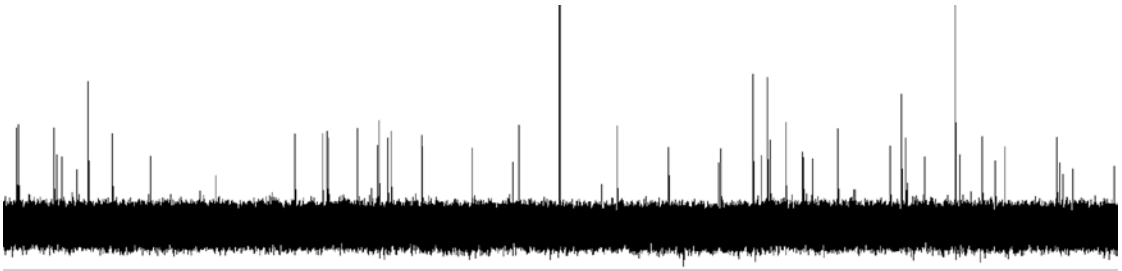
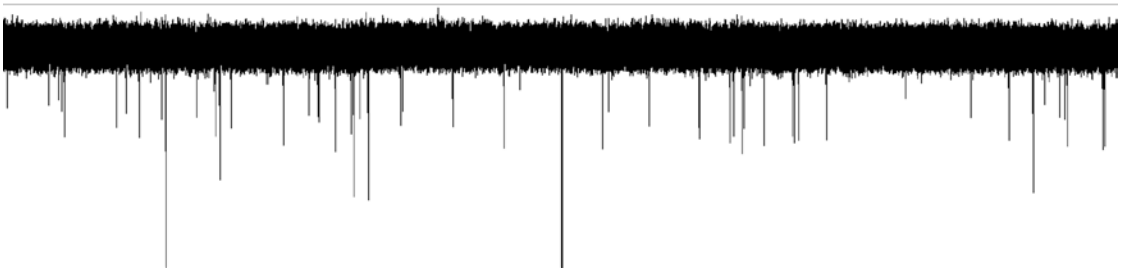
ADVANCED BIOENGINEERING METHODS LABORATORY
NANOPORES
Aleksandra Radenovic



NANOPORES



Abstract

Modern biotechnology increasingly uses single-molecule techniques to detect, quantify and in some cases to determine the detailed structure of biomolecules. Nanopores, which are nanometer sized holes, have emerged as one of the most elementary methods to interface directly with biomolecules. The ion current through the nanopore provides a direct electronic measurement of an individual molecule as it passes through the pore. For optimal sensitivity, the physical properties of the pore such as width, length or material must be adapted to the molecular measurement of interest. To date, the perhaps most sophisticated and spectacular application of nanopores has become sequencing of DNA. Besides single-molecule sensing, nanopores have impact in such diverse areas as drug discovery, power generation or water desalination. In this lab the student will be introduced to how nanopores work. Emphasis will be given to develop an understanding of the intrinsic physical processes and to practical aspects. The student will learn to understand its strengths and challenges. The lab participant will be setting up a solid-state nanopore (silicon nitride pore) and a biological protein nanopore (MspA). Translocation experiments to discern individual DNA molecules will be conducted. The goal of this module is to familiarize yourself with the underlying physics of nanopores (both biological and solid-state nanopores) and have hands-on practical experience of handling and measuring the electronic signals that characterize individual molecules through these “nano-sensors”.

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1. Introduction

Nanopore sensing is derived from the concept of a Coulter counter, an instrument which has become a standard equipment in many medical applications. In Coulter counters an ion current through a small opening is obstructed by an object, such as a red blood cell, or a biomolecule and the reduction of the ion current is used to identify the object or the molecule. Coulter counters were pioneered by Wallace H. Coulter in the 1940's. Smaller holes are used for smaller things and nanopores are used for nanoscopic objects such as biomolecules. With microtechnology advancing to nanotechnology it became feasible to make smaller and smaller devices bringing the possibility closer to use the Coulter principle to detect single molecules. In the 1996's Kasianowicz *et al.* showed that biological pores could be used to detect single molecules such as DNA, and nanopore-based DNA sequencing was proposed (Kasianowicz et al., 1996). In 2012, the first paper was published that showed actual sequencing of DNA using biological pores, which then led to development of 3rd generation DNA sequencing machines(Manrao et al., 2012). At present, intensive research at EPFL and other labs is under way to harness the substantial progress made in solid-state physics and material science to incorporate non-biological nanopores into electronic chips.

This teaching lab concentrates on nanopore technology and will introduce you to the basic concept of ion transport as well single-molecule detection using nanopores (both biological and solid-state types of nanopore).

1.1 **Nanopore concept**

In Nanopore sensing or sequencing a membrane is prepared that separates two sides of a conducting solution (electrolyte), which is often a simple salt solution(e.g. KCl). The membrane has a single nanometer-sized hole that connects the two sides. With an electrode on each side of the membrane a voltage is applied. This causes an ion current to flow through the pore. Generally, if something in the midst of the pore is obstructing the passage of the ions, then the current in the circuit is diminished. This reduction of current can be used to detect small objects such as biomolecules. The obstruction of the ion current depends on the size and shape of the object, but also on other aspects such as its charge. DNA, which has a negative charge at every unit of its backbone can be drawn through the pore. With the right type of nanopore the remaining ion current depends on the type of a nucleotide that is in the narrowest part of the pore; this makes nanopore sequencing of DNA possible.

