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4. Muhammad Sajeer, and Manoj M. Varma. "TEM based applications in solid state nanopores: From fabrication to liquid in-situ bio-imaging." *Micron* 162 (2022): 103347. DOI: https://doi.org/10.1016/j.micron.2022.103347

5. Boya, Radha, Ashok Keerthi, and Muhammad Sajeer Parambath. "The wonderland of angstrofluidics." *Physics Today* 77, no. 8 (2024): 26-33. DOI: https://doi.org/10.1063/pt.frik.vxpk

Science & Society

TRANSPARENT PROCESS

EMBO reports

Disruptive technology

Exploring the ethical, legal, political, and societal implications of nanopore sequencing technology

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Nanopore technology has attracted much interest during the past years, mainly owing to its applications in DNA and protein sequencing. However, the fast uptake of nanopore sequencing as an affordable and easy-to-use technology and the rapid technological developments could have major ethical, social, legal, and security ramifications. This article explores these sociological implications of nanopore sequencing technology with the hope that it will generate interest in the social science and policy domains, and inspire discussions on unintentional consequences with the aim of preventing negative impacts on society.

Nanopores

Nanopores, irrespective of whether they are man-made or naturally occurring, are defined as holes with a diameter between 1 and 100 nm and are used in a variety of applications from DNA and protein sequencing (Johnson *et al*, 2017; Hu *et al*, 2020) to filtering (Li *et al*, 2016) and nanomedicine (Majd *et al*, 2010). The versatility and scalability of nanopore technology have raised significant interest in research as demonstrated by the increasing number of publications on the topic (Fig 1) and in industry with a number of companies focusing on various aspects of nanopore technology, including fabrication, measurement, or DNA and protein sequencing. The latter in particular has become popular in the life sciences as one of the Next Generation Sequencing (NGS) methods with applications in genomic mapping, population genomics, and evaluating genetic risk factors for diseases. Recently, it has also been used to detect SARS-CoV-2 variants (Smith, 2021). In contrast to other sequencing technologies, nanopore-based sequencing is cheaper, very easy-to-use, does not require much computational and lab infrastructure, and only needs a small amount of DNA in a test sample.

> *"... nanopore-based sequencing is cheaper, very easy-to-use, does not require much computational and lab infrastructure, and only needs a small amount of DNA..."*

It is, in fact, these characteristics that would make nanopore sequencers an ideal consumer product. Oxford Nanopore, which pioneered the technology, has already developed small handheld devices (Fig 2) that can be used in the field or the clinic for direct sequencing. As Gordon Sanghera, the CEO of Oxford Nanopore Technologies, commented, the popularity of the nanopore technology will contribute to a "large and terrific shift" in the usage of genetic information similar to the computer revolution during the past decade (Riding Unicorns, 2022).

There are clearly opportunities but also risks when consumers outside a professional research environment get access to powerful DNA sequencing technology. As nanopore-based sequencing as a consumer technology has both positive and negative implications, a discussion through the lens of social science will help to map the challenges and unintentional negative consequences it may create for society in the same way the social sciences have contributed to analyzing the potential impact of nanotechnology and synthetic biology (Shapira *et al*, 2015). These in turn would inform politicians and lawmakers when it comes to regulating the use of consumer sequencing technology. Studies and suggestions from the humanities and social sciences will also help to guide responsible research and direct the trajectory of growth of this technology.

DNA sequencing technology for consumers

The high portability and affordability of nanopore sequencers could lead to pervasive DNA sequencing. Along with many bona fide applications—for instance, testing for the presence of pathogens or food contaminants—it could also encourage illegal or at least harmful uses such as unauthorized ancestry tracking, self-testing for genetic risk factors, or parental tests without the consent of those whose DNA is being sequenced.

> *"The high portability and affordability of nanopore sequencers could lead to pervasive DNA sequencing."*

In many cases, the discussion of the ethical, legal, or societal implications (ELSI) only took place after a new technology was nearly or fully developed and reached the market, which left little room for change, policies, or regulation at this late stage. This was the main reason why, in the late 1990s, the funders of the Human Genome Project decided to involve the humanities and social sciences in the research stage and established an active ELSI program. These scholars explored and mapped the positive and negative societal impacts of DNA and full-genome sequencing and made recommendations to funders and policymakers (Hanna, 1995). Most of the ethical, legal, and societal issues related to DNA sequencing per

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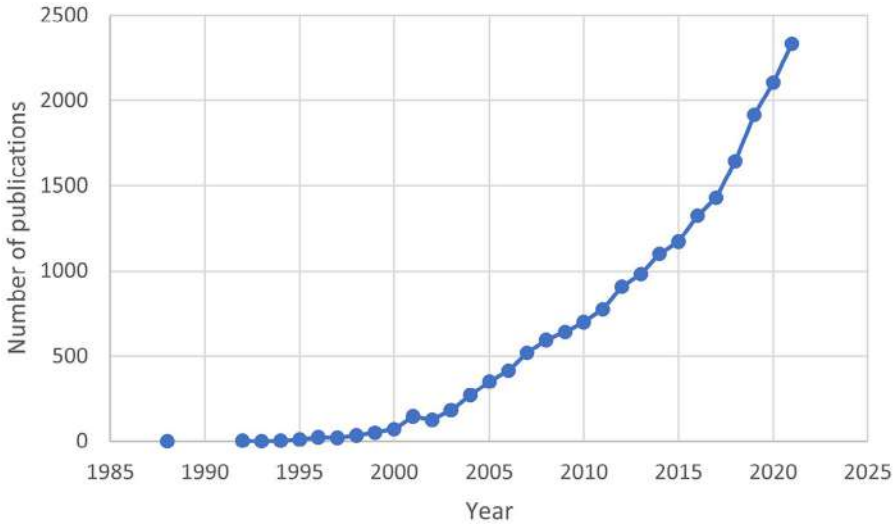

Figure 1. The increase in the number of publications related to nanopores.
Data from Web of Science using the keywords "nanopore" or "nanopores" in all domains.

Figure 2. A hand-held nanopore sequencer.

se have been widely discussed and addressed in the form of regulations and laws.

The advent of consumer DNA sequencing technology raises many of the same ELSI that were discussed more than 20 years ago in the context of the Human Genome Project and subsequently about full-genome sequencing (McGuire *et al*, 2008). However, it also creates novel concerns since the laws and regulations that have been put in place may not be sufficient to prevent potential abuse of nanopore sequencing once it becomes a widely accessible consumer technology. These concerns—loss of privacy, sharing of genetic information, confidentiality of personal data or consent by the elderly, children, or other dependants, and so on—are not novel per se, but nanopore sequencing would move them out of the realm of research and clinics into wider society.

Nanopore-based sequencing technology

Nanopore sequencing technology is based on measuring current fluctuations along the pore, which sits between two reservoirs of the same electrolyte. A voltage applied over the pore causes a small current to flow. When a molecule, say DNA, translocates through the pore, the ionic current changes; the analysis of these fluctuations reveals which nucleotide passed through the pore. As nanopore sequencers move a single DNA molecule through the pore, they can determine the DNA sequence directly based on the current fluctuations without the need for presequencing PCR amplification and post-sequencing computation to assemble the sequence from a microarray read.

There are mainly three types of nanopores used for sequencing: biological, solid-state, and hybrid nanopores. The biological variety is usually pores such as MspA or α-hemolysin in a lipid bilayer membrane while solid-state nanopores are fabricated from artificial membranes such as silicon nitride by dielectric breakdown, laser-assisted milling, ion beam milling, or transmission electron microscopy. So far, only biological nanopores have been used for nanopore DNA sequencing. There is much interest and research in solid-state nanopores as these are potentially superior to their biological counterparts owing to their chemical and mechanical stability, scalability, and integration capacity with electronic devices. DNA sequencing using solid-state nanopores still faces limitations though, such as controlling the speed of molecule translocation.

The increase in the number of patents in any particular field is an indicator of the increasing industrial interest and potential commercial value. A search on lens.org, a free patent search engine, yielded 38,758 records for the term "nanopore" and 21,731 records for the term nanopore sequencing in early 2023. Moreover, the number of patents related to nanopore sequencing has been increasing exponentially during the past 20 years (Fig 3).

Nanopore sequencing is not limited to DNA though: Academic and industry are further developing the technology to expand it to protein sequencing for use in research and diagnostics. Generally, the potential ability to detect single molecules from a sample holds great potential for applications in research, clinical diagnostics, food safety, environmental monitoring, biosafety and biosecurity, and so on.

