Published in final edited form as:

Methods Mol Biol. 2012 ; 870: 79–97. doi:10.1007/978-1-61779-773-6_5.

DNA characterization with Ion Beam Sculpted Silicon Nitride Nanopores

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Abstract

Solid state nanopores are emerging as robust single molecule electronic measurement devices and as platforms for confining biomolecules for further analysis. The first silicon nitride nanopore to detect individual DNA molecules were fabricated using ion beam sculpting (IBS), a method that uses broad, low energy ion beams to create nanopores with dimensions ranging from 2 to 20 nm. In this chapter, we discuss the fabrication, characterization, and use of IBS sculpted nanopores as well as efficient uses of pClamp and MATLAB software suites for data acquisition and analysis. The fabrication section will cover the repeatability and the pore size limits. The characterization discussion focuses on the geometric properties as measured by low and high resolution transmission electron microscopy (TEM), electron energy loss spectroscopy (EELS), and energy filtered TEM (EFTEM). The section on translocation experiments focuses on how to use tools commonly available to the nanopore experimenter to determine whether a pore will be useful for experimentation or if it should be abandoned. A memory efficient method of taking data using Clampex's event-driven mode and dual channel recording will be presented, followed by an easy to implement multi-threshold event detection and classification method using MATLAB software.

Keywords

Ion beam sculpting; silicon nitride nanopore; ionic current blockage; DNA size; DNA conformation

1. Introduction

The measurements of single DNA molecules with thin silicon nitride nanopores were published in 2001 and significantly extended in 2003 (1,2). The silicon nitride nanopore were fabricated using ion beam sculpting (IBS), a method that uses broad, low energy ion beams to create nanopores with dimensions ranging from 2 to 20 nm. The results of these studies demonstrated that the earlier DNA translocation studies with biological nanopores could be performed with a synthetic material in which the diameter and surface properties of the pores could be engineered. It was also shown that current blockages in this synthetic material obeyed Ohm's law and mean translocation times could be modeled by a simple viscous drag model. The analysis of DNA translocation through synthetic nanopores revealed that the DNA could pass through the pores while folded, forming discrete blockage levels and demonstrating that the nanopores could be used to measure cross sectional changes along the translocating molecule.

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Shortly thereafter studies using alumina coated IBS pores demonstrated that DNA folding during translocation could be decreased by using higher voltages, suggesting that an inhomogeneous dielectrophoretic ‘tidal force’ on the DNA molecule can cause it to unfold as it diffuses near the pore (3). Optical observations of λ -DNA fluorescently labeled with YOYO-1 further established that molecules diffusing within a micrometer scale radius of the pore would most likely be captured. In another experiment with IBS pores, the diffusion characteristics and the capture radius were measured by rapidly reversing voltage polarity to cause the same DNA molecule to translocate back and forth through the pore, varying the time the molecule was allowed to drift before reversing the polarity for recapture (4). These experiments strengthened the conclusion that the capture process had a characteristic capture radius where the applied electric force would overcome the thermal forces on the DNA molecule and that the electric field was responsible for the unfolded, linear translocation of the molecule.

In addition, experiments that focused on different types of DNA molecules rather than the translocation process itself demonstrated that denaturation of the molecule could be detected on the basis of the translocation properties of ssDNA versus dsDNA (5). The ssDNA had roughly half the current drop of dsDNA (Fig. 1A), yet the more flexible DNA would still pass through the nanopore in an unfolded state in the majority of the translocation events. Since ssDNA is the preferred molecule for nanopore-based DNA sequencing applications, this study was a technologically important step towards achieving this goal. The detection of folded ssDNA also indicated that regions tagged to the width of dsDNA could be localized on a DNA molecule, suggesting possible applications in DNA genotyping.

Research with IBS pores have also demonstrated that the event charge deficit (*ecd*), the integrated area of an event and equivalent to the total displaced charge during the event, was conserved for molecules of the same length (6), regardless of conformation (Fig. 1B) and could be used to measure the length of DNA (Fig. 1C). Circular relaxed, supercoiled, and linear DNA molecules of the same length were found at different positions along the same constant *ecd*/hyperbolae (Fig. 1D). These results allowed the simultaneous determination of conformation, number of superhelical turns, and length from a mixture of DNA types and lengths. This approach would be more effective with longer DNA molecules and should provide improved resolution over the standard size separation used with traditional gel electrophoresis for molecules over 40kb. The pitch and total number of superhelical turns of the supercoiled molecule can be determined by comparing the current blockage of nicked and supercoiled molecules since the superhelical conformation increases the current drop over the relaxed form as shown in Figure (1E).