$$UU = RR \times II \qquad \text{Ohm's law ….(Equation 1)}$$

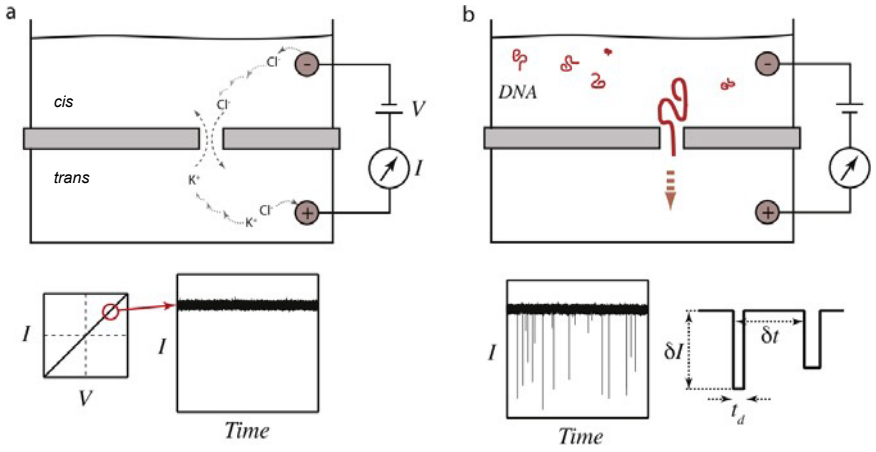

Figure 1. Nanopore experiments are set up with two electrolyte containing compartments titled *cis* and *trans*. The sole connection between them is a nanometer sized opening: the nanopore. (a) When a voltage is applied, an electrochemical half-cell reaction occurs and ions of opposite poles move causing the flow of the electrical current. (b) When an analyte such as negatively charged DNA is added to the chamber, under an electric field the DNA electrophoretically moves through the nanopore temporarily blocking the flow of ions (or in other words flow of current) leading to current blockades (also called as resistive pulses). Such resistive pulses are used for electronic sensing and characterization of biomolecules. The figure is adapted from (Wanunu, 2012).

Question 1: *You are conducting an experiment with a 3 nm sized pore and applying a positive voltage to the trans compartment. What current signal response do you expect after adding the following theoretical analytes to the cis compartment? Draw a hypothetical current-signal response in each case (assuming that all the analytes are spherical).*
A: diameter(4 nm); charge(+3)
B: diameter(1 nm); charge(+3)
C: diameter(2 nm); charge(+1)
D: diameter(2 nm); charge(-3)

1.2 **Ion current**

Different from an electron current in a conductor where electrons carry the current, ion currents consist of the positive and negative ions diffusing through the solution. The positive ions (cations, such as K^+) migrate from the positive electrode to the negative electrode; at the negative electrode they undergo a “reduction” reaction which neutralizes the cation by drawing an electron from the electrode and therefore the power supply. The negative ions (anions, such as Cl-) move in the opposite direction and each anion deposits an electron into the positive electrode in an oxidation reaction (Figure 1). Similar to electrons in a metal wire, the ions in a solution have a very small drift velocity, i. e. it takes quite some time to move from one electrode to the other, but for every ion arriving at an electrode an ion passes through the pore practically at the same instant. Therefore, the current measured in the circuit is equal to the number of ions flowing through the pore (more on the literal correctness of this statement later). The obstruction of the ion current by an object, and therefore the signal, also depends

strongly on the size of the pore, the length of the pore, the salt concentration, the viscosity of the liquid and definitely on the applied voltage.

1.3 **Wet electronics, macro-to-nano world**

In the following we will review some simple electrical properties of liquids and ionic conductors and help you to expand your intuition for the molecular nano world. To first approximation, the ion current conductance through a nanopore can be approximated by modeling it as a cylindrical tube. The conductance, *G*, of a tube is simply proportional to the tube's cross sectional area $A = \pi d^2/4$, inversely proportional to the tube's length L and proportional to the bulk conductivity σ of the salty water:

$$\pi d^2 \sigma /4 L \quad \text{....(Equation 2)}$$

The bulk conductivity σ of the water depends entirely on the ions in the water. (Distilled water that was not subjected to the CO_2 in the air is actually a very bad conductor). A good value for σ to remember is that for 1M KCl-solution is ~10 $\Omega^{-1}m^{-1}$. In salty water, the ions struggle in a Brownian motion through a dense forest of water molecules making collisions with the water all the time. Therefore, the conductance is proportional to the ions *mobility*, i.e. how well the ions can move through all the water molecules. The mobility depends on the ions size, mass and importantly, how many water molecules form around the ions, called the hydration shell. It is quite natural to assume that the bulk conductance is also roughly proportional to the density of ions i.e. the concentration of the salt that is being used, therefore the conductance for KCl is:

$$\sigma = (\mu_K + \mu_{Cl})\, n_{K+Cl}\, e \quad \text{....(Equation 3)}$$

Here n_{K+Cl} is the salt's number density of cations or anions per m^3, and μ_K and μ_{Cl} are the cation and anion mobilities ($\mu_K = 7.6 \times 10^{-8}$ m^2/Vs and $\mu_{Cl} = 7.9 \times 10^{-8}$ m^2/Vs) and e is the electric charge, 1.6×10^{-19} C. People like to use solutions that have similar mobilities of cation and anions because it makes the positive and negative currents about equal. The mobilities of K+ and Cl- are about equal because potassium and chloride have about the similar diameter and mass (and both are monovalent). Water is a much more complex substance than one generally assumes. Because of water molecules's large dipole moment and ability to form hydrogen bonds with each other, water forms clumps of several water molecules. There are more and more (and also bigger) clumps as the temperature of liquid water is lowered until all water molecules form one clump and water freezes. Because of this clumping the viscosity of water is larger at low temperatures and therefore the mobility of the ions is also less good at lower temperatures. The upshot is, that the conductivity of water is not as simple as it may seem initially, but understanding what happens at the molecular scale is helpful in building some intuition. For more ingredients in what goes into the intuitional model you should familiarize yourself also with terms such as "hydration radius", charge screening, Debye length, also, review the lab on Brownian dynamic, etc.

