DNA strand nucleotide order is determined by using Nano pore sequencing method can this method is under development since 1995. Many porous membranes proteins and used as Nano pores, Each nucleotide base is pass through this small hole of almost 1 nanometer and the peak is recorded in the form of chromatograph. This method is considered more accurate than gel electrophoresis because the chances of sample damage are low and more authentications of sequences were previously reported. Now a day's manual methods are replaced by automated methods for less time consumption and more accurate results.

Keywords: DNA, Nanopore, nucleotide.

1. Introduction

A Nanopore is just a small hole its internal diameter is 1 nanometer. many porous transmembrane cellular proteins act as Nanopore, and they have also been made by etching a somewhat bigger hole in a piece of silicon, and then gradually filling it in using ion-beam sculpting methods which results in a much smaller diameter hole the Nanopore. Graphene is also being explored as a synthetic substrate for solid-state Nanopore [1]. DNA could be passed through the nanopore for various reasons. For example, electrophoresis might attract the DNA towards the nanopore, and it might eventually pass through it. Enzymes attached to the nanopore might guide DNA towards the nanopore [2]. The scale of the nanopore means that the DNA may be forced through the hole as a long string, one base at a time, rather like thread through the eye of a needle. As it does so, each nucleotide on the DNA molecule may obstruct the nanopore to a different, characteristic degree. The amount of current which can pass through the nanopore at any given moment therefore varies depending on whether the nanopore is blocked by an A, a C, a G or a T. The change in the current through the nanopore as the DNA molecule passes through the nanopore represents a direct reading of the DNA sequence. Alternatively, a nanopore might be used to identify individual DNA bases as they pass through the nanopore in the correct order this approach has been shown by Oxford Nanopore Technologies [3].

Figure 1: Nanopore
(Source: https://allsales.guru/goto?id=1504347359)

2. Principle

When a Nanopore is immersed in a conducting fluid and a voltage is applied across it, an electric current due to conduction of ions through the Nanopore can be observed [4]. The amount of current is very sensitive to the size and shape of the Nanopore. If single bases, strands of DNA or other molecules pass through the Nanopore, this creates a characteristic change in the magnitude of the current through the Nanopore [5].
3. Types of Nanopore

3.1 Alpha hemolysin

Alpha hemolysin (αHL) is a Nanopore from bacteria that causes the lysis of red blood cells. It has been studied for over 15 years. To this point, studies have shown that all four bases can be identified using ionic current measured by the αHL pore. The structure of αHL is advantageous to identify specific bases moving through the pore. The αHL pore is ~10 nm long, with two distinct 5 nm sections [6]. The upper section consists of a larger, vestibule-like structure and the lower section consists of three possible recognition sites (R1, R2, R3), and is able to differentiate between each base [7].

3.2 Sequencing using αHL

Sequencing using αHL has been developed through basic study and structural mutations, moving towards the sequencing of very long readings. Protein mutation of αHL improved the detection abilities of the pore [8]. The next proposed step is to bind an exonuclease onto the αHL pore. The enzyme would periodically cleave single bases, enabling the pore to identify successive bases. Coupling an exonuclease to the biological pore would slow the translocation of the DNA through the pore, and increase the accuracy of data acquisition [9].

A recent study has pointed to the ability of αHL to detect nucleotides at two separate sites in the lower half of the pore [10].

The R1 and R2 sites enable each base to be monitored twice as it moves through the pore, creating 16 different measurable ionic current values instead of 4. This method improves upon the single read through the nanopore by doubling the sites that the sequence is read per nanopore [11].

3.3 Mycobacterium smegmatis porin A (MspA)

Mycobacterium smegmatis porin A (MspA) is the second biological nanopore currently investigated for DNA sequencing. The MspA pore has been identified as a potential improvement over αHL due to a more favorable structure. The pore is described as a goblet with a thick rim and a diameter of 1.2 nm at the bottom of the pore. A natural MspA, while favorable for DNA sequencing because of shape and diameter, has a negative core that prohibited single stranded DNA (ssDNA) translocation. The natural nanopore was modified to improve translocation by replacing three negatively charged aspartic acids with neutral asparagines [12].

3.4 MspA is more suitable for sequencing than Alpha hemolysis

The electric current detection of nucleotides across the membrane has been shown to be tenfold more specific than αHL for identifying bases. Utilizing this improved specificity, a group at the University of Washington has proposed using double stranded DNA (dsDNA) between each single stranded molecule to hold the base in the reading section of the pore [13]. The dsDNA would place the base in the correct section of the pore and enable the identification of the nucleotide or bases. A recent grant has been awarded to collaboration from UC Santa Cruz, the University of Washington, and Northeastern University to improve the base recognition of MspA using phi29 polymerase in conjunction with the pore [14].
Figure 5: The electric current detection of nucleotides across the membrane

3.5 Solid state Nanopore sequencing

Figure 6: Solid state Nanopore sequencing

3.6 Electron tunneling

Measurement of electron tunneling through bases as ssDNA translocates through the nanopore is an improved solid state Nanopore sequencing method. Most research has focused on proving bases could be determined using electron tunneling. These studies were conducted using a scanning probe microscope as the sensing electrode, and have proved that bases can be identified by specific tunneling currents. After the proof of principle research, a functional system must be created to couple the solid state pore and sensing devices [15].

Figure 7: Electron tunneling

Researchers at the Harvard Nanopore group have engineered solid state pores with single walled carbon nanotubes across the diameter of the pore. Arrays of pores are created and chemical vapor deposition is used to create nanotubes that grow across the array. Once a nanotube has grown across a pore, the diameter of the pore is adjusted to the desired size. Successful creation of a nanotube coupled with a pore is an important step towards identifying bases as the ssDNA translocates through the solid state pore. Another method is the use of Nanoelectrodes on two sides of pore. The electrodes are specifically created to enable a solid state nanopore's formation between the two electrodes. This technology could be used not only to sense the bases but to help control the base translocation speed and orientation [16].

3.7 Fluorescence

An effective technique to determine a DNA sequence has been developed using solid state nanopore and fluorescence. This fluorescence sequencing method converts each base into a characteristic representation of multiple nucleotides which bind to a fluorescent probe strand-forming dsDNA. With the two color system proposed, each base is identified by separate fluorescence's, and will therefore be converted into two specific sequences. Probes consist of a fluorophore and quencher at the start and end of each sequence. Each fluorophore will be extinguished by the quencher at the end of the preceding sequence. When the dsDNA is translocating through a solid state nanopore, the probe strand will be stripped off, and the upstream fluorophore will fluoresce [17].

3.7.1 Capacity

This sequencing method has a capacity of 50-250 bases per second per pore, and a four color fluorophore system (each base could be converted to one sequence instead of two), it can sequence over than 500 bases per second.

3.7.2 Advantages of fluorescence sequencing method

Advantages of this method are based on the clear sequencing readouts using a camera instead of noisy current methods (detectors). However, the method must require sample preparation to convert each base into an expanded binary code before sequencing. Instead of one base being identified as it translocates through the pore, ~12 bases are required to find the sequence of one base [18].

3.8 Epigenetic DNA modifications and Nanopore DNA sequencing

Sequencing reveals genetic variations, which determines each person risk for many diseases, as well as which drugs will work best for each individual. Cancer centers are already sequencing tumors in search of variations that make some resistant to chemotherapy. And global sequencing studies seek to find the genetic contributors to a variety of conditions from autism to diabetes. The nanopore technology also can be used to identify subtle DNA modifications that happen over the lifetime of an individual. Such modifications, referred to as
epigenetic DNA modifications and may take place as chemical reactions on the DNA within cells and tell the cells how to interpret their DNA. While essential for proper cellular functioning, epigenetic modifications can also be the underlying causes of various undesired conditions. Epigenetic modifications are important for things like cancer, and being able to provide DNA sequencing that can directly identify epigenetic changes is one of the charms of the nanopore sequencing method [19].

4. Nanopore sequencing apparatus

The development of solid state nanopores and the studies of DNA translocation through these nanopores suggest how a nanopore could be the core of an instrument capable of inexpensive de novo sequencing. The investigation and development of basic science and technology required to build a nanopore based instrument that should be able to sequence a mammalian genome for <$1,000 and that meets the following requirements [20]:

a) High-speed sequential identification of the DNA nucleotides directly on the basis of their distinct physical or electrical properties.

b) Very long, indefinite length reads. Analysis and assembly is a bottleneck in de novo sequencing and limits re-sequencing when copy number polymorphism or variable indels are to be identified in heterozygous genomes.

c) The requisite sequence coverage (7.7-fold coverage, 6.5-fold coverage in Q20 bases) using genomic DNA from <10^6 cells with no amplification and minimal preparatory steps. Otherwise, amplification or other preparatory steps become limiting.

Propose to investigate and develop the science and technology required to build a nanopore based instrument that meets the above requirements. Among the unique capabilities of this instrument, four well demonstrated features as follows:

1) A nanoscale device that translocates polymer molecules in sequential monomer order through a very small volume of space, a small pore in an electrically biased membrane.

2) A single molecule detector that is also a very high throughput device. A nanopore can probe thousands of different molecules or thousands of identical molecules in a few minutes.

3) A detector that directly converts characteristic features of the translocating polymer into an electrical signal. Transduction and recognition occur in real time, on a molecule-by-molecule basis.

A device that can probe very long lengths of DNA that can be analyzed as it translocates through a nanopore and it is not aware of any theoretical limits.

5. Advantages of nanopore sequencing technology

The potential is that a single molecule of DNA can be sequenced directly using a nanopore, without the need for an intervening PCR amplification step or a chemical labelling step or the need for optical instrumentation to identify the chemical label [21].

6. Drawbacks of nanopore sequencing technique

As of July 2010, information available to the public indicates that nanopore sequencing is still in the development stage, with some laboratory-based data to back up the different components of the sequencing method. Despite these advancements, nanopore sequencing is not currently commercially available and routine zed, it is not cost-effective enough to compete with next generation sequencing methods. Nanopore-based DNA analysis techniques are being industrially developed by Oxford Nanopore Technologies (developing direct exonuclease sequencing and strand sequencing using protein nanopores, and solid-state sequencing through internal R&D and collaborations with academic institutions), NabsYs (using a library of DNA probes and using nanopores to detect where these probes have hybridized to single stranded DNA) and NobleGen (using nanopores in combination with fluorescent labels. IBM has noted research projects on computer simulations of translocation of a DNA strand through a solid-state nanopore, but no projects on identifying the DNA bases on that strand [1].

7. Challenges to nanopore technique

One challenge for the strand sequencing method is in refining the method to improve its resolution to be able to detect single bases. In the early papers methods, a nucleotide needed to be repeated in a sequence about 100 times successively in order to produce a measurable characteristic change. This low resolution is because the DNA strand moves rapidly at the rate of 1 to 5μs per base through the nanopore. This makes recording difficult and produce background noise, failing in obtaining single-nucleotide resolution. The problem is being tackled by either improving the recording technology or by controlling the speed of DNA strand by various proteins engineering strategies. More recently effects of single bases due to secondary structure or released mononucleotides have been shown. Professor Hagan Bayley, founder of Oxford Nanopore, recently proposed that creating two recognition
sites within an alpha hemolysin pore may confer advantages in base recognition. One challenge for the exonuclease approach, where a processive enzyme feeds individual bases, in the correct order, into the nanopore, is to integrate the exonuclease and the nanopore detection systems. In particular the problem is that when an exonuclease hydrolyzes the phosphodiester bonds between nucleotides in DNA, the subsequently released nucleotide is not necessarily guaranteed to directly move in to, a nearby alpha-hemolysin nanopore [22]. One idea is to attach the exonuclease to the nanopore, perhaps through biotinylation to the beta barrel hemolysin. The central pore of the protein may be lined with charged residues arranged so that the positive and negative charges appear on opposite sides of the pore. However, this mechanism is primarily discriminatory and does not constitute a mechanism to guide nucleotides down some particular path.

8. Commercialization

Agilent Laboratories was the first to license and develop nanopores but does not have any current disclosed research in the area. The company Oxford Nanopore Technologies in 2008 licensed technology from Harvard, UCSC and other universities and is developing protein and solid state nanopore technology with the aim of sequencing DNA and identifying biomarkers, drugs of abuse and a range of other molecules. They revealed their first working device in February 2012.Sequenom licensed nanopore technology from Harvard in 2007 using an approach that combines nanopores and fluorescent labels. This technology was subsequently licensed to Noblegen. NabSys was spun out of Brown University and is researching nanopores as a method of identifying areas of single stranded DNA that have been hybridized with specific DNA probes.

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"Harvard University and Oxford Nanopore Technologies Announce License Agreement to Advance Nanopore DNA Sequencing"