2. Materials

2.1. Free Standing Si₃N₄ Membranes

Fabrication procedures to produce free standing membranes are discussed at length elsewhere (7). In short, 275 nm thick low tensile stress silicon rich Si_{3.5}N₄ is deposited by low pressure chemical vapor deposition (LPCVD) on both sides of 4 inch silicon wafers (100). Photolithography, reactive ion etch, and then wet chemical etching with 30% w/v KOH at 90°C to produce pyramid-shaped recesses that release a roughly 30 μ m x 30 μ m freestanding membrane of SiN 275 nm thick (Fig. 2A). In the center of this membrane a submicron hole is formed either by focused ion beam (FIB) milling or electron beam lithography (Fig. 2B). FIB milling of a 100 nm hole through the 275 nm structure is facilitated by higher energy systems such as the 50keV Ga⁺ beam of the Micrion 9500. Electron beam lithography can be done at the Cornell Nanofabrication Facility. After creation of the submicron holes, the wafer can then be diced or cleaved to release individual

chips (*see* Note 1). Chips should be stored individually in a desiccator box until the area of the submicron hole can be imaged (*see* Note 2).

2.2. Ion Beam Sculpting Apparatus (IBSA)

Many parts of the IBSA can be purchased off the shelf, but must be custom assembled into a dual chamber vacuum system. The sample carriage and electron optics for the feedback mechanism must also be custom fabricated. In depth design information of this system can be found in the literature (8,7,9). In brief, a high vacuum system capable of operation in the 10^{-8} mbar range or lower, with a load-lock system to prevent contamination of the main chamber, is fitted with an ion beam source from the Thermo Electron Corporation and electron beam source from Kimball Physics, custom sample mounting and electrostatic lenses and an energy analyzer, and a Channeltron style single ion detector made by Burle. The ions passing through the forming pore are detected by the Channeltron and the signal is amplified by a custom made pulse amplifier and counted by an Agilent Universal Counter connected to a data acquisition card controlled by a PC-LabVIEW system.

2.3. Nanopore Characterization

Nanopores can be imaged on any TEM with at least 1 nm resolution, such as the JEOL 100-CX (*see* Note 3). Voltages from 60kV to 300kV give good contrast for basic imaging purposes. Thickness mapping and materials properties investigation must be performed on high resolution TEM fitted with scanning TEM (STEM) mode and EELS or EFTEM modes such as the FEI Titan 80–300 kV S/TEM fitted with a Gatan post-column energy filter.

2.4. DNA Molecules for Translocation Studies

1. 5.4 kbp DNA from PhiX174 (New England Biolabs; Ipswich, Massachusetts, USA www.neb.com)
2. 3.0 kbp plasmid pSP65 (Promega Corporation; Madison, Wisconsin, USA www.promega.com)
3. NoLimits linear DNA fragments of almost any size up to 20 kbp (Fermentas Inc.; Glen Burnie, Maryland, USA www.fermentas.com)

2.5. Nanopore Fluid Chamber and Electrodes

2.5.1. Electrochemical electrodes

1. Silver wire (World Precision Instruments; Sarasota, Florida USA, www.wpiinc.com).
2. Bleach (Clorox; Oakland, California USA)
3. Luer-Lock fittings (Small Parts, Miami Lakes, Florida, USA, www.smallparts.com)
4. Polydimethylsiloxane (PDMS) (Ellsworth Adhesives, Germantown Wisconsin, USA, www.ellsworth.com)

¹Since several TEM steps will be required, having a sample compatible with the TEM will greatly ease processing. Since most TEM holders can accommodate only 3mm circle, the TEM holder may require custom modification by milling out a recess capable of holding the chip. We have modified TEM holders to accommodate both 3mm x 3mm and 4 mm x 6 mm chips for the JEOL 100-CX, and for 3 mm x 3mm chips for the FEI Titan.

²Samples should be stored such that the membrane does not come in contact with another solid surface. This prevents contamination and breakage. Samples can be stored in hinged plastic containers such as those offered by Mr. Plexi (Ithaca, NY www.mrplexi.com) with a small amount of PDMS cured in the bottom. Samples placed membrane side up will adhere to these sample holders even during light movement.

³A high throughput TEM imaging system will save many hours waiting for vacuum pumpdown time and increase the feedback loop when troubleshooting fabrication problems.