1.4 **Biological nanopores**

Biological cells have many types of pores that permit passage of nutrients, passage of water, chemicals, signaling etc. As can be seen in Figure 2 these pores typically have sizes in a range from 1 nm to 4 nm. Biological pores are protein pores, assembled from chains of amino acids/polypeptides. Many biological pores are assembled from several identical chains of amino acids giving them a high degree of symmetry. Since biological life relies on a high degree of repeatability, protein pores of a given type are automictically identical, despite their chemical complexity. Their nature makes them highly repeatable sensors and also provides the exciding option of precisely tuning their sensing properties. E.g. by mutating their amino acid sequence their interaction with the object of measurement can be optimized. Surprisingly, protein pores can also be very robust, meaning that the pore itself is compatible with a wide temperature range and a wide range of chemical environments.

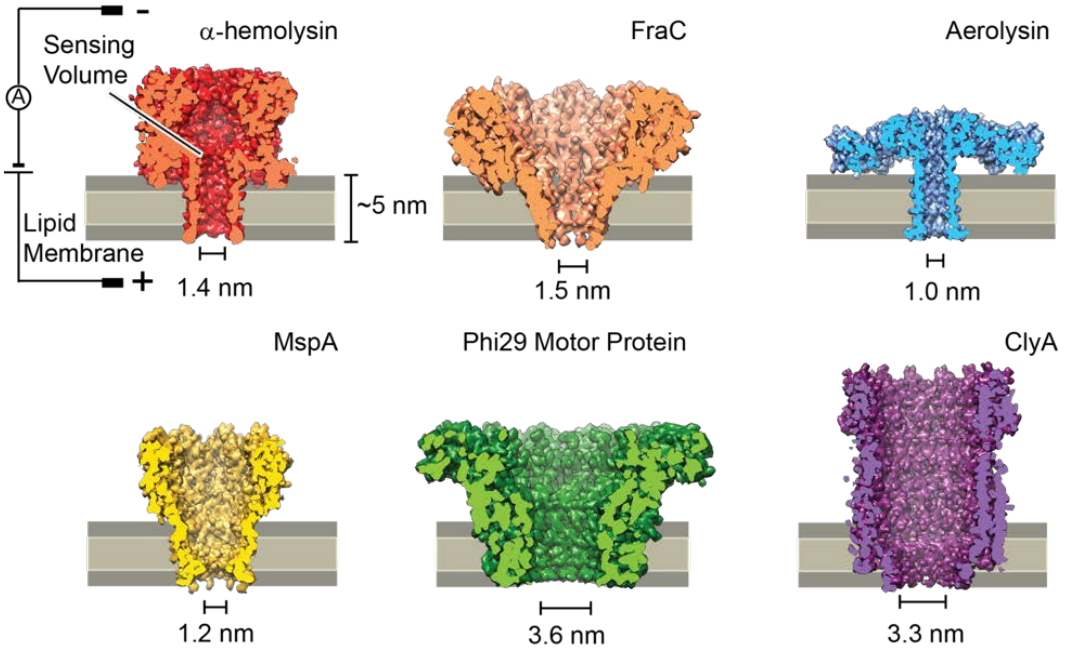

Figure 2. Different examples of biological nanopores with their respective pore size. The figure is adapted from (Houghtaling et al., 2018).

Lipid Bilayers: Most protein pores insert or assemble into lipid bilayers. These bilayers form the link between the nanometer scale of the pores to the several-micrometer scale that can be manipulated under a microscope. While the protein pores themselves are mechanically almost unbreakable the bilayers are very fragile and are the most finicky ingredient in a biological nanopore setup (you will see that during the lab). Not only are the lipid bilayers fragile mechanically, but they also limit the temperatures at which a device can be operated, they limit the chemicals that can be used (A little bit of detergent dissolves lipid bilayers). Despite being only 4-5 nanometers thick, the biological bilayers are surprisingly good insulators. However, the lipid bilayers limit the voltage which can be applied across the nanopore. The conventional lipid bilayers (Dphpc) have a very limited lifetime at voltages higher than 300 mV. Because of these difficulties, lipid bilayers (or other synthetic membranes)

are the most critical obstacle in making long-lasting devices with biopores, thereby strengthening the case for solid-state pores that do not have these limitations.

In the hands-on section you will learn how to establish lipid bilayers, and (probably) this sometimes frustrating process will stimulate your inventiveness.

A picture-book example of a protein pore is α-hemolysin (more on that below) from *Staphylococcus aureus*,. α-hemolysin consists of seven identical amino acids long chains that assemble into the bilayer. We mention α-hemolysin here so we can test our tube model for the pore conduction. The α-hemolysin has a narrow, 1.7 nm average inner diameter tube in its lower 5 nm long stem. Assume 1 M KCl and 100 mV applied voltage in the following expression:

$$\pi V d^2 \sigma /4 L = x \ldots \text{(Equation 4)}$$

While this calculation comes within an order of magnitude, you will find that the calculated ion current is quite a bit larger than the measured current of about 100 pA indicating that the macroscopically derived picture leaves out important details relevant for and the microscopic physics. For example, points of active debate are the effects of charged amino acids and layers of immobile water, or areas within the pore where hydrophobic amino acids repel the water molecules. Nowadays molecular dynamics simulation, which tracks the motions of all atoms and their interactions, is the theoretical bottom-up approach to understand the complex dynamics of the microscopic picture.