The biological nanopore DNA sequencing technology was first commercialized in 2005 by Oxford Nanopore Technologies (ONT), a

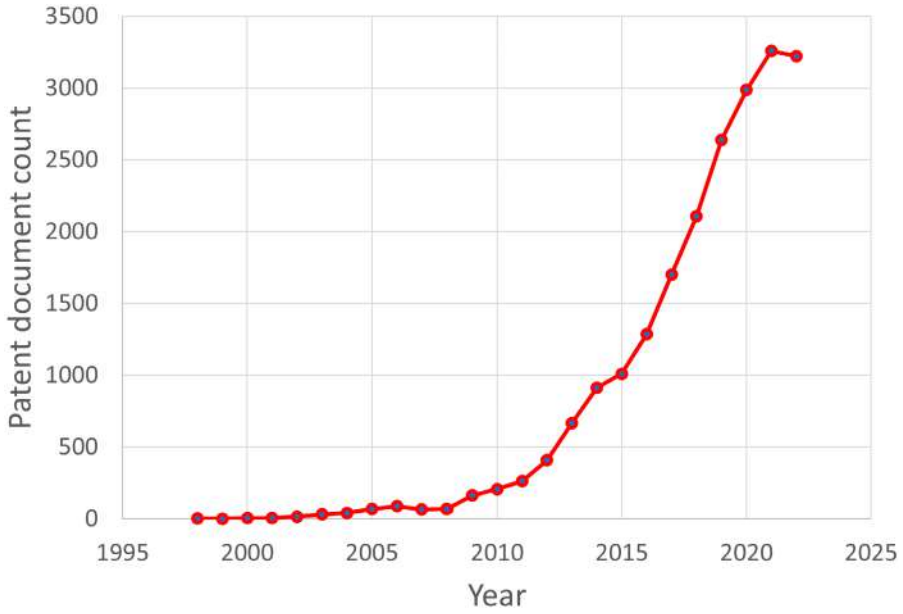

Figure 3. The increase in the number of patent publications yielded by the search term "nanopore sequencing".
Generated from Lens.org (https://www.lens.org/).

spinout from Oxford University in the UK. Backed up with more than 2,000 relevant patents (https://nanoporetech.com/about-us/intellectual-property), the company has become a major player in DNA sequencing technology. New startups are now developing instruments based on solid-state nanopores, such as Northern Nanopore Instruments, Quantapore or Ontera. Other companies supply essential parts such as current amplifiers or flow cells; according to nanoporesite.com, 27 companies are involved in this area. The global nanopore technologies market is projected to reach more than US$ 600 million by 2030 (https://www.alliedmarketresearch.com/nanopore-technologies-market-A11864.). Nonetheless, nanopore sequencing is still a smaller player in the global DNA sequencing market, which is anticipated to reach a value of US$ 29 billion by 2028 (Research and Markets, 2022).

The ethical issues of nanopore sequencing

Since the human genome project with a price tag of US$2.7 billion in 2000, sequencing has become cheaper and faster—a full-genome sequence nowadays costs less than US$ 1,000 and takes less than a day using massive parallel microarray NGS. Nanopore sequencing makes it much more affordable and easy-to-use in the field with small handheld devices—Oxford Nanopore's portable MinION Mk1C costs less than US$ 5,000 compared with Illumina's NovaSeq Series sequencers, which start at more than US$ 800,000. This enables new applications without the need for sending samples to a laboratory, such as direct sequencing in the field of ecology or population genetics research, rapid diagnostics in the clinic, or surveillance of the presence and distribution of pathogens.

It also could turn portable nanopore sequencers into a consumer product rather than a research or diagnostic tool. Consumer DNA sequencing would give anyone access to a technology that so far has been limited to research, clinical diagnostics, law enforcement, and private companies. This has enormous potential for applications: Consumers could test themselves for pathogens, for bacterial contaminants in food, or monitor their homes for pathogenic bacteria or viruses. Interested citizen science laypeople could greatly contribute to ecology or population genetics research projects by sequencing samples in the field. Biology education in schools would also greatly benefit from affordable and easy-to-use DNA sequencers. But it would also enable parents to test whether their children have an inherited genetic disorder or to conduct a paternity test. It would enable anyone to test him or herself or anyone else for genetic risk factors for diseases or determine their ethnic background. It could allow any user to test whether a particular individual has been at a certain location, say the bedroom or bathroom.

These possibilities raise serious concerns about privacy, discrimination, informed consent, the 'right to know' versus the 'right not to know' in case of severe inherited diseases, sequencing the DNA of children or the elderly who are not able to give informed consent or what to do with an unexpected diagnosis without adequate counseling. Given that nanopore sequencers are not 100% accurate and require several reads and careful analysis to generate reliable sequence data, this may lead to false assessments. Moreover, the reuse of sequence data generated this way by third parties, their storage, and sharing raises additional ethical concerns (Cambon-Thomsen, 2004; Dove *et al*, 2014; Sherkow *et al*, 2022; Wan *et al*, 2022).

None of these issues are new and have been intensely discussed during and since the Human Genome Project. These have also been addressed in laws and regulations that govern the generation and use of DNA sequence data by research institutions, clinics, law enforcement, and private companies. However, as these mostly apply to institutional actors, it is not clear whether the existing regulatory and legal framework would be sufficient to prevent abuse and misuse by individual actors.

> *"Consumer DNA sequencing would give anyone access to a technology that so far has been limited to research, clinical diagnostics, law enforcement, and private companies."*

Political and legal issues

Nanopore technology gives the ability to sequence anything, anywhere, anytime. Hand-held nanopore sequencers have been successfully tested under harsh conditions such as Antartica[2]. The technology's portability and affordability can equip underrepresented communities with affordable sequencing capabilities for onsite diagnostics or pharmacogenetics, which would greatly strengthen developing countries' capacity to fight infectious diseases. For

example, British and Brazilian researchers deployed nanopore sequencers in Brazil to analyze clinical samples for the presence of the zika virus (Quick *et al*, 2017). This easy and rapid onsite diagnosis will contribute to the early detection of diseases or help to track mutations of important pathogens such as SARS-CoV-2. It will allow governments and healthcare experts to quickly develop preventive measures to stop the spread of diseases and reduce causalities. This technology can also contribute to education giving teachers the means to practical-oriented teaching of genetics and biology.

If nanopore-based sequencing becomes ubiquitous, this may lead to political problems or even national security matters. Portable sequencers can be easily abused by the state for identifying people from underrepresented communities, refugees, or ethnic minorities who would be the target of discrimination or oppression. Even though this is already possible with other sequencing technologies, portable and low-cost nanopore-based sequencers would make it much more easy.

> *"Portable sequencers can be easily abused by the state for identifying people from underrepresented communities, refugees, or ethnic minorities who would be the target of discrimination or oppression."*

Related to this, nanopore sequencers could challenge existing laws that ensure privacy and the safety of genetic information. In the USA, the 2008 Genetic Information Nondiscriminatory Act (GINA) specifically prohibits health insurances and employers from discriminating people using genetic data. The 1996 Health Insurance Portability and Accountability Act further prohibits any healthcare providers and business from sharing such information without the consent of the person. In Europe, the General Data Protection Regulation (GDPR) protects individuals' personal data, including genetic information. There are similar laws in other countries such as the Personal Information Protection and Electronic Documents Act (PIPEDA) in Canada, the Privacy Act in Australia, and the DNA Technology Regulation Bill, which is under consideration in India. There are also specific laws that regulate the storing, sharing, and analyzing the genetic information of criminals in Ireland, the UK or South Africa.

An easy-to-use sequencing technology could challenge this legal framework. For example, a child could secretly conduct a paternity test or a father could test his child whether he is really the biological father. Before the advent of nanopore sequencing, paternity tests were usually done by specialized laboratories only after authorization by a court. Similarly, someone could use a customer DNA sequencer to track an individual and test for the presence of this person in certain locations or secretly test for genetic risk factors related to diseases or behavior. Hence there is a need for extending existing laws and regulations that protect the genetic information of individuals to prevent unauthorized usage of genetic data by individual users.

> *"... the existing laws and regulations that were mostly created to cover businesses and institutions may no longer be effective to prevent abuse and misuse by individuals."*

Again, these concerns are not new, but as nanopore sequencing greatly expands the potential users and customers of DNA sequencing, the existing laws and regulations that were mostly created to cover businesses and institutions may no longer be effective to prevent abuse and misuse by individuals. As the science and the applications are complex and constantly evolving, it will be challenging to draft the laws and regulations that cover these and potential future applications of DNA sequencing by people. Hence, there is a need for intense discussions that involve natural and social scientists, ethicists, legal experts, and politicians.

Conclusion

The rapid growth and diverse applications of nanopore DNA sequencing technology have made it a valuable tool for scientific research. The development of portable sequencing devices is a paradigm shift in sequencing technologies. However, the easy accessibility and potential of a consumer sequencer raise serious concerns about the ethical, social, legal, political, and security implications of this technology. Specific policies and regulations that are fair, responsible, and respectful of the values and rights of individuals need to be put in place to regulate the use of this technology and mitigate any negative impacts on society.

Despite many scientific publications on nanopore technology, there have not been many efforts to explore the social science and ethical implications of nanopore sequencing technology and its implications. As this technology penetrates more into the market for individual consumers, we may have to involve research centres such as the Centre for Nanotechnology in Society or the Science Justice research centre to start and organize a debate on how to safely employ this technology while ensuring its benefits for society. This is not novel terrain: The human genome project, synthetic biology, or nanotechnology have been accompanied and their public acceptance strengthened by involving social scientists, ethicists, and lawyers during the research and development of these technologies.

I hope this article will generate interest among experts in social science, policy, and law to explore these aspects of nanopore technology and generate productive discussions. This will provide opportunities such as sharing knowledge across the natural and social sciences, help to project unintentional negative consequences of nanopore sequencing technology, and help to shape the growth and trajectory of nanopore technology with ethical and humanitarian values.

Expanded View for this article is available online.

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Author contributions

Muhammad Sajeer P: Conceptualization; formal analysis; investigation; visualization; methodology; writing – original draft; writing – review and editing.

Disclosure and competing interests statement

The author declares that he has no conflict of interest.