5. 2 mm and 1 mm connection plugs to fit with Axon 200B headstage (World Precision Instruments; Sarasota, Florida USA, www.wpiinc.com)

2.5.2. Sample holder

1. Polydimethylsiloxane (PDMS) (Ellsworth Adhesives, Germantown Wisconsin, USA, www.ellsworth.com)
2. Aluminum or plastic mold with pins (Mcmaster-Carr, Elmhurst, Illinois, www.mcmaster.com)
3. 3 ml syringes (VWR international, Radnor Pennsylvania, USA, www.vwrsp.com)
4. 3-way Luer-Lock valves (Small Parts, Miami Lakes, Florida, USA, www.smallparts.com)
5. Intramedic Polyethelene Tubing for fluid input, silicone tubing for fluid output (VWR international, Radnor Pennsylvania, USA, www.vwrsp.com)
6. Blunt needles (Small Parts, Miami Lakes, Florida, USA, www.smallparts.com)

2.6. Chemicals and Filtering

1. 18M Ω water, can be dispensed from Waterpro PS polishing station (Labconco Corp., Kansas City Missouri, USA)
2. KCl (VWR international, Radnor Pennsylvania, USA, www.vwrsp.com)
3. Tris HCL (VWR international, Radnor Pennsylvania, USA, www.vwrsp.com)
4. EDTA (VWR international, Radnor Pennsylvania, USA, www.vwrsp.com)
5. KOH (VWR international, Radnor Pennsylvania, USA, www.vwrsp.com)
6. Whatman 0.02 μ m nucleopore filters with Swin-Lok holder (VWR international, Radnor Pennsylvania, USA, www.vwrsp.com)

2.7. Data Taking and Software

1. Axon Axopatch 200B (Molecular Devices Inc., Sunnyvale, California, USA, www.moleculardevices.com)
2. Digidata 1332A Digitizer (Molecular Devices Inc., Sunnyvale, California, USA, www.moleculardevices.com)
3. PC running Windows (*see* Note 4)
4. pClamp 9 or 10 (Molecular Devices Inc., Sunnyvale, California, USA, www.moleculardevices.com)
5. MATLAB (MathWorks, Natick, Massachusetts, www.mathworks.com)

3. Methods

3.1. Pore Fabrication

1. Take a low resolution TEM image of the submicron hole, contrast should be high. Store sample in desiccator until ready to be closed.

⁴See requirements for pClamp software on Molecular Device's website, www.moleculardevices.com. Hard drive size should be chosen so that space is always available for experimentation. Continuously saving data can fill a hard drive of any size and files of many GB are not unheard of.

2. Load a nanopore chip (Fig. 2A) or sample onto carriage.
3. Align sample on carriage using a microscope. Building a replica of the carriage holder in the main chamber onto a dedicated inverted microscope saves time and significantly increases reproducibility. If this is not done, the ion beam spot can be scanned across the surface until maximum counts are detected. Since this procedure depends on the alignment abilities of the user, it can take variable amounts of time, making beam exposure time variable. Sample loading and alignment should take as short a time as possible to prevent contamination and typically lasts only a few minutes.
4. Vent load lock with dry N₂. Mount carriage in load lock.
5. Pump load lock to a pressure that will prevent contamination of the main chamber, usually in the 10⁻⁷ mbar range.
6. Load carriage into main chamber under vacuum.
7. Turn on all electrostatic lenses. Turn on ion beam with beam diverted to metal plate connected to an ammeter to measure beam current. Monitor the beam current until it becomes stable. For the Thermo Scientific EX05 ion gun this is usually about 5 min.
8. Turn on electron beam and monitor the current on the same plate until stable.
9. Using the TEM image, calculate the area of the submicron hole for flux calculation. Areas of digitized TEM images can be measured using ImageJ (rsbweb.nih.gov/ij) by tracing the edges of the hole. Most submicron holes made by FIB are slightly elliptical and calculating the area by measuring the semimajor diameters of the ellipse by ruler usually get one within 5% error of the area calculated by imageJ and within unknown errors elsewhere in the closing process.
10. Using LabVIEW, divert the beam to the sub-micron hole (see Note 5). Using the initial number of counts per second and the measured area, calculate the flux F from $F = \frac{C_0}{A_0}$, where C_0 is the initial number of counts per second, A_0 is the initial area of the pore. Waiting for the beam current to stabilize before closing the pore ensures flux is constant, so the final number of counts for the desired pore area can be found from $C_f = A_f F$. When the final number of counts is reached, the beam is automatically deflected from the nanopore.
11. (optional) For pores smaller than approximately 10nm, the flux can be increased and the pore can be closed by manual pulses. Deflect the beam when the number of counts corresponds to a 20 nm diameter. Increase the flux 10X and pulse for a sort time on the order of 100 μ s. Using the new number of counts and assuming the pore size did not change for the first pulse, estimate the new flux. Calculate the new final number of counts and pulse until this value is reached.
12. Remove sample and store sample in desiccator box until final TEM image can be taken.