1.5 **Solid-state nanopores**

For pores made from solid-state materials, the macroscopic approach promises better understanding. Compared to biopores, solid-state pores are generally made from less heterogeneous materials and are therefore amenable to better understanding based on bulk material properties. Solid-state nanopores leverage onto manufacturing processes of the computer industry and can be directly integrated with electronics on a silicon (Si) chip. However, solid state pores do not yet have the reproducibility developed by 4Gy of biological evolution inherent in biological pores. It can be shown that the conductance (G) of a circular pore in a thin membrane of thickness L becomes:

$$G = \sigma (4L / \pi d^2 + 1/d)^{-1} \ldots \text{(Equation 5) (“Conductance model”, (Kowalczyk et al., 2011)}$$

where the second term is called “access resistance.” The access resistance becomes dominant for pores where L is smaller than d, which is usually the case for pores in 2D materials e.g. molybdenum disulfide (MoS_2).

1.6 **Measuring small currents**

As we learned from calculating the current through a nanopore, the currents are quite small. Such small currents are not easy to measure and require special current meters called amplifiers. Fortunately, such equipment was developed for patch clamping. The important properties of the amplifier are its gain, its noise level and noise characteristics and its

bandwidth. The effective gain (the conversion of current to voltage) is given by the feedback resistor. In every measurement gain, bandwidth and the noise level are tightly connected. This amplifier does not only convert the current to a voltage, but it also applies the voltage that drives the current.

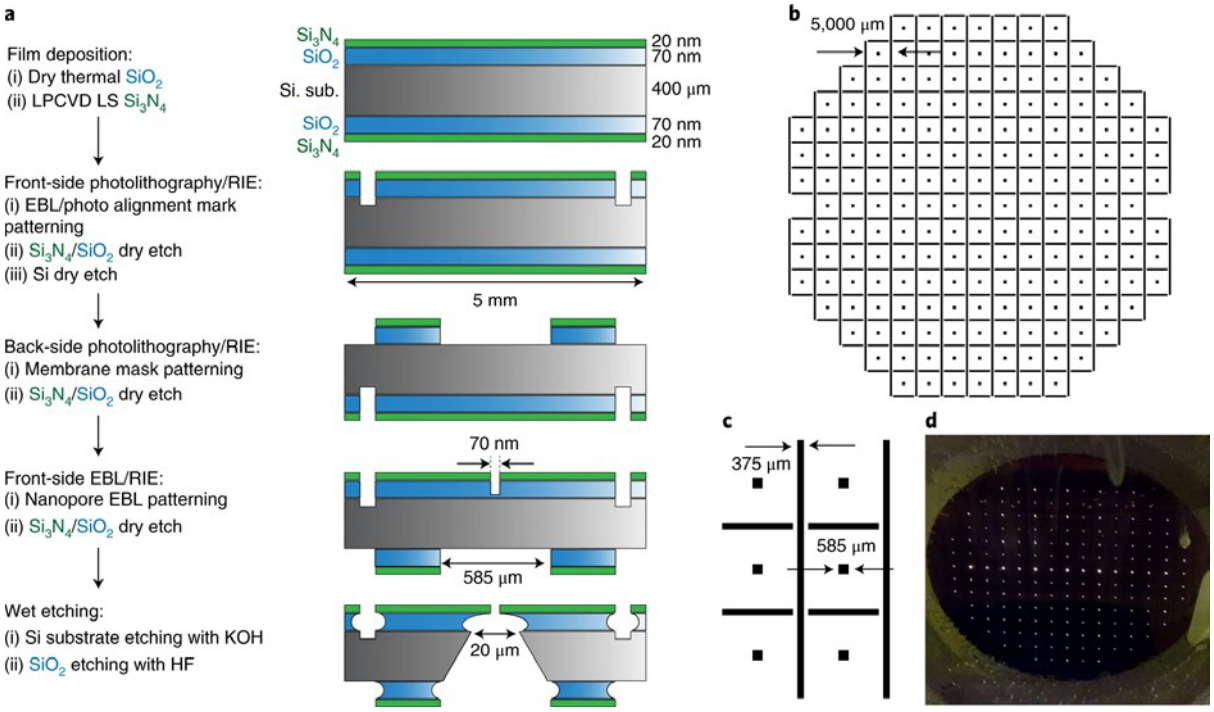

Figure 3. Microfabrication of solid-state devices for nanopores and their architecture. (a) Clean-room process flow. (b) Back-side-lithography design. (c) Dimensions of the membrane openings and the dicing lines. (d) Back-side illumination during KOH etching to verify membrane formation. The figure is adapted from (Graf et al., 2019). The reader is advised to refer to this paper for more information on substrate microfabrication for nanopores.

The amplifier that we will be using in the experiments consists of two components: a headstage which contains all the delicate current amplification and a signal conditioning unit. It is advantageous to put the headstage as close to the nanopore as possible to reduce the effect of ambient electromagnetic fields such as from radio stations or nearby electric machinery, etc. Bringing the headstage close also reduces stray capacitances and thereby permits that faster signal changes can be recorded. To reduce noise the headstage and the nanopore are generally operated in a Faraday cage, which in our setup is a grounded metal box. To understand the proper grounding is very important, but elaborating also exceeds the framework of this experimental tutorial. The other part of the amplifier is a signal conditioning unit which applies appropriate analog filters to the data and amplifies the signals further so that the signals are ideally adapted to the computer interface that records the data.

1.7 **Noise**

In delicate measurements such as these nanopore measurements it is important to maximize the signal-to-noise ratio. The size of the signal is given by the change in current when a molecule moves through the pore. For example, a segment of single-stranded DNA reduces the ion current in a biological pore operated at the best conditions from 150 pA to 50 pA. The individual bases within the DNA change the ion current by 1 to 10 pA, so for DNA sequencing this is the signal amplitude. The noise in these measurements consists of the electromagnetic disturbances and of stochastic noise. While much of the electromagnetic noise can be shielded (Faraday cage) and in many cases subtracted after the recording was done, the stochastic noise cannot be subtracted out. Let's look at the stochastic noise sources:

Shot noise: electric and ion current is quantized because it consists of individual electric charge carriers, e.g. electron charges. Within a microsecond a 50 pA current is on average made up of N=313 electron charges, but that number fluctuates by +-sqrt(N) = ±18 charges or ±3 pA. This number fluctuation is called shot noise. Shot noise is a fairly fundamental noise that also occurs in many measurements, such as measuring the intensity of very dim light. In the power spectral density (PSD), shot noise is "white" meaning that the power density of shot noise is independent of frequency.