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Practical guide for in-house solid-state nanopore fabrication and characterization

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ABSTRACT

Solid-state nanopores are considered a better alternative to biological nanopores for several sensing applications due to their better chemical, mechanical, and temperature stability. In addition to sequencing, nanopores currently also find applications in education, biomarker identification, quantification, single-molecule chemistry, and DNA computing. Nanopore technology's simplicity and wide interdisciplinary applications have raised further interest among industry and scientific community worldwide. However, further development in solid-state nanopore technology and exploring its applications presents the need to have the capability to fabricate them in-house. This will be a more financially viable and flexible approach, especially in resource-limited situations. In order to do an in-house fabrication of solid-state nanopores, two key steps are involved. The first step is to fabricate suspended thin films, and the second one is the drilling of pores in these suspended thin membranes. Successful implementation of these two steps involves tedious optimization and characterization of the fabricated chips and nanopores. In this work, we describe the nanopore fabrication process in a ready-to-follow step-by-step guide and present solutions for several practical difficulties faced during the silicon nitride pore fabrication process. This work will help anyone new to this field and make the pore fabrication process more accessible.

I. INTRODUCTION

Solid-state nanopore technology has received much interest due to numerous advantages, such as robustness and large-scale integration capabilities over their biological counterpart. The solid-state nanopores are currently being explored in various applications, such as protein and DNA sequencing, DNA computing, biomarker identification, quantification, and single-molecule chemistry.[1] Owing to the diversity in applications, low complexity of the technique, and expected enormous impact on science and society, several research groups and industries worldwide have begun working in solid-state nanopore technology, including biology and material science, to computing streams.[2–4] However, the complete process, including nanopore fabrication and translocation measurements, is riddled with enormous practical challenges. Other problems include relatively high prices and increased waiting time for the delivery of commercial solid-state nanopores. Overcoming these challenges requires developing in-house nanopore fabrication expertise, which helps to quickly customize the nanopore to meet the specific research requirements. Optimizing the fabrication protocols and conducting successful translocation measurements will take time, money, and effort, as several practical challenges could arise in the fabrication process (Fig. 1). These are usually not explicitly discussed in the existing solid-state nanopore-related literature, increasing the difficulties for novices in the field. This work addresses these problems by giving a ready-to-follow, step-by-step protocol to fabricate a silicon nitride nanopore starting from a bare silicon wafer.

II. FABRICATION PROTOCOLS

The entire fabrication process of silicon nitride nanopores involves two major steps (Fig. 2), which are (1) suspending a thin silicon nitride membrane and (2) fabrication of nanopores (in this case sub-10 nm diameter). Proper optimization of these two steps is essential for precisely controlling the pore size and thickness. This helps achieve better spatial and temporal resolution, enabling a high signal-to-noise ratio during translocation measurements for single molecular sensing.

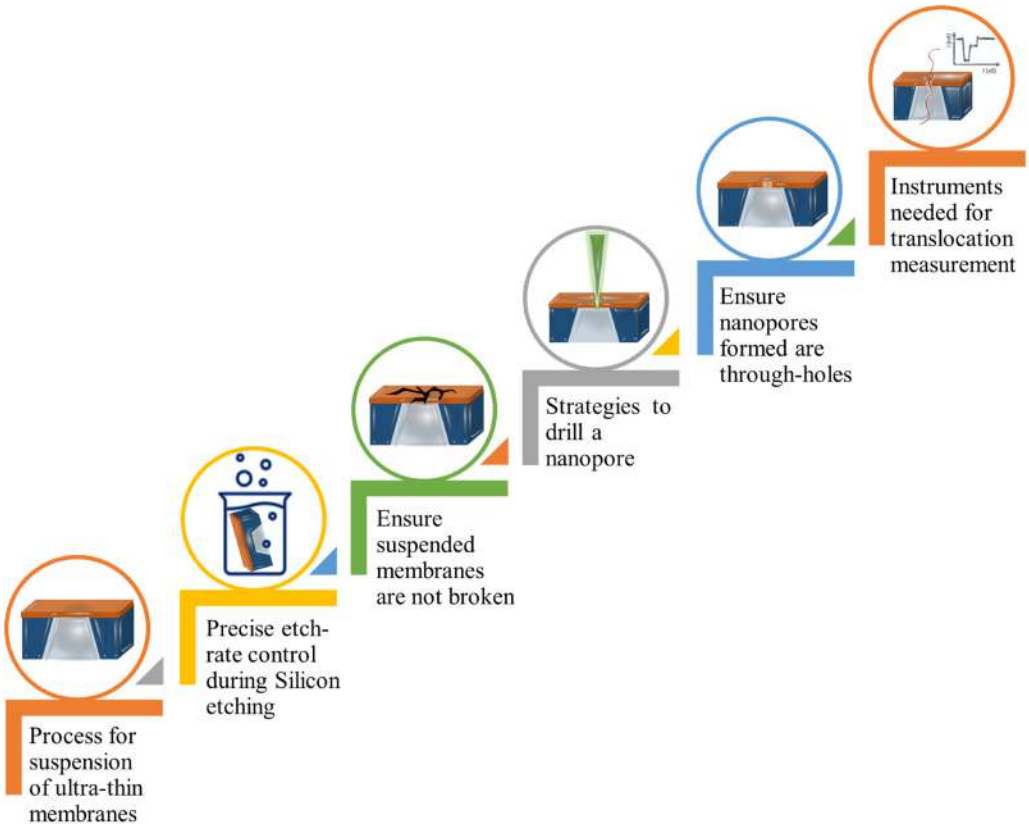

FIG. 1. Graphical representation of the challenges encountered when establishing an in-house solid-state nanopore fabrication process.

A. Suspending silicon nitride membrane

The thin silicon nitride membrane can be successfully released by careful optimization and tuning of parameters involved in this step, which otherwise will lead to membrane failures. Sections II A 1–II A 5 have detailed the silicon nitride membrane suspension starting from a bare silicon wafer.

1. Procuring silicon wafers and cleaning techniques

Silicon wafers are available in a wide range of sizes, generally from 2- to 12-in. wafers. These wafers can also be customized and procured as per the requirement. The details of standard silicon wafers are given in Table I.

For the current fabrication protocol, 2-in. n-type Si (100) wafers were chosen taking into consideration the fabrication tool constraints and the ease of wafer handling. For instance, choosing a wafer of lower thickness gives the advantage of less time required for etching bulk silicon using KOH. Also, the TEM (transmission electron microscopy) sample holder in our facility can easily accommodate the thickness of ~300 um silicon substrate for the fabrication of nanopores. The wafers were procured from Silicon Valley Microelectronics (Single side polished wafers) and WaferPro (Double side polished wafers). The membrane fabrication process is undertaken in the National Nanofabrication Centre at the Centre for Nano Science and Engineering (CeNSE), a research facility located at the Indian Institute of Science, Bangalore.

Before starting any device preparation processes, the newly procured wafers are recommended to be cleaned using a standard cleaning procedure used in the semiconductor industry. This standard cleaning procedure is a two-step process. The first step involves performing an RCA-1 clean or Standard clean-1 on the freshly procured wafers. This step comprises immersing these wafers in a mixture of 1:1:5 vol. % H_2O_2:NH_4OH:H_2O at 80 °C for 10 min. This procedure helps in cleansing organic residues and films from the silicon wafers. The second step in the cleaning procedure is called RCA-2 or standard clean-2. This step comprises immersing these wafers in a mixture of 1:1:5 vol. % H_2O_2:HCl:H_2O at 80 °C for 10 min. This step ensures that the wafers are cleansed of any metallic contamination that could have been present. The cleaning of the wafers is followed by a 30 s dip in a mixture of 1:50 vol. % of HF:H_2O to eliminate any thin silicon dioxide film that would be present on the surface of the Silicon wafers. It is to be noted that after each step, the wafers are thoroughly rinsed with de-ionized water (DI) water and blow-dried well with nitrogen gas before going for the membrane deposition process.

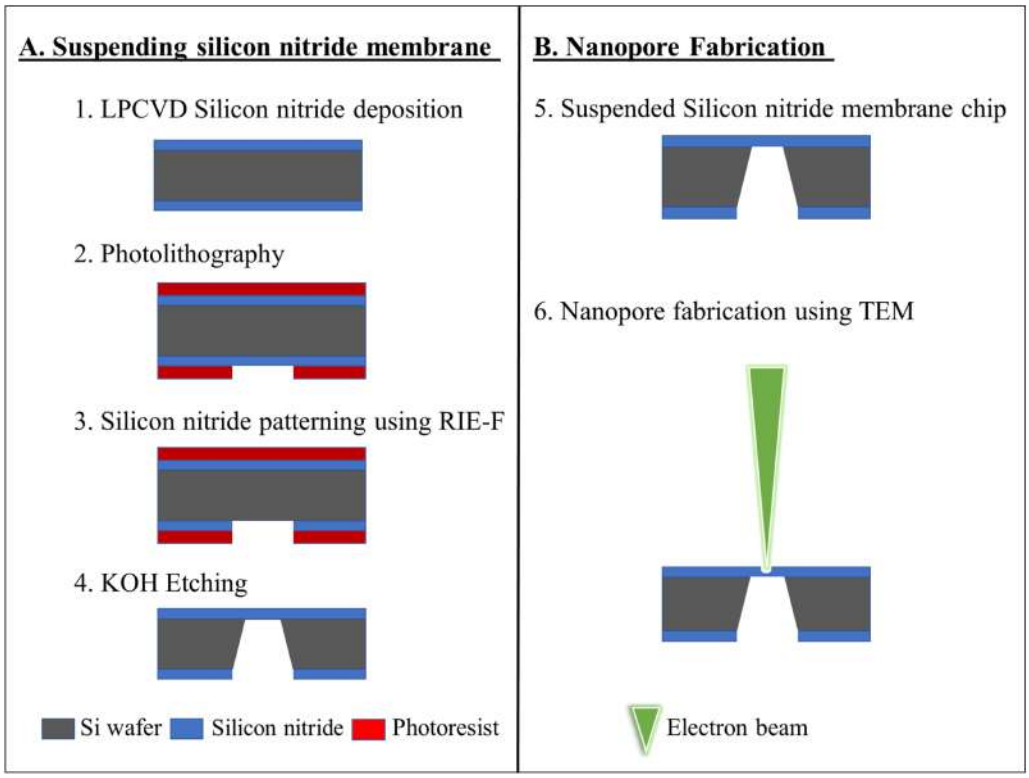

FIG. 2. Schematic illustration of the complete silicon nitride nanopore fabrication process. (a) Process flow for suspension of thin silicon nitride membranes. (b) Process flow for fabrication of nanopores in the suspended silicon nitride membranes using TEM.