The resolution of the system is fundamentally shot noise limited by the number of ions passing through the pore. A 5 nm pore has an area of 19.6 nm², so for a typical flux of 1 ion/

⁵Because the closing process usually takes on the order of minutes, completely automated control of closing does not need to be done. Using pen and paper to calculate flux and desired final number of counts can be done while waiting for the pore to close and manually diverting the beam by a switch has been done. In order to foster flexibility and avoid program complexity, the user can calculate flux and desired shut off counts, but have the computer take control and divert the beam once the desired number of counts has been reached.

sec/nm² and one second of integration, 19–20 counts are expected through the pore. The number of ions passing through the pore in a fixed amount of time can be modeled as a Poisson process, which means estimates of the mean number of counts over that time have an RMS noise equal to the square root of the number of counts, in our case 4.4 counts corresponding to diameters between 4.3 and 5.5 nm. A 2 nm final diameter is similarly limited to sizes between 1.3 and 2.5 nm. In practice, a higher range of variability is experienced with more pores completely closing than expected. Since each measurement of counts is found by integration over a timescale between 0.1 and 1 sec, and that diameters shrink with a rate of 1–10 nm/sec, the assumption that the measurement is instantaneous becomes invalid during the final few, important seconds of pore fabrication, usually yielding smaller than expected pores. It has also been shown that the pores close even after the beam is turned off and may ‘coast’ to a smaller size (10). A flexible method to decrease signal to noise ratio and increase time resolution is to manually pulse the beam for short periods of time at fluxes an order of magnitude higher than those used to close the pore. In practice, the smallest reproducible nanopore size is 2 – 4 nm.

3.2. Pore Characterization

A final picture by TEM (Fig. 2C) is usually necessary to determine the diameter of the pore because of the variability of the IBS method. Almost no sample preparation is required to image nanopores in the TEM if the chip is designed to fit in a TEM holder or a holder is modified to accept the chip and the TEM (*see Note 1*). Using a device with pump down times of about 1 min, an experienced user can take a nanopore image in less than 10 minutes. Manual focusing and astigmatism correction can be done by underfocusing a submicron hole and correcting astigmatism seen in the Fresnel ring inside the hole, similar to the procedure for astigmatism correction using holey carbon films.

Electron energy loss spectroscopy (EELS) and energy filtered transmission electron microscopy (EFTEM) can be used to characterize the pore’s thickness profile. The thickness of pores made by the IBS method have been measured using destructive ion beam sputtering at low temperature to be between 8–20 nm depending on fabrication parameters (11). This method relies on accurate estimates of sputter profile rates and cannot be done on pores that are later used for DNA translocation experiments. Modern TEMs capable of computer controlled EELS and EFTEM measurements make nondestructive thickness profiles of the pores possible.

Most electrons that impinge upon a sample either pass straight through the sample or lose energy through inelastic collisions (12). The thickness of the sample can be measured from the spectrum of electron energy loss using Kramers-Kronig analysis or the log ratio method. The log ratio method requires an experimental calibration, but is much simpler and very close to the Kramers-Kronig method. The log ratio thickness can be found by

$$t = \lambda \ln \left(\frac{I_{total}}{I_{zlp}} \right) \quad \text{Equation (1)}$$

where λ is the mean free path of electrons with at zero loss energy, I_{total} is the total integrated number of electrons measured, and I_{zlp} is the total number of electrons in the zero loss peak. The one calibration parameter, λ , can be estimated theoretically, but the known thickness of the freestanding membrane near the pore provides an excellent calibration. For 300 keV electrons in Si_{3.5}N₄, λ is 185 ± 2 nm. For samples less than a few mean free paths, separation of the zero loss peak from the remaining spectrum is trivial as it is almost completely separate from the rest of the spectrum. The thickness measured is averaged over the entire region interacting with the beam. In order to build 1-D thickness profiles or 2-D

thickness maps, the beam is typically controlled in STEM mode and the spectrum measured point-by-point as the beam is moved.

EFTEM thickness maps are measured in a similar manner and can quickly produce 2D thickness maps (Fig. 2D) with thousands of positions measured in a very short time. To do this, an energy filtered zero loss electron image is taken in addition to an unfiltered image. Only a narrow pass band around the zero loss peak is allowed to strike the detector in the filtered image before removing the energy filter and immediately taking the unfiltered image. The number of electrons recorded by each pixel of the CCD becomes the integrated areas of the zero loss peak and total peak. After cross correlating the images to adjust for any sample drift, the log ratio of each pixel is calculated and multiplied by the mean free path digitally. The choice of energy range used to determine the zero loss peak intensity is determined by the inherent peak width and the proximity to the first plasmon loss peak. Since this method cannot deconvolute the plasmon and zero loss peaks, the assumption that the narrow energy range selected for the zero loss peak leads to less reliable results for material less than 10% of the mean free path and will report nonzero thicknesses for the vacuum at the center of the pore. This thickness baseline can lead to inaccuracies in samples without a vacuum region, but the vacuum region of nanopore thickness maps gives the calibration point necessary to avoid this problem. Simply subtracting the measured vacuum thickness from all pixels removes this problem.

