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Electro-Opto-Fluidics: Nanopore-Gated Devices For Multi-Modal Analysis Of Single Biomolecules

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ELECTRO-OPTO-FLUIDICS: NANOPORE-GATED DEVICES