2. Silicon nitride coating on a silicon wafer

After cleaning the silicon wafers, the immediate process is depositing low-pressure chemical vapor deposition (LPCVD) silicon nitride on both sides of the cleaned silicon wafer. This is crucial to prevent the formation of a thin silicon oxide layer on the bare wafer. LPCVD is a high throughput method for fabricating stable silicon nitride membranes. The silicon nitride layer deposited is mostly uniform and conformal at pressures below 1 Torr.[5] Silicon nitride has been chosen as the membrane material due to its popularity and compatibility with most semiconductor processes. These films are amorphous, stable against oxidation, dense, and provide a good barrier against moisture penetration.[6] As specified earlier, we will be using a 2-in. silicon wafer given that it is easy to handle, cheap, and of less thickness (~290 um), which will help reduce the overall process time to achieve membrane suspension. The LPCVD deposition process in the current fabrication protocol involves depositing silicon nitride film of thickness 30 nm on both sides of the bare Si wafer. The deposition is done at a temperature and pressure of 750° and 200 mTorr. Dichlorosilane (DCS) and ammonia (NH_3) are the source gases used with a gas flow rate of DCS: NH_3 = 10:70 sccm. The deposited silicon nitride film of thickness 30 nm was measured and verified to be uniform throughout the Si wafer using Ellipsometry (J. A. Woollam) with a standard deviation of 0.2 nm. The LPCVD can be followed by an annealing process, which can significantly reduce the residual stress as reported.[7] A high temperature annealing (~1000 °C) is required to reduce the residual stress considerably. We have not carried out an annealing process after LPCVD, as it can possibly increase the roughness of surface.[7]

TABLE I. Details of standard silicon wafers.

Types of wafers (in.)	Thickness (um)
2	275
3	375
4	525
6	675
8	725
12	775

3. Optical lithography for pattern transfer

After the silicon nitride thin film is deposited on the wafers, the next step is the process of creating patterns on silicon

nitride-coated wafers. This can be done by transferring patterns using optical lithography. For this, a positive photoresist (PR) (AZ5214E) is coated on the wafer using a spin coater (Laurell H6-23 Spin Coater). The spin coating process is performed with the conventional parameters of 4000 rotations per minute (rpm) for a duration of 40 s. After coating, the photoresist-coated wafer is soft baked for 1 min at 110 °C to remove photoresist solvents. The designed mask is then patterned on to the photoresist-coated wafer using a direct writing tool (Heidelberg UPG 501) that writes the pattern directly onto the substrate using a focused laser beam (exposure wavelength: 390 nm). This mask is designed to fabricate chips of 3 $\times$ 3 mm^2 throughout the 2-in. wafers having circles of diameter ~500 um at the center. This chip dimension has been chosen to make it compatible with our TEM facility for pore fabrication. The standard TEM sample holder can contain a sample of 3 mm diameter size and can be customized to hold larger sizes such as 8 mm. The layout for pattern writing can be designed on any layout editor tool, such as CleWin.[8] If required, a model mask design for nanopore can be downloaded from our research group website (https://sites.google.com/view/nanoporegroup/resources). Once the direct writing is completed, the substrate is dipped in the developer solution to remove the PR from exposed areas. We have used the MIF726 developer for the same, and it may take nearly 30 s for the development process to complete. These recipes were optimized for a temperature of 20 °C and relative humidity of 45 in a class 100 cleanroom. Once the development is complete, the PR-patterned samples are hard baked for 3 min at 110 °C to drive off any remaining solvents or moisture from the sample surface.

4. Backside silicon nitride removal prior to wet etching

The LPCVD silicon nitride is deposited on both sides of the bare Si wafer. To release membranes, we need to pattern the thin silicon nitride film from the PR-patterned side of the wafer to facilitate Si etching. Reactive ion etching based on fluorine chemistry can be used for patterning the thin layer of Silicon nitride. The Plasma lab systems 100 from Oxford Instruments are used for the dry etching using CHF_3 and O_2 gases (1:10 gas ratio). The recipe was optimized for a temperature and pressure of 20 °C and 55 mT. Once patterned, this thin silicon nitride layer will act as a hard mask during silicon etching.

5. Wet etching for suspending silicon nitride membrane

Once the etching patterns are formed on the silicon nitride hard mask, the membranes can be suspended by etching the silicon from the backside using dry or wet etching techniques or an optimized combination of both. The classic dry etching technique to etch out all 280 um or a significant part of the 280 um thick silicon is to use a deep reactive ion etching (DRIE) tool. The process involves using gases SF_6 and C_4F_8 and is done at a temperature and pressure of −10 °C and 200 mT with a gas ratio of 1:2.4. The etch rate of silicon was measured as ~37 um/min using a Dektak XT Surface profiler. This high etch rate gives us less control over the etching when we approach the membrane, causing membrane failure for almost all chips. This is a key argument for not utilizing the dry etching technique to completely etch the silicon out of the etch pattern.

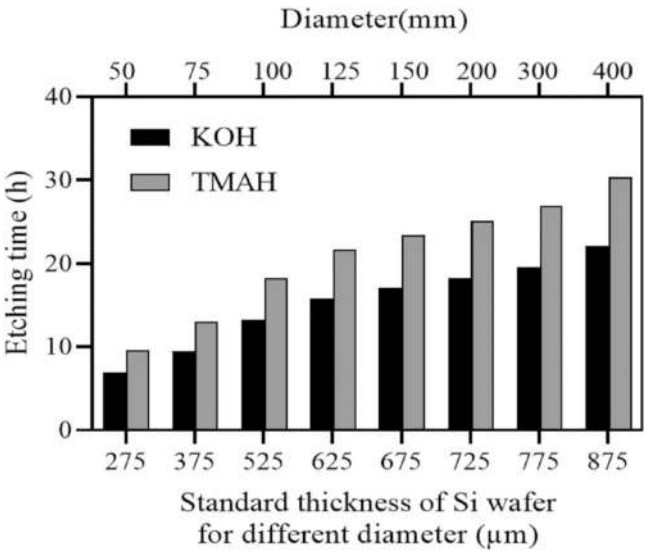

FIG. 3. Etching time to form through holes in different thickness silicon wafer using 5 wt. % TMAH and 20 wt. % KOH at 70 °C. Reprinted from Pal *et al.*, Micro Nano Syst. Lett. **9**(1), 4 (2021). Copyright (2021) Springer Nature under a Creative Commons License.

The typical technique for silicon wet etching is to use the potassium hydroxide solution (KOH)/tetramethylammonium hydroxide (TMAH) solution. The etching times of both solutions are compared in Fig. 3.[9]

These wet etching techniques are well-established anisotropic etching processes due to the dependence of the silicon etch rate on the temperature and concentration of the etchant solution and the crystal plane orientation, allowing precision in the geometry of the structures to be fabricated. This gives us better control over the etching than the dry etching technique when we etch close to the membrane and helps us ensure successful membrane suspension in most devices. We have used the KOH etching protocol to remove the silicon from the patterned area. A 30% KOH solution by weight is used for the process (EMPARTA Potassium Hydroxide Pellets, DI water). The standard KOH etching process requires the solution to be consistently maintained at 70 °C for approximately 7 h, as shown in Fig. 3. To avoid this time-consuming procedure, we have divided the process into two steps of KOH etching carried out at two different temperatures. The first step involves etching at 90 °C for 1 h 45 min, followed by etching done at 80 °C for 1 h 50 min. The temperature specified here is the temperature of the solution. This is controlled using standard hot plates (IKA C MAG H7 Hotplates). A single etching step of maintaining the solution at 90 °C for 2 h 30 min also works given that you control the stirring well, reducing the chances of membrane damage (Fig. 4). The details of the etching setup and devices used for precise temperature controller are discussed in Sec. IV.