Capacitive and dielectric noise: This noise arises because there is a small voltage fluctuation coming out of the amplifier input, (yes, coming out of the input). This changing voltage drives charges in and out of all the stray capacitances of the system according to i=C dV/dt. The stray capacitances, C, are the membrane capacitance or the capacitances or wires, or even capacitances within the amplifier etc. The amplitude of these capacitance current fluctuations grows with frequency, f, and therefore in the PSD this noise grows with f^2. This noise is the main reason why the bandwidth should be aggressively rolled off at high frequency. (The amplifier that you will be using has a 4-pole filter to reduce this noise that rises with frequency). The dielectric noise dominates at 1–10 kHz. These frequency regimes are closely related to the heat dissipation in the system.

1/*f*-noise: This noise has power spectral density that is higher at low frequencies (e.g. 1/f). It occurs in many technical applications and has many names: flicker noise, pink noise, fractional noise, ... This is a noise that every experimentalist hates for several reasons. First, there are many mechanisms that can be the root cause of this noise. In the nanopore system it could be charges near or at the pore that rearrange themselves and modulate the current, it could be chemical interactions at the pore or other thermally activated processes. The truth of the matter is that the cause of the 1/f-noise in nanopores is not well understood and is subject to ongoing research. Secondly, for noise $1/f^n$, $n \leq 1$ one can average over some time and the averaged value becomes a stable value; not so with 1/*f*-noise averaging, the value averaged over longer times scatters just as much as when it is averaged over shorter intervals. (The stock market exhibits 1/*f*-character which makes it impossible to predict simply on statistical grounds.)

2. Practical work

2.1 Conducting a biological nanopore experiment

2.1.1 Material requirements

Handling: Lab coats, safety goggles, gloves, pipetting equipment (various sizes), cleanroom wipes, MECA 4 Recording Chips 50 µm aperture (Ionera)

Products/chemicals. Buffer: 1M KCl buffered with 10 mM HEPES, pH 7.4; 8 mg/ml DPhPC in Octane.

Machines: Orbit mini (Nanion technologies) with temperature control.

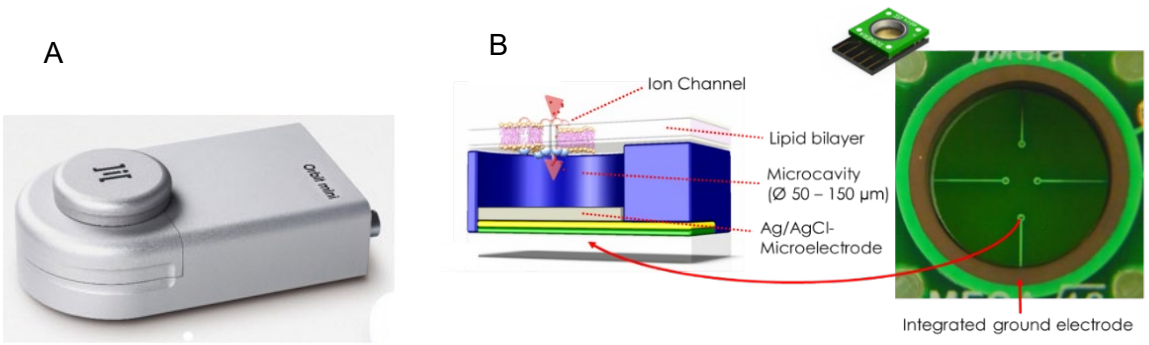

Figure 4: (A) Outside view of the orbit mini device (B) Illustration of an MECA 4 Recording chip (https://www.nanion.de/en/products/orbit-mini.html)

2.1.2 **Prep. Work**:

Lipid handling (done by TA)

1,2-Diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) [Avanti CAT#850356] is suspended in chloroform (10mg/ml) aliquoted in 250 µL vials, dried in vacuum (chloroform is evaporated leaving a lipid film on the bottom of the vial) the aliquots are capped under argon and stored in the freezer at -20 °C.

Question 2: Which pipette should be used to pipette chloroform?

2.1.3 **Single pore experiments**

The insertion of biological nanopores into lipid bilayers happens spontaneously following their addition to the reaction chamber. The main goal in setting up the nanopore experiment is to establish a single pore in the bilayer over an extended time period. The parameters that you will try to controlled to achieve this are: (1) the quality of the lipid membrane and (2) the concentration of the pore in solution. Conveniently, the orbit mini set-up allows to operate 4 channels simultaneously (figure 4 B) which increases the chances of a successful single pore insertion. Each channel has its own aperture over which a membrane can be formed and is connected to its own circuit for voltage application and current readout.

2.1.3.1. **Setting up the experiment**

The TAs will show you in detail how to set up the system before starting the experiment. Here is a check-list of the necessary steps:

a) Plug in the connecting USB cable from the Orbit mini to the computer
b) Connect the temperature control by flipping the on switch and opening the channels for water cooling.
c) Open the EDR software and connect the device
d) Perform compensation of all channels with the calibration test cell
e) Insert the recording chip and add 150 µl of recording buffer in the chamber.
f) Apply voltage protocol 1 and use the blunger (as instructed by TA) until all the channels are wetted

2.1.3.2. **Membrane formation**

In the orbit mini set up four channels can be operated at the same time. Each channel has an aperture over which a membrane can be established. In order to do so the following steps are necessary:

a) Take a P2 pipette set to 0.5 µl and use it to dip a single-use tip in the provided solution of 8 mg/ml DPhPC.
b) Before moving to the reaction chamber make sure all the solvent has dried from the pipette tip
c) Move the pipette into the reaction chamber and form a air bubble over one of the apertures on the MECA chip. In this step you are spreading a thin layer of lipids over the aperture. This leads to spontaneous membrane formation (if done successfully).
d) If a membrane is formed you should see that the current signal is now stably at 0 pA.
e) If no membrane is forming take a fresh pipette tip and start over until you have formed a membrane.