3.3. Single Molecule Measurements

3.3.1. DNA preparation—To obtain linear DNA, circular DNA can be linearized or linear DNA such as NoLimits can be purchased directly. To produce linear DNA from circular DNA, the supercoiled and relaxed circular PhiX174 DNA molecules is diluted to the appropriate concentrations (typically 10 nM) using TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). A restriction enzyme digestion is performed using an enzyme that cuts the circular plasmid only once. For example, the digestion of pSP65 can be performed using SmaI. A good choice are restriction enzymes that yield blunt ends (i.e. SmaI, EcoRV, PvuII) versus sticky ends (i.e. BamHI, EcoRI, HindIII) to avoid single-stranded ends on the DNA molecules. After restriction, the DNA is purified by two sequential phenol:chloroform extractions (1:1 ratio), followed by one chloroform extraction, and finally precipitation of the DNA with two volumes of ethanol. The restriction digestion and quality of the recovered DNA can then be confirmed by agarose gel electrophoresis and UV absorbance. The basic methods for DNA digestion, agarose gel electrophoresis, and the determination of DNA purity and concentration by UV spectrophotometry have been described in numerous molecular biology manuals including Sambrook and Russell (13). For denaturation of the linear dsDNA to linear ssDNA, adjust the solution to pH 13 using KOH.

3.3.2. Fabrication of chambers—A set of disposable fluid chambers with an integrating chip sealing face can be made by PDMS. The chambers can incorporate press fit seals for electrodes and fluid inlets and outlets. The design of such a device (Fig. 3) needs to consider the efficiency of fluid flow across the chip to avoid dead regions where solution may not be exchanged and avoid bubble formation. One successful mold design uses stainless steel machine pins of the diameter desired for the interior chambers that can slide through holes in a mold machined from aluminum or plastic. In order for the PDMS chambers to press against the SiN surface and form a seal, holders with clamps are needed to press the PDMS components together.

1. Prepare PDMS: hardener mixture in a 10:1 ratio by mass and stir until well mixed in disposable plastic cup.

2. Place PDMS in bell jar for approximately 20 min under vacuum or until all bubbles are gone.
3. While degassing, clean mold. An aluminum mold can be sonicated in acetone for 15 min. followed by isopropyl alcohol for 15 min.
4. Assemble mold with pins in place and pour degassed PDMS over mold. Bubbles that form while pouring can be removed with a pipette tip.
5. Heat mold at 70 °C for 3 hours.
6. Disassemble mold by first removing the pins, and then forcing out the molded fluid chamber.
7. Using a dissecting microscope, narrow tip tweezers, and sharp hypodermic needle as a cutting tool, clean up any unwanted films or other artifacts.
8. Clean fluid chamber by sonicating in 10% ethanol for 15 minutes. Ethanol is added for weak cleaning and to wet the interior of the PDMS. Replace solution with 18 MΩ water and sonicate for 15 minutes.
9. Blow dry chambers with clean N₂ and store in clean place until ready to use.

3.3.3. Electrode fabrication

1. Insert enough Ag wire into the Luer-Lock fitting such that it fits several millimeters past the end of the Luer-Lock head.
2. Use laboratory tape to affix the end to be soldered to the Luer-Lock in a water tight fashion.
3. Mix and degas several grams of PDMS similar to chamber fabrication procedure and fill the empty space between the Luer-Lock interior and the electrode with unhardened PDMS. Arrange the Luer-Lock fitting so that it holds the PDMS in a cup-like fashion.
4. Heat electrode head in an oven at 70 °C for 3 hours, or leave one week at room temperature.
5. Solder and heat shrink about 10 cm of thin, flexible wire to the small end of the electrode head.
6. On the opposing side of the wire, solder a 1mm male pin to mate the electrode with the headstage.
7. Sand the electrode tip with fine sandpaper, 600 grit works well.
8. Sonicate the electrode head in 18 MΩ water for 15 minutes.
9. Bleach the Ag tip in household Clorox bleach to form an Ag/Cl coating.
10. Electrode tip can be stored in bleach or washed with water and dried with clean N₂ and store in clean place until ready to use.