Alternatively, a combination of DRIE and KOH etching processes can be optimized and adapted to achieve successful silicon

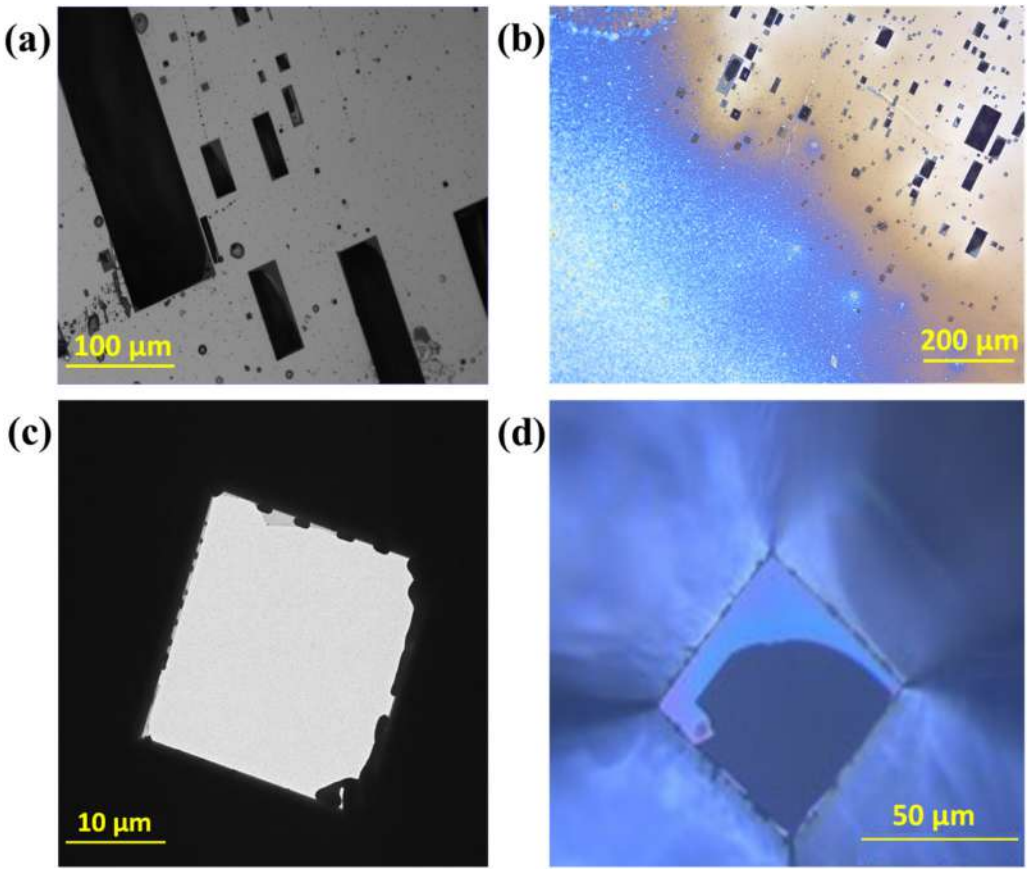

FIG. 4. (a) and (b) Optical microscope images of multiple microslit formations. (c) TEM image of the broken membrane. (d) Optical microscopy image of the broken silicon nitride window.

etching. For this, it is recommended to perform dry etching using DRIE [SPTS LPX Pegasus DRIE system] for 6.5 min and then wet etching in 30% vol KOH solution at 80 °C for 45 min. The etching time required for DRIE and KOH Etching has been measured using a Dektak XT Surface profiler. The etching patterns in the hard mask need to be designed accordingly. This method ensures a quick initial etching that saves time for the fabricator and then a slow and more controlled etching as we etch closer to the membrane, thus taking care that fewer membranes are damaged.

Once the membrane is suspended successfully (Fig. 5) and is confirmed by observing through an optical microscope, dipping the sample in isopropyl alcohol for 3 min and in DI Water for 1 min is advised to clean the chip.

B. Nanopore fabrication using TEM

TEM-based nanopore fabrication is the most precise nanopore fabrication technique currently available,[10] and it is a well-established technique.[2,11] Even though dielectric breakdown-based nanopore fabrication[12] is emerging, lack of control over the location and size of the nanopore is still a significant problem. Unlike other tools, TEM provides live visual confirmation of pore formation and has several applications in the solid-state nanopore domain, from pore drilling to bioimaging.[10] The conventional sample size for TEM is 3 mm, even though it can be customized up to 8 mm diameter samples. We have used a transmission electron microscope (Titan Themis 300 kV from Thermofisher Scientific) for pore fabrication, which is available in the Micro and Nano characterization facility at CeNSE, Indian Institute of Science, Bangalore.[13] The nanopore can be drilled in approximately half a minute by electron beam exposure (Fig. 6). Although operational values of TEM to drill a nanopore vary from instrument to instrument, it is helpful to have an idea about the parameters, such as the size of the beam, dosage (number of electrons falling on a unit area of the thin film), and intensity for the reproduction of exact pore sizes. Usually, an accelerating voltage of 200 or 300 kV is commonly used for the fabrication of nanopores. The electron beam with an intensity of 10^8–10^9 e/nm^2 s with full width half maxima of 2–10 nm is tightly focused on the silicon nitride membrane to drill a nanopore of size ranging 3–6 nm. Defocused beams can be used

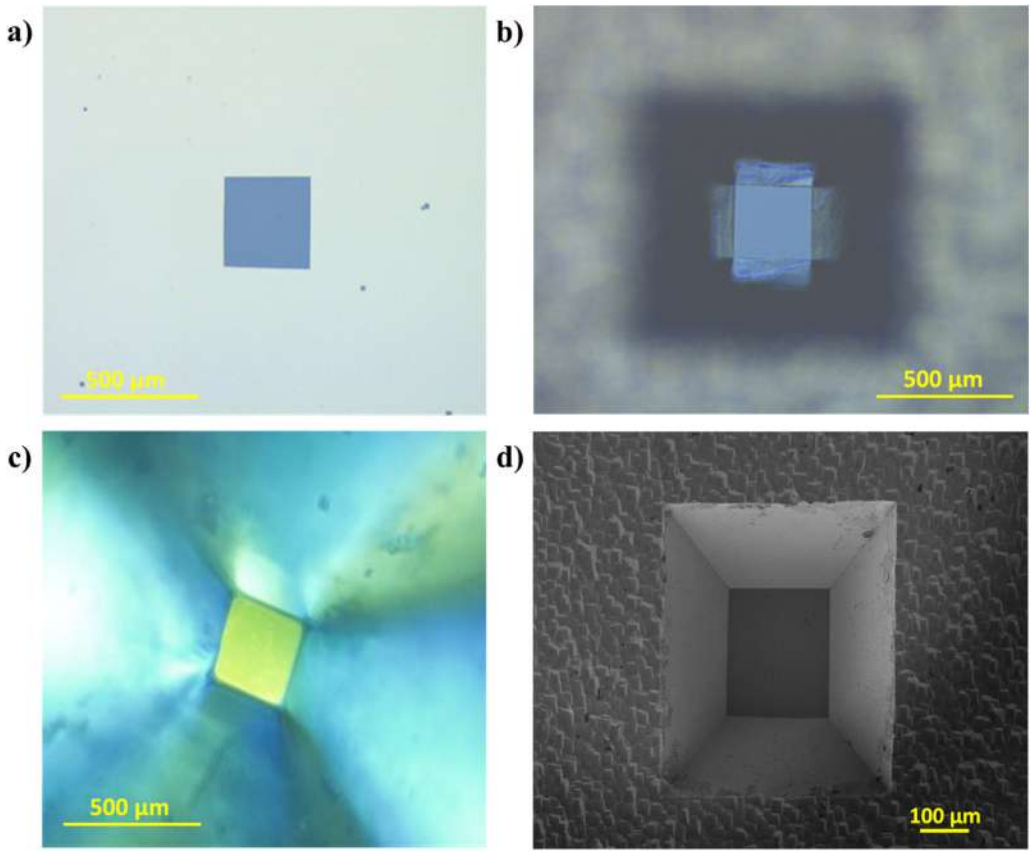

FIG. 5. Suspended silicon nitride membrane under the optical microscope: (a) top view, (b) bottom view, (c) suspended membrane imaged using differential interference contrast mode of the optical microscope, and (d) SEM image of KOH etched area.

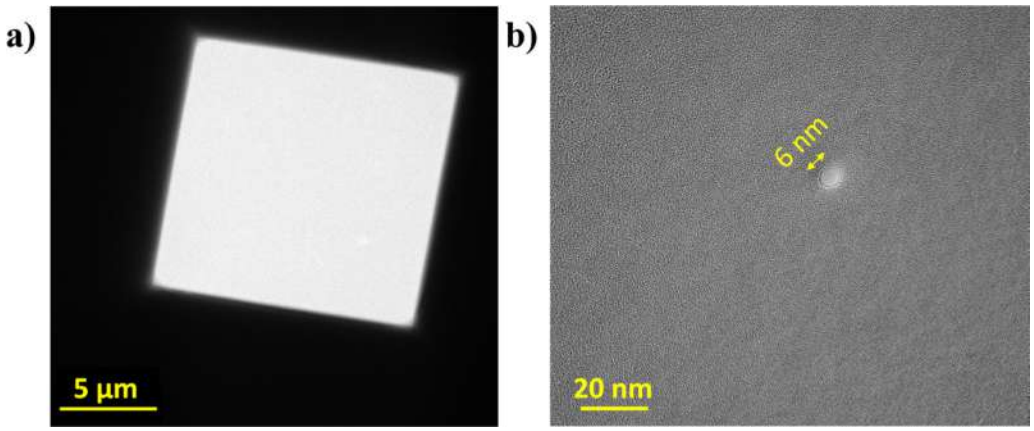

FIG. 6. (a) TEM image of the suspended silicon nitride window. (b) 6 nm pore fabricated using TEM.