2.1.3.3. **Membrane-test**

When establishing a membrane there can be several layers of lipid or residual solvent inside the membrane making it difficult for the pores to form ion-conducting channels. On the other hand, the membrane can be unstable and rip after short periods of time. In order to prevent both scenarios, we assess the quality of our membranes.

a) Apply a voltage of ~150 mV to see if the current signal stays at 0 pA. If it fluctuates → reform the membrane

b) If a) was successful: test the capacitance using the elements EDR software (will be instructed by TA). Applying a triangular voltage protocol (Figure 8 C). A purely capacitive signal is desirable (Figure 8 A), if you observe a resistive signal on top, your bilayer has leaks (Figure 8 B). Rule of thumb: In 200 pA gain mode a capacitance of >8 pF is good, < 4pF is much too thick.

c) If the capacitance is very low. Try to "zap" the membrane (will be instructed by TA) and reform the membrane

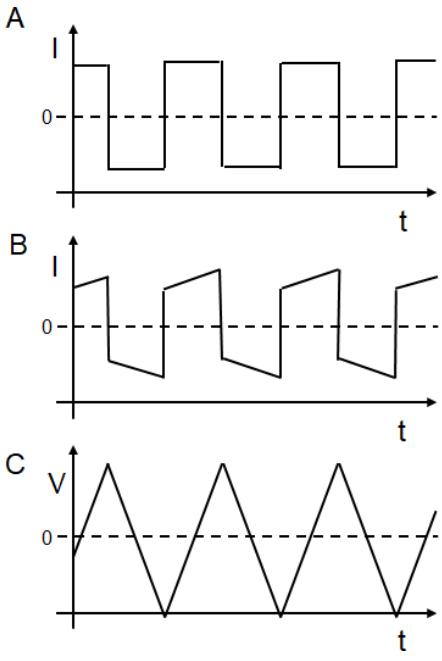

Figure 5. Current response of a stable bilayer (A) and a leaky bilayer (B) when a triangular voltage wave is applied (C).

**The capacitance depends on the area of your bilayer (for a teflon film with a 100 µm aperture the capacitance will be higher), its thickness, the dielectric constant of the membrane and the absolute dielectric constant.*

Question 3: Draw the simplest electrical equivalent circuit of a bilayer.

2.1.3.4. Pore formation

The biological nanopores used in this course are designed by nature to insert themselves into membranes. Therefore, in principle, they accomplish this step for you. However, the concentration of protein pore in the reaction chamber is critical for the experiment. If the concentration is too low it will take a very long time for a pore to insert itself. If it is too high there will be several pores in the same channel very quickly. The following steps should be started after good membranes have been formed and will help you to accomplish single pore insertions:

a) Add 0.5 µl of the nanopore stock you will be provided with
b) Mix the reaction chamber by carefully pipetting up and down with a volume of 50 µl
c) Apply a voltage of 150 mV. In this way you will see the moment of pore insertion as the measured current will suddenly jump to a stable higher value
d) If after 8 minutes there are no pore insertions the steps a) to c) should be repeated
e) If within a few minutes several pores insert themselves the concentration of pore is too high. In this case the reaction chamber can be diluted by replacing part of the volume with fresh recording buffer

2.1.3.5. Checking the pore quality and IV curve

A pore can be distinguished by the sudden jump in current level. The channel should be stable at 180 mV and should not exhibit excessive amounts of gating, meaning the current should be steady and not fluctuate suddenly. The channel should conduct similar with negative voltage, although gating will be more frequent at negative voltages.
Once a clean (no current spikes), stable channel (lifetime greater than 5 minutes) has been formed, record 10 seconds of data at -100 mV to 100 mV in 10 mV steps. If the pore gates too badly at a negative voltage do not bother with data at that voltage. Once this is complete record 2-3 min of baseline data at 100 mV. The EDR software allows you to record the current-voltage dependence (IV-curve) of your pore while alternating the voltage.

At this point you have successfully set up a stable nanopore and are ready for the experiment to start. From now on you are collecting data, the exciting part can start!!

2.1.3.6. Analyte sensing

Now you can add single stranded DNA to the reaction chamber (TAs will provide stock solutions). Reflux with pipette to mix thoroughly. Event rates of .5-10 current obstructions/second (resulting from passage of individual ssDNA molecules through the nanopore) should be observed. Close the Faraday cage!

When making recordings keep in mind that you want to collect enough data to do statistical analysis on data from each individual pore.

<u>Question 4:</u> Having the applied bias for data recording (+180 mV) in mind, why is the DNA added to the cis compartment?

2.1.3.7. **Cleaning:**

There are many issues that can derail nanopore experiments and carrying out the proper cleaning procedures is key to success. The smaller an experimental setup is the larger the surface-to-volume ratio and cleaning is all about the surfaces. For example, a small amount of detergent residue and it will be impossible to form bilayers. Of course, contamination (dirt) relevant in nanotechnological experiments is not visible even with very good optical microscopy. Therefore, for experiments with many consecutive steps, as the ones described here, it is advisable to use only the highest-grade chemicals and materials. The cost (and frustration) of an experiment that does not work is much higher than the cost of the extra high purity chemicals. Always keep in mind that time is your most valuable resource!

The recording chips can be used up to 10 times and need to be replaced afterwards. For cleaning between experiments the chamber should be thoroughly rinsed with water and ethanol in alternating steps. Three rounds of this should be performed starting with water and ending with an ethanol step.