3.3.4. Alignment and establishing open pore current

1. Since the freestanding membrane is transparent, alignment with a microscope is straightforward. Place one of the PDMS chambers with sealing surface facing upwards illuminated from below. Since light can pass through the PDMS and the freestanding membrane it can be aligned by hand on the PDMS surface. In the design presented in Figure (3), light can pass unobstructed through the center of the PDMS piece. By looking down the open tube on the top PDMS chamber, it can be

aligned on top of the chip. Using the clamps, affix the PDMS chambers to each other and mount the electrodes and tubing

2. Flow 18 MΩ water taking special care to avoid bubbles in the system. This can be facilitated by only fitting the fluid inlet valves and flushing liquid such that a few drops of liquid pass through the hole for the fluid outlet and electrode holes. Carefully inset the fluid outlet tubing and electrodes.
3. Flush the system with 1 M KCl, 10 mM Tris, 1 mM EDTA and hook up to the headstage of the Axon 200B.
4. If the pore is wet (*see* Note 6) and current flows, measure and determine the IV curve and the RMS noise at 120 mV to determine if pore resistance and noise are within specification. The pClamp software can perform an automated IV curve, but a single current measurement at roughly 120 mV yields a good estimate of the pore resistance. The Axon 200B can measure the RMS noise over a 5 kHz bandwidth on the front panel. If unavailable, the RMS noise can be determined by roughly 1/6th the peak-to-peak distance of the noise at 120 mV.

3.3.5. Pore usability classification by RMS noise and resistance—Once conducting, some nanopores show anomalous noise or resistances different from that expected by the measured geometry of the pore. IBS fabricated nanopores at 0 mV bias have RMS noise very close to the baseline thermal noise of an equivalent resistor. Pores with 120 mV bias tend have 1/*f* noise as well as a small amount of white noise above the 0 mV limit that depends heavily on applied bias (14,15). In practice, good pores have a 5 kHz bandwidth RMS value within 2–5 pA above the baseline thermal noise limit (*see* Note 7). Pores that have noises higher than this range are usually rejected or are for further treatments to reduce the noise.

Predicting the open pore current depends on the precise geometry and would require a numerical solution of the Poisson-Boltzmann equation. A useful approximation of the open pore current treats the pore and submicron pore vestibule as cylindrical resistors in series and includes the access resistance on either side of the pore open to bulk solution (Fig. 4) (16,17). For very thin pores, the pore resistance becomes small enough that the resistance due to ions converging from the bulk to a small disk must also be considered—the access resistance—yielding a total resistance

$$R_{total} = R_{a,n} + R_n + R_v + R_{a,n} \\ = \rho \left(\frac{1}{2d_p} + \frac{4L_p}{\pi d_p^2} + \frac{4(L_m - L_p)}{\pi d_v^2} + \frac{1}{2d_v} \right) \quad \text{Equation (2)}$$

where ρ is the fluid resistivity, d_p and L_p are the pore diameter and length respectively, d_v is the diameter of the vestibule facing the bulk solution, and L_m is the membrane thickness. The first and last terms represent the access resistances of the nanopore and vestibule sides, respectively, while the second and third terms represent the resistance of the pore and vestibule (FIB hole), respectively. Varying the parameters of this equation within their uncertainties can usually account for the open pore current measured, and give open pore current predictions smaller than nanopore resistance only model. Pores with currents above

⁶Pores soaked from several hours to days in 10%–50% ethanol tend to wet within minutes of applying voltage and most blockages are due to macroscopic bubbles inside the chamber or fluid line. For pores that do not wet, KOH is an effective wetting agent. A 0.1 M KOH, 1M KCl solution flushed over the pore for a few minutes tends to help open the pore.

⁷A wide variety of chemical treatments have been attempted to clean noisy pores with only occasional success. KOH solutions in the 0.1M–1M range have been tried the most often and occasionally help reduce noise.

the nanopore only model are suspected of breaking or dissolving and are rejected if the experiment requires precise size control of the pore. Pores that are not open or have a current that is too small are suspected of being clogged by contamination and may be cleaned by chemical means.

3.3.6. Pore failure—A nanopore can become unusable when its open pore current is too big, too small, or too noisy. During an experiment, the pore often becomes clogged as seen in discrete current blockages that do not return to the original baseline current. Manually reversing the applied potential for a few seconds often ejects the molecule and clears the pore, but clogging tends to become more frequent as the experiment continues. Reversing the potential for longer amounts of time and at higher reverse biases helps, but eventually the pore remains at the lower current value or only briefly maintains the original open pore current. This situation is often—but not always—accompanied by very high 1/f noise and the inability to translocate any more molecules. In some situations the pore current will fluctuate rapidly between the open pore current and a current near the previously measured current drop, probably due to part of an adsorbed biomolecule partitioning into and out of the pore. Our multiple attempts at recovery of the pore functionality by chemical denaturation of the adsorbed molecules have been met with some success using chemical denaturants such as Urea and KOH.