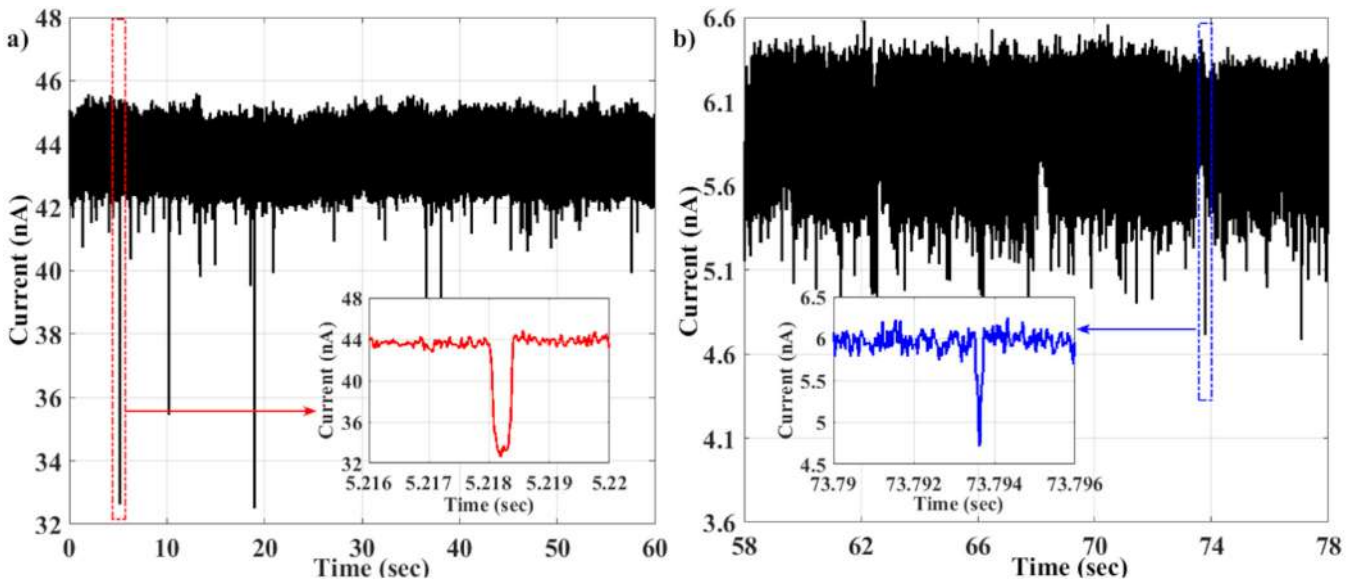

FIG. 7. (a) Current measurement data of translocation of 6HB through the nanopore (raw data). Inset shows the zoomed in detail of a single event. (b) Current measurement data of translocation of DNA Duplex through the nanopore (raw data). Inset shows the zoomed in detail of a single event.

to enlarge (if needed) and to fine-tune the size and shape of the pore. The theory and experimental aspects of electron beams assisted nanopore fabrication are explained in detail in an earlier review paper from our group.[10]

III. DETECTION OF TRANSLOCATION OF BIOMOLECULES USING THE FABRICATED NANOPORE

The sensing capability of solid-state nanopores fabricated using the protocol explained in this work is shown in this section.

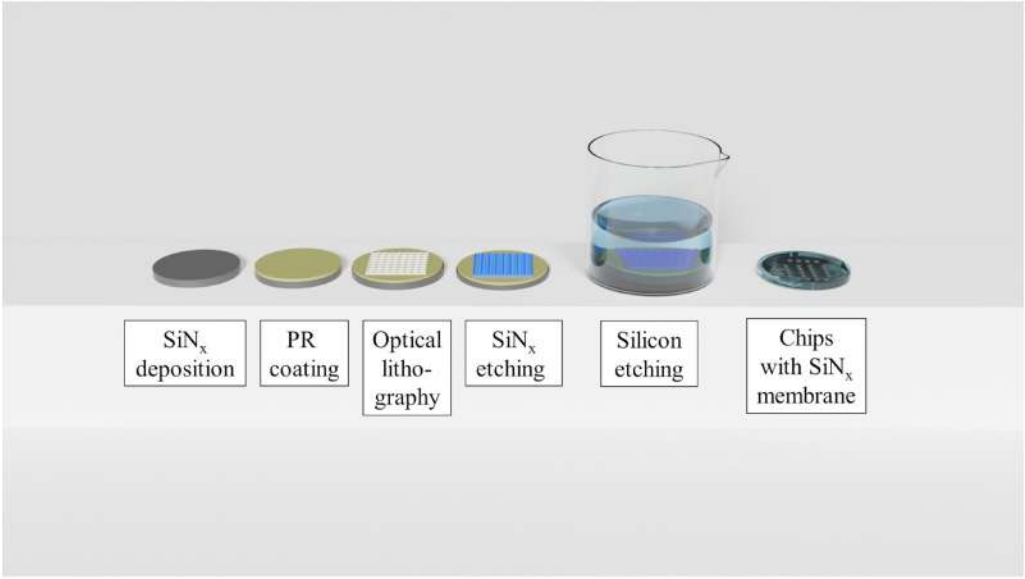

FIG. 8. Simple schematic of the cleanroom fabrication process for membrane suspended TEM compatible chips.

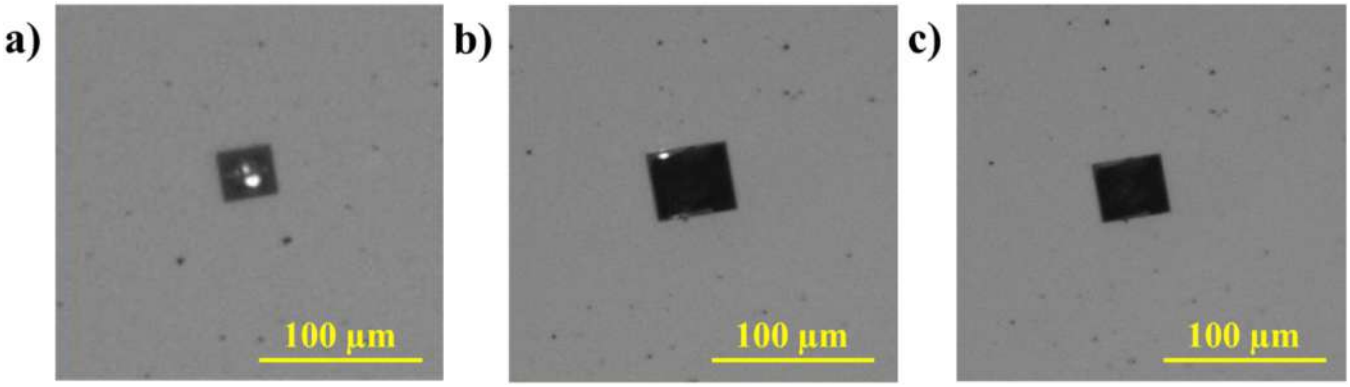

FIG. 9. Images captured using the laser Doppler vibrometer. The central black square is the membrane region. (a) Reflection of the laser (see white dot) from the membrane region confirms the presence of an intact membrane. (b) Laser dot reflects from an edge of a partially broken membrane. (c) No laser reflection can be seen in the membrane region when the membrane is completely broken.