Question 5: *Why do we use different cleaning solvents, and what do you think is the role of each solvent?*

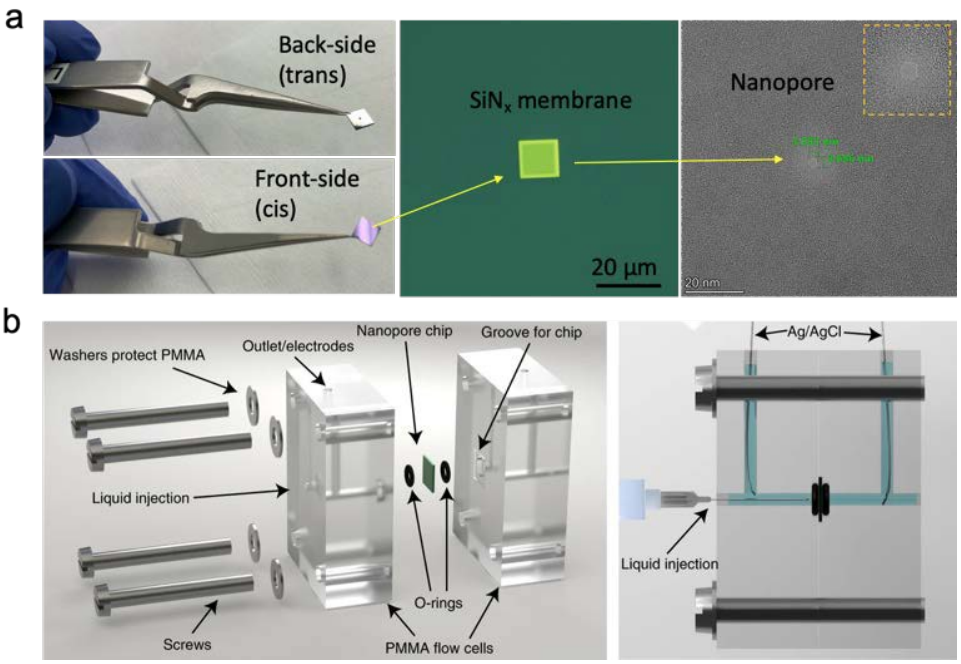

Figure 6. The silicon nitride nanopore chip logistics, design and assembling into a flow cell. (a) Pictures showing front and back-side of the SiN_x chip. The front side faces the cis-compartment while the back side faces the trans-compartment of the flow cell. An optical micrograph of the front side with freestanding silicon nitride membrane of thickness ~20 nm. The nanopore is drilled inside this freestanding membrane. TEM image showing a nanopore of size ~3.4 nm is drilled in the membrane using focused electron beam, highlighted in inset. (b) The components used to assemble flow cell. The final device after mounting the chip is shown on the right panel and the chip is now ready for measurement.

2.2 **Conducting a solid-state nanopore experiment**

2.2.1 Material requirements

Handling: White lab coats, Safety goggles, gloves, pipette tips.

Products/chemicals. Buffer (e.g. 1M KCl buffered with Tris (10 mM) and EDTA (1 mM) at pH 8) and the DNA sample to be analysed (100 bp).

Silicon-nitride chips: The chips with pre-formed nanopore will be made available by the TA during the session.

Nanopore setup and Data acquisition: Faraday cage, Patchclamp amplifier (Axopatch 200B), NI-PXI-4461 (National Instruments, Austin TX, USA) to digitize the analog data from Axopatch 200B, chlorinated Ag/AgCl electrodes (0.2 mm diameter).

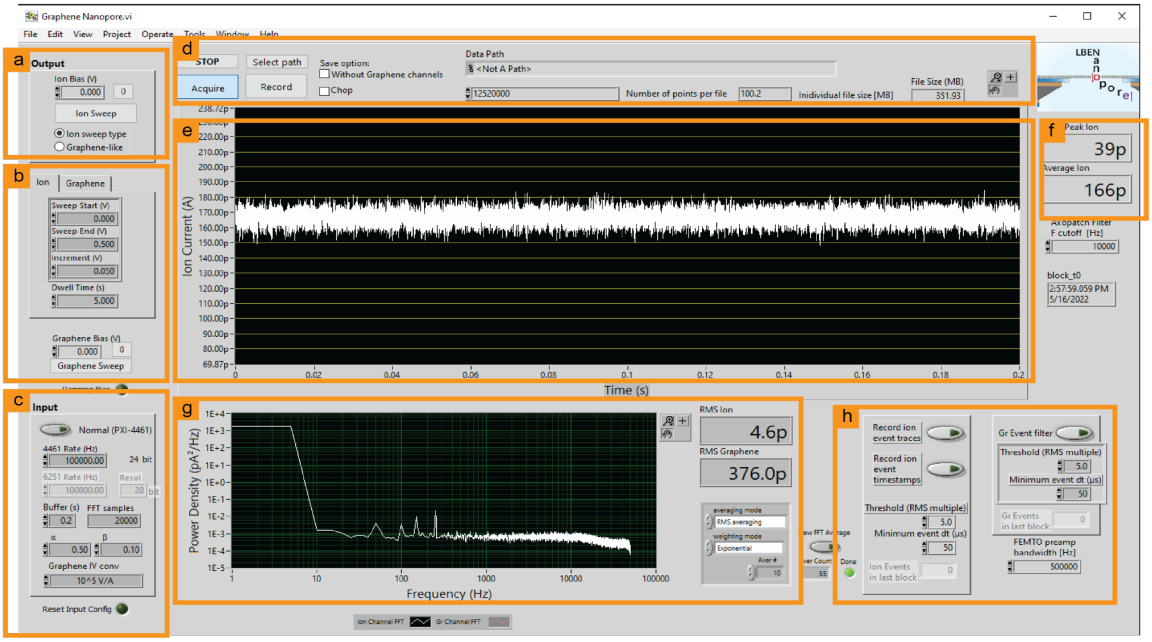

Figure 7. Snapshot of custom-built LabView program for recording nanopore experiments. The labels denote following: (a) manual voltage-bias input, (b) I-V sweep inputs, (c) digitization frequency input, (d) defining recording data inputs, (e) live time trace of current, (f) average current output from e, (g) power spectral density from e, (h) options for the option to turn on/off filtering or timestamps of the events.