3.3.7. Adding DNA

1. Before opening the pore, prepare a pre-diluted experimental sample of DNA at the desired pH and salt concentration. DNA concentrations between 5–10 nM work well. If the DNA concentration used is too high, events come very frequently and are hard to separate. Moreover, clogging of the pore can occur soon after the experiment begins (*see* Note 8).
2. Once a stable, low noise open pore current is established, carefully remove the waste outlet and pipette out most of the fluid from the *cis* chamber, making sure a layer of fluid remains over the nanopore at all times and back fill the entire chamber with the pre-diluted sample. Use the pipette to stir the solution.
3. Apply a voltage of roughly 120mV and wait for up to a few minutes for translocations. This wait time depends on how well the *cis* chamber was mixed and nanopore quality (*see* Note 9).

3.3.8. Recording data—Data can be recorded using the Axon Instruments Clampex program with the Axon 200B and digitizer. Although this software is capable of many different operational modes, two modes are most frequently used for nanopore experiments. The simplest is a continuous, or gap-free, recording of the signal. This mode is simple, but for experiments lasting hours can create cumbersome files that are slow to move and analyze. Slightly more complicated is the event-driven mode where a single trigger level is set on the fly to capture events that one observes by eye. In this method, a user defined number of points up until the current moment is buffered, but only committed to permanent storage when an event falls below a trigger level. This mode benefits from the small files saved, roughly 30MB for approximately 10,000 events at a 2 μ s sample rate, and can be

⁸Pores can sometimes clog without warning, so preparing and freezing samples ahead of time can save effort since they are ready to immediately add to the sample chamber once thawed.

⁹Take note of the open pore current and noise levels, some pores can clog very quickly and then stop translocation, dropping slightly in current. Observe the pore current while quickly reversing polarity and returning to the original bias, sometimes a few translocations can be seen as the bias is reversed, indicating molecule are adhered to or very close to the pore. If this is the case, denaturant can be added to clean the pore. If no molecules are detected, re-stir the solution and try a higher voltage if the molecule is near the size of the pore.

configured to save the moment in time when the event occurred, allowing event frequency analysis. As a rule of thumb, to detect an event falling below a trigger line, the event amplitude must be greater than the peak-to-peak noise of the system. To ameliorate this problem, the Axopatch provides a dual channel mode where the event can be triggered from a band pass filtered channel. Setting the lower edge of the pass band to around 5 Hz removes most of the slow drift and $1/f$ noise and makes data acquisition much easier.

3.3.9. Analyzing data—Molecular Devices will aid parsing binaries and importing data into MATLAB or similar software for analysis by readily providing users documentation on the header and binaries stored in their proprietary Axon Binary Files. At this time they do not provide MATLAB scripts for this purpose, but several can be found for free in the MathWorks File Exchange (see Note 10). The header stores important scaling information and details on the location of the binary data that must be extracted in order to properly interpret the saved current values that make up most of the file. Molecular devices will also provide example MATLAB scripts that are of great use to the experimenter in understanding the header and following binary file.

Experiments are often taken over the course of hours and are sometimes accompanied by a very slow drift that can change the open pore current between events and can cause problems with data analysis later on. In order to better compare these events side by side and then later by automated analysis, it is useful to adjust the drifting baseline to a constant value. In event driven mode this becomes easy since the drift is negligible for a single event. Once binaries are parsed into data formats that are easier to handle, event detection can be based on the events that were used by pClamp to define the time to record the event. By taking the mean of pre-event and post-event data, an estimate of the baseline can be made. If event detection is left to the data analysis algorithm, the experimenter can define an amount of time before and after the analysis algorithm to average to estimate the current. The length of time used depends on the event frequency and noise encountered during the experiment. For typical experiments with event frequency no more than 10 Hz and a RMS current noise of less than 10 pA, times of 1 ms are often used.