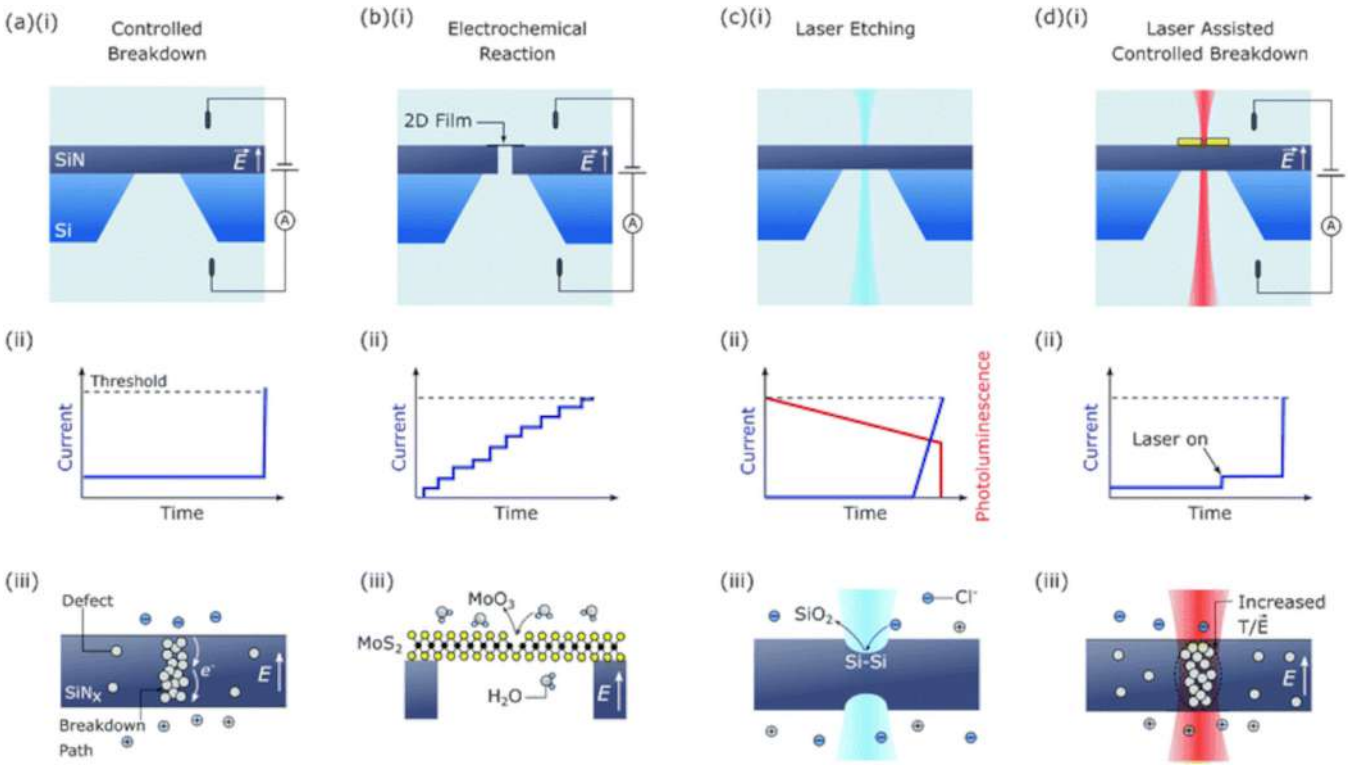

FIG. 10. Schematics of the experimental setup (top), measurement trace (middle), and pore formation mechanism (bottom) for the four *in situ* pore fabrication techniques. (a) Controlled breakdown (CBD) whereby nanopores are formed by applying a large electric field (~0.6–1 V nm^{-1}) across a dielectric membrane. Such electric fields result in charge trap accumulation that forms a percolation path and results in the physical breakdown in the membrane as a result of Joule heating. (b) ECR where nanopores are formed in suspended two-dimensional films due to electrochemical reactions originating from a defect in the film. (c) Laser etching whereby nanopores are formed as a result of photochemical etching of Si-rich SiN_x membranes in solution. (d) la-CBD whereby a laser is focused on the membrane simultaneous to the application of an electric field to induce breakdown at the focal point of the laser. This can be due to either localized heating, laser etching, or enhanced electromagnetic fields. Note that the threshold current shown in the experimental measurement trace schematics will not be the same magnitude for each technique. Reprinted from Fried *et al.*, Chem. Soc. Rev. **50**(8), 4974–4992 (2021). Copyright (2021) Royal Society of Chemistry under a Creative Commons License.

Translocation signals of six helix bundles (6HB) (using approx. 20 nm pore) and 29 bp DNA (using approx. 5 nm pore) through the fabricated nanopore at an applied voltage of 200 and 600 mV, respectively, are shown in Fig. 7. The 6HB is made by DNA origami technique using M13mp18 (New England Biolabs). Buffer with the concentrations of 1 M KCl, 10 mM Tris-HCl, 1 mM EDTA at pH 7.5 is used for the experiment. Data were collected using Elements SRL nanopore reader at 50 kHz.

IV. SOLUTIONS FOR PRACTICAL DIFFICULTIES DURING FABRICATION

We have discussed in detail the step-by-step fabrication protocol for membrane suspension and nanopore fabrication (Fig. 2) in Sec. II. Several practical issues arise during different steps of the nanopore fabrication process. These are not usually mentioned in the existing literature. With the view that this work serves as a practical guide, the major challenges encountered and their proposed solutions are listed in this section.

A. How to ensure that the silicon nitride membrane is suspended successfully?

The first major step toward fabricating nanopores is the successful release of thin silicon nitride membranes (Fig. 8).

The cross-sectional SEM is an excellent tool for the optimization of KOH etching. Once the membrane is suspended, confirmation of the presence of an intact suspended membrane is one of the major issues both during fabrication and transportation. As the color of silicon nitride membrane varies with respect to thickness and becomes more transparent at ultralow thickness, this can be challenging. The membrane can break due to several reasons if we are not careful enough, even during transportation, as shown in Figs. 4(c) and 4(d). If the membrane has a high aspect ratio, the chances of membrane breakage are higher. Along with ionic current measurement, there are several rough and ready ways to check if membranes are intact. Optical microscope [Figs. 5(a) and 5(b)], the differential interference contrast mode of the optical microscope [Fig. 5(d)], and shining laser on the membrane and imaging the same (the laser Doppler vibrometer instruments are equipped with this facility as shown in Fig. 9) can be used to check if membranes are still intact.

B. How to avoid breakage of thin silicon nitride membrane?

One of the major challenges in successful membrane fabrication is the failure of the membrane due to loss of mechanical integrity in the thin film deposited. Literature as early as the 1960s suggests that the fracture responses of samples from the same material with different thicknesses are different.[14] This causes a crack to require different amounts of energy to stretch and damage along different planes of maximum shear stress. Since fabricating an ideal nanopore device requires releasing membranes as thin as possible, the breakage issue is even more severe. Silicon nitride is brittle, and its strength is directly correlated with the defects in the membrane. The defects can be reduced either by reducing the window area or by applying residual compressive surface stress to counteract the applied pressure, which causes the membrane to break.[15]

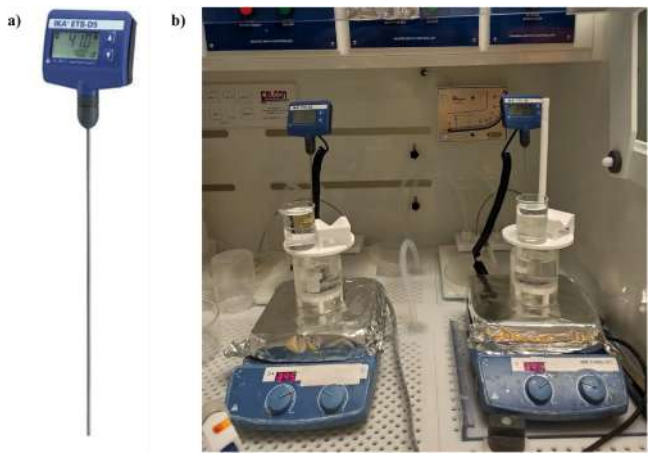

FIG. 11. (a) Automatic temperature controller. (b) Whole KOH etching setup.

Decreasing the silicon nitride membrane residual stress can also contribute to the same. This intrinsic residual stress in silicon nitride films can be altered by changing the silicon component of the films. This is usually done by adjusting the relative concentration of the gases, dichlorosilane, and ammonia, to achieve different ratios of silicon and nitrogen components in the film. Studies indicate that the intrinsic residual stress of Si-rich silicon nitride films is lower than the standard stoichiometric silicon nitride films due to reduced strain in the Si–N bond.[16] Additionally, LPCVD silicon nitride deposition at 700–900 °C is preferred over plasma enhanced chemical vapor deposition silicon nitride, as this film deposited is a denser and more stable thin film with fewer defects.[7]

Moreover, any amount of optimization of process conditions does not entitle us to even minor chip mishandling. Things like gel packs, lint-free cleanroom wipes, and polydimethylsiloxane (PDMS) -lined boxes can be used to carry them around to avoid membrane breakage during transportation. We would recommend against creating a vacuum within the desiccator as the potential pressure difference created could cause membrane failure.

C. How to ensure no silicon remains at the backside of the membrane?

The thin silicon nitride membrane is transparent when observed through an optical microscope. A brownish color in the optical microscope image is an approximate indication of the presence of silicon remaining most of the time. This is one of the ways we can ensure that no silicon remains on the suspended silicon nitride membrane. Characterization techniques, such as Raman spectroscopy and TEM EDS, can also be used to check if any Silicon remains.

D. What are the cost-effective alternative methods for TEM-based nanopore fabrications?

The accessibility of the field of nanopore research gets restricted to many researchers, especially in developing and underdeveloped countries, due to the complexity, low throughput, and

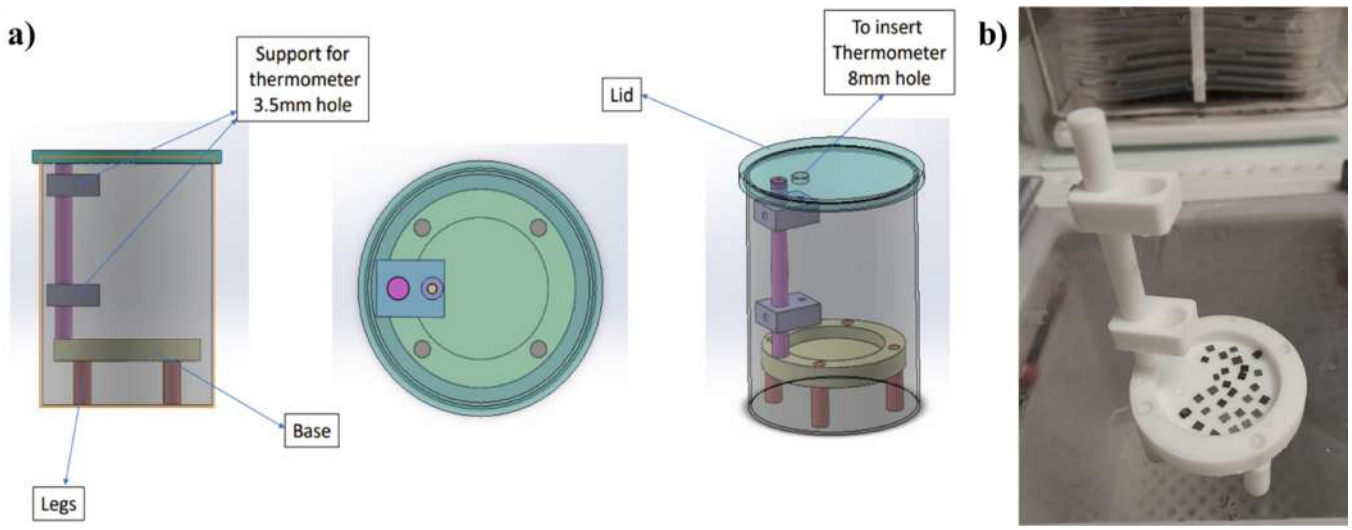

FIG. 12. (a) Design of sample holder with provisions to keep thermometer and enough gap for the magnetic rotator. (b) Fabricated Teflon holder with 3 mm chips.

expensive tools and techniques required. This restricts the research opportunities in this field to a relatively small community. TEM and FIB (focused ion beam milling) are not common facilities in labs worldwide, especially in universities and colleges in underdeveloped and developing countries. So, alternative, less complex, and low-cost strategies need to be developed to improve the reach of this research field.