2.2.2 Solid-state nanopore measurements

Nanopore drilling in a silicon nitride (SiNx) membrane (20 nm thickness) can be performed precisely using transmission electron microscopes (TEM) using focused electron beam(Graf et al., 2019; Hout et al., 2010). Another method is using dielectric breakdown(Kwok et al., 2014) to make nanopore in situ using high voltage. For the measurements in this course, a nanopore will be drilled beforehand by the TA and provided to you. **The nanopore chip should be handled with care as the SiN_x membrane is very fragile. Always place the front side of the chip facing upwards.**

Now your first goal is to successfully wet the pore (liquid contact from *cis-to-trans* via nanopore) and measure ion-transport through this nanopore. To do this, measure a current-voltage (I-V) response of the chip in given conducting solution (KCl). Using this data, you will estimate the size of the nanopore using the conductance model equation 5. Follow the following steps to perform this operation.

2.2.2.1 Chip mounting and assembling the flow cell

You can also check out an online video on how to mount a chip and assemble a flow cell here: (Video 1 from (Graf et al., 2019)).

1. Place an O-ring in a groove of the flow cell followed by carefully placing the chip on the O-ring. Place another O-ring on the chip followed by gently assembling another half of the flow cell. Make sure you mark “cis” and “trans” side on the flow cell.

2. Prepare a single Ag/AgCl electrodes with both ends chlorinated (The TA will assist you chlorinating the electrodes).
3. Insert the chlorinated end of the electrode through the top electrode outlet in respective the flow cell chambers (Figure 6).

2.2.2.2 I-V measurements

4. The next step is to wet the nanopore. Using a syringe needle, inject the degassed salt-buffer solution (1M KCl in Tris-EDTA buffer) to either side of the flow cell. Make sure there are no air bubbles in the chamber.
5. Plug the flow cell to the headstage. Make sure you connect the ground electrode to the cis-compartment and active electrode to the trans-compartment.
6. Apply a potential of 100 mV on the custom-built LabView program by applying a voltage bias, section 'a' labelled in Figure 7.
7. Using the Nanopore LabView software (Figure 7), in section 'b', apply appropriate range of voltage (e.g. 500 mV), increment of 100 mV and dwell time of 5 seconds. In section 'c', the data can be sampled at 6250 Hz. (Note: The low-pass filter is set to 10 kHz on Axopatch amplifier).
8. Press record and give an appropriate title for your file. Once the voltage scan is finished, press record once again to stop recording and manually revert back 0 mV by pressing zero. You can ask for help from the TA for quickly plotting the IV plots.
9. If the IV curve is not linear, the nanopore might not be wet properly. In such a case, repeat step 4, and measure again.
10. Extract the conductance of the IV and calculate the diameter of your nanopore using the $G = \sigma (4L / \pi d^2 + 1/d)^{-1}$ ….(Equation 5) ("Conductance model", (Kowalczyk et al., 2011).

2.2.2.3 Translocation and sensing of DNA using nanopore

11. Prepare a stock DNA (e.g., 100 bp double stranded DNA, 0.5 µg μL^{-1}) in buffer (1 M KCl) in a ratio of 1:50 in a PCR tube to reach a final concentration of ~10 ng/µL. The aliquot is kept at -20°C. You might need to thaw the DNA.
12. To do this, heat the DNA/buffer mix at 40°C for 10 min on a block heater to thaw the DNA.
13. Use a micropipette (p20) and gently load the mix into the cis-chamber of the flow cell.
14. The data acquisition is made using a custom-made Nanopore LabView program. In section (c), the data can be recorded at 100 kHz. Make sure the filter (h) is turned on and the signal to be detected is at least 5 times the standard deviation and minimum dwell time of 50 µs.
15. Set-up the flow cell as before and apply a voltage (e.g. 100 mV, 200 mV, 300 mV). You can also measure the DNA translocations through your nanopore with different voltages. **Note: With higher voltages, the translocation frequency increases however the nanopore stability might decrease and the pore may grow with time.**
16. You can record and save the files (.dat) with different titles. You will be required to analyse these files in the next session.

2.2.3 Data analysis

For event detection and data analysis, we will use our Python-based OpenNanopore toolkit (https://www.epfl.ch/labs/lben/opennanopore-python). The step-by-step example guide for various functions that you will be using for analysing your data will be provided to you by the TA during the session. The two main objectives of this section would be to plot and interpret the pore size of the solid-state nanopore using the I-V response that you measured during last session and compare with the TEM image of the same nanopore. Secondly, using the Open Nanopore python scripts, analyse the DNA translocation data. Extract median current drop and dwell time from the recorded events. Plot a scatter plot of current drop versus dwell time for the data. Document all the steps and comment appropriately in the Jupyter notebook. If you are not familiar with Python programming, you can always refer to example copy of the step-by-step analysis or ask the concerned TA.

Questions

- Do you see any variation in the size of the nanopore from TEM image and the one estimated using conductance model? Can you comment on the reasons that might cause this variation?
- Can you comment on ways on how to increase the signal-to-noise ratio of the translocation events you recorded?
- What is access resistance and in what case will it dominate your conductance of the nanopore?
- Can you comment on the 1/f noise, sources and some ways to reduce it in solid-state nanopores?
- Compare biological nanopores and solid-state nanopores in terms of sensitivity, stability, robustness and scalability?

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