Once baseline drift is removed, a multiple trigger level method can be used to detect the events and define a range of parameters that can be used to classify events. Depending on the application, two or more trigger levels can be used. In a single trigger level scheme, an event begins and ends when it crosses below and back above the trigger. In a situation where the event has a magnitude larger than the peak-to-peak value of the noise and very fast rise and fall time, this is adequate. The high bandwidth required for a fast fall and rise time also increases the noise, ultimately limiting the temporal resolution of the measurement for even high amplitude events. Almost as simple and much more useful is a two trigger level scheme, where a lower event-reject trigger level is added (Fig. 5). The original trigger level is moved to or just below the baseline current and registers the beginning and ending of possible events, but only events that also cross the lower trigger level are classified as good events. A further benefit of this scheme is that event duration as defined by the first point the event drops below the baseline current can be measured by the first trigger, while event amplitude can be estimated by only those points that fall below the second trigger. For Gaussian noise, where the peak-to-peak noise level is defined as 6 times the RMS value, the signal to noise ratio for the single trigger level method is roughly equal to the peak-to-peak noise since the base of the event must completely clear the noise of the baseline. For the double trigger level method the signal can be half the peak-to-peak noise since the only requirement is that the noise at the base of the event be below the first trigger. For partially

¹⁰The authors have not used the software available on the MathWorks website. <http://www.mathworks.com/matlabcentral/fileexchange>

folded dsDNA or molecules with large variations in cross section, the event can be viewed as a series of nested square pulses. The sub-event can be approached in the same manner as the first event, using an added trigger to determine the location, duration, and amplitude of the sub event.

The quality of events found by the trigger level methods depends on the signal to noise ratio of the experiment, but even in high signal-to-noise situations many events are false positives and need further classification and removal. We have developed a variety of heuristic approaches that classify these events to ease downstream data analysis. Clear examples are inductive type noise spikes that cause a swing in measured signal down and then returning upwards. To remove these, the experimenter finds the upper limit of noise of the baseline and sets a trigger to classify and remove events that rise above this level. By far the most frustrating situation is when the baseline drifts slightly such that the noise range triggers the baseline and noise reject triggers, creating thousands of false events. These events are often very short, occur near each other, and do not have a baseline near the adjusted value. Filtering by duration and proximity is straightforward and useful, but classification based on the deviation of the pre and post event current from baseline produces the best results for the widest variety of ‘bad looking’ events. By comparing the absolute deviation of the mean of the pre and post event current from the overall average baseline current, events that do not begin or return to the baseline current can be removed. Since downstream data analysis typically assumes that the event can be broken into a series of a few nested square pulses, events that do not meet this assumption, although interesting, should not be sent through the same translocation time and current drop feature extraction procedures. Luckily, these events occur infrequently and the focus can remain on the simpler event types usually discussed in the literature.

Acknowledgments

This work was supported by the National Human Genome Research Institute (NIH) R21HG004776, and partially supported by NSF/MRSEC 080054, ABI1114, and 1P30RR031154-01 from the National Center for Research Resources (NCRR) of NIH.

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Figure 1.

DNA conformation and length characterization. A) Density plot of current drop magnitude versus translocation time for 3 kbp ssDNA at pH 13. Inset i, ii, and iii show completely folded, partially folded, and unfolded event traces. Outline of density plot above and to the right is for the same length of dsDNA at neutral pH. B) ecd of 5.4 kbp DNA in several conformations. C) ecd of dsDNA ladder, numbers at each peak are length in bases. D) Density plots of 5.4 kbp in several conformations. E) Nanometer scale geometry of linear, circular relaxed, and supercoiled DNA. Panel A reproduced from Fologea, 2005 with permission from the American Chemical Society. Panels B, C, D, E reproduced from Fologea, 2007 with permission from Wiley.

Figure 2. Nanopore characterization. (A) Cross section and top view of a nanopore chip. (B) Ion Beam Sculpting nanopores by keV noble gas ions by closing a larger FIB hole. TEM images (C) and corresponding thickness maps (D) measured by EELS and EFTEM.

Figure 3.

Cross section of PDMS chip holder apparatus. Fluid inlet tubing at left side press fits into PDMS cavity. At top and bottom, fluid outlets and electrode ports are wider, so luer lock adapter press fits into PDMS cavity. The cis (top) fluid outlet has been removed to load the sample manually via pipette. PDMS potted luer fittings with chlorinated silver wire are connected on the right side of the apparatus. Voltage is applied and current measured using the Axopatch headstage, with digital output sent to the control electronics. Clamps used to hold entire system together not shown.

Figure 4. Cross sectional view of nanopore and vestibule left over from sub-micron hole. Four-resistor resistance model. From top to bottom, access resistance of nanopore, resistance of nanopore, resistance of sub-micron hole vestibule, access resistance of vestibule.

Figure 5.

Single event recorded at 120 mV. Three candidate events fall below the baseline trigger, only the event that falls below the event-reject trigger is classified as an event. Double-level trigger level finds when a double-level drop occurs.