 100 nm down to 10 nanometers or less **[50]**. In this way significant advantages are achieved because the effectiveness of a solid state nanopore increase as a detection technique for single molecules when pore diameter is nearly the same as the molecule diameter that is needed to be detected. Usually this diameter is 2–10 nanometer **[47, 51]**.

Conclusion

Sequencing term basically refers to the determination of primary structures of Biological molecules from their unbranched biopolymers. Advance sequencing techniques being used in bioinformatics helps biologists in various aspects including DNA Sequencing, RNA Sequencing and Protein sequencing for the determination of mutations, variants calling, expressional analysis and the structural behaviors of biomolecules. Sequencing of biomolecules was first introduced in 1977 by Sanger who proposed the chain termination method for DNA sequencing. This technique is called first generation sequencing followed by second generation sequencing and third generation sequencing respectively. In the middle of late 1990s, second generation sequencing (NGS) emerged and was commercially implemented for DNA Sequencing especially. NGS techniques helped biologist widely due to its high-throughput sequencing methodology where large genomes were cut down into small pieces ("reads") by fragmentation processes and named as massive parallel sequencing. After NGS, third generation sequencing has been introduced and currently is under development. This advanced sequencing technique read the nucleotide sequences at a single molecule level by breaking down long fragments into smaller ones and them by amplification and synthesis process, determines the nucleotide sequence.

Nanopore sequencing is another advance and emerging technology which can be broadly defined as the use of nanometer scaled devices for the identification of larger as well as small biomolecules. These devices can be of artificially produced nanopore chips or the biological membranes. The advance nanopore-based devices use the principle of electrophoresis to detect the molecules by driving them in solution through Nano-scaled pores. Many pore-forming proteins like MspA porin and hemolysin are being used as biological nanopores while artificially produced nanopores which are known as Solid-State Nanopores are made by a thin film of silicon nitride like as in "Deploying and sculpting with ion beam" and "fabrication by electron beam". As compared to biological membranes commercially produced dielectric material nanopores chips like SiN shows more chemical and thermal stability due to its adjustable range of nanopore sizes. Soli-State Nanopores carries a range of sub nanometers to hundreds of nanometers according to the molecule to be detected. Different techniques in Sloid-State Nanopore sequencing have been introduced like Anodic Oxidation method on the metal aluminum, Ion track-etching technology, Ion-beam sculpting, Ion current rectification and Electron-Beam Fabrication. Anodic. Among all these five techniques, Ion-beam sculpting techniques offer great deals to the biologists, chemists and in electronics too. This technique offers direct patterning techniques due to its less shattering rate of ions and good firmness.

In Pakistan High throughput sequencing and Nano-pore sequencing has not yet been introduced properly. This is due to the fact that biologists in Pakistan prefer to send their wet lab products to foreign for massively parallel sequencing & analysis and focus on the results interpretation only which is reducing the scope of Next Generation Sequencing in Pakistan. At most our universities have is Sanger sequencing machines which are not so supportive now a

days due to not producing sequenced data with high coverage rate. Some institutes in Pakistan i.e. COMSATS University Islamabad, Quid e Azam University, Virtual University of Pakistan, Lahore University of Management Sciences and the University of Karachi Pakistan have established their bioinformatics department and working on NGS data analysis but lacking the advance sequencing techniques. Higher Education Commission should focus on the deployment of advance sequencers to promote Next Generation Sequencing and Nanopore Sequencing in Pakistan so that we could compete with the world.

Authors' contributions

Conceived and designed the idea: MT Pervez, T Hussain; Corrections: MT Pervez; Proof Reading: T Hussain; Wrote the paper: MJ Hasnain, B Afzal, T Anwar.

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