One of the cost-effective alternatives for nanopore fabrication using TEM or FIB is controlled dielectric breakdown (CBD).[12] Figure 10(a) shows the typical dielectric breakdown setup, current measurement during nanopore fabrication, and pore formation mechanism. An electric field applied across the ultrathin membrane causes a small leakage current through the charge traps in the dielectric membrane. Over a period, depending on the thickness of the membrane, a sudden increase in the current is noted due to the formation of a percolation path through the charge traps signifying breakdown and formation of nanopore. At this point, the voltage supply is quickly turned off to prevent any further damage to the membrane. Despite its advantages like reduced cost and complexity, CBD exhibits low yield in forming nanopores of similar features for the same protocol.[17] Each run could present possibilities of forming multiple nanopores, thinned regions, or irregularly shaped nanopores along with the correctly fabricated nanopores.

Like the CBD method, the electrochemical reaction (ECR) method utilizes a similar experimental procedure. The major difference that can be pointed out between the two methods is that CBD is helpful for thicker dielectric materials like silicon nitride, and ESR is helpful for thinner conductive materials, such as MoS_2 and

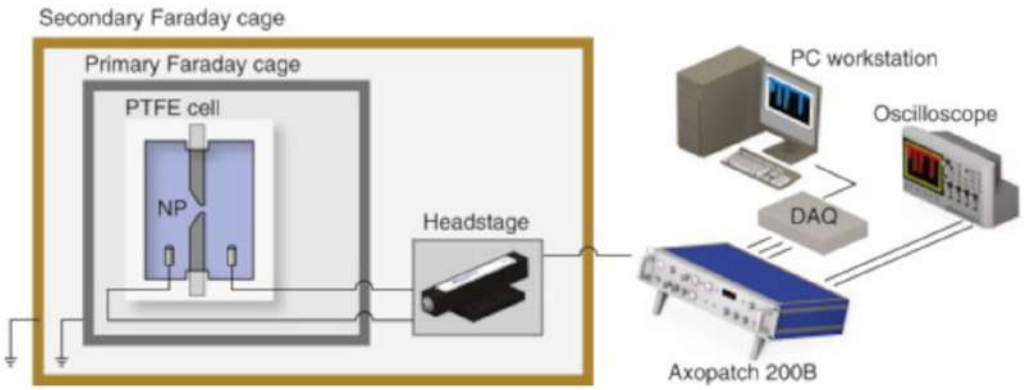

FIG. 13. Schematic diagram of typical nanopore sensing setup. Reprinted with permission from V. Tabard-Cossa, *Engineered Nanopores for Bioanalytical Applications* (Elsevier, ■, 2013). Copyright 2013 Elsevier.

TABLE II. List of tools required to perform nanopore translocation experiments.

Instruments/accessories	Commercially available products
Amplifier	Axopatch 200B (Ref. 20), AM2400 (Ref. 21), Element SRL (Ref. 22)
DAQ	DAQ Card, Axon Digitizer (Ref. 23)
Dielectric breakdown setup	Northern Nanopore (Ref. 24)
Flow Cell	Teflon flow cell
O rings	Seals Shop (Ref. 25)
Solid-state nanopore starter kit	Zimmer and Peacock (Ref. 26)

graphene. The details of the typical setup, current measurement while fabrication of nanopore, and the mechanism of pore formation are shown in Fig. 10(b). The nanopore formation in this method is expected to be due to the electrochemical removal of exposed atoms near the defect in the film.

Other than these, laser etching can also be used for the fabrication of nanopores. This method uses a low-power, highly focused blue-green laser to destabilize Si–Si bonds in Si-rich silicon nitride membranes, causing the eventual formation of nanopores. The nanopore formation can be monitored by applying a small transmembrane potential simultaneously with the laser illumination and keeping tabs on the measured ionic current. The experimental setup here is slightly more complex than the CBD technique but can still replace the traditional methods of TEM or FIB. The details of the typical setup, current measurement, and fabrication of nanopore and the mechanism of pore formation are shown in Fig. 10(c).

Another method built on these existing low-cost methods is laser-assisted controlled breakdown (la-CBD). The breakdown in the membrane can occur owing to photochemical etching at the focal point of the laser, localized heating in the membrane, or the effect on the charge traps in the membrane because of the enhanced electromagnetic field. The details of the typical experimental arrangement, current measurement while fabrication of nanopore, and the mechanism of pore formation are shown in Fig. 10(d).

E. How to get good control of the KOH etch process?

As mentioned in Sec. II A 5, the etching process to release the silicon nitride membrane requires continuous monitoring over a long time. The process involves etching in two steps, each performed at different temperatures. This requires precise control of the temperatures for each step to avoid unmanageable etch rates of the Si substrate. Our setup uses an automatic temperature controller (IKA ETS D5) with a hot plate (IKA HS MAG 7) to achieve precise control (Fig. 11). This allows us to set the required temperature for the solution with high precision and does not require manual intervention.

F. How to handle 3 mm-sized samples during etching?

Even though all nanofabrication facilities are equipped with wafer processing carriers, there will not be many options to deal with samples as small as 3 mm. A proper enclosure is also required to prevent KOH liquid evaporation, which otherwise will change the volume ratio. We have custom designed a holder that can be put in a standard 500 ml beaker and the provision to keep the thermometer, as shown in Fig. 12.

G. How to prevent pore expansion over time?

The expansion of solid-state nanopores over time is a major challenge. The nanopores expand over time in electrolyte solutions, varying from ~0.2 to 3 nm/day. Atomic layer deposition of 1 nm hafnium oxide has been reported to prevent this long-term pore expansion.[18]

H. What are the typical solid-state nanopore-based tools that can be used for measurement?

Once the nanopore chips have been successfully fabricated, we can move on to testing them for different applications. To do the DNA/protein translocation measurements, it is essential to set up dedicated amplifiers and filters optimized for nanopore measurements. Figure 13 shows a schematic diagram of all the building blocks required to assemble a nanopore setup optimized for low-noise and high-bandwidth current measurements.[19]

The nanopore chip is mounted in a fluidic cell made of Teflon with a tight seal between the two electrolyte reservoirs on either side of the chip. This flow cell is connected to a patch-clamp amplifier with good capacitive feedback to reduce the noise to as low as possible in the current recordings. The signal data can be directly observed on an oscilloscope or be recorded using a PC Workstation. A computer controlled DAQ setup helps digitize analog data signals obtained from the amplifier. We have presented the instruments required to carry out these low-noise nanopore experiments in Table II.

V. CONCLUSIONS

Nanopore technology is recently gaining research interest due to its futuristic industrial applications and high societal impact. Its existing applications range from healthcare technologies (e.g., DNA sequencing) to DNA computing. Hence, researchers from multiple domains are increasingly working on solid-state nanopores. This work explains the step-by-step protocol to follow and address the common practical challenges and their solutions during the fabrication of silicon nitride nanopore. We have also shown the translocation data of DNA duplex and 6HB structures through these fabricated nanopores, as well as listed the typically used instruments required for nanopore experiments. This work will help anyone new to this field and make the pore fabrication process more accessible.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Anumol Dominic and Muhammad Sajeer P contributed equally to this work.

Anumol Dominic: Investigation (equal); Methodology (equal); Validation (equal); Writing – original draft (equal); Writing – review & editing (equal). **Muhammad Sajeer P:** Investigation (equal); Methodology (equal); Writing – original draft (equal); Writing – review & editing (equal). **Simran Nasa:** Methodology (supporting). **Manoj Varma:** Funding acquisition (lead); Investigation (equal); Methodology (equal); Resources (lead); Supervision (lead); Writing – review & editing (equal).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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[22]See https://elements-ic.com/ for information about "Elements—Miniaturized, Easy to use, Affordable Instruments."
[23]See https://www.moleculardevices.com/products/axon-patch-clamp-system/digitizers/axon-digidata-1550b-plus-humsilencer for information about "Axon Digi data 1550B Low-Noise Data Acquisition System plus HumSilencer, Mol. Devices."
[24]See https://www.solidstatenanopore.com/ for information about "Northern Nanopore Instruments."
[25]See https://www.seals-shop.com/eu/en/o-rings for information about different O-rings available.
[26]See https://www.zimmerpeacocktech.com/partner-products/nanopore-solutions/ for information about "Zimmer and Peacock—Solid State Nanopore Starter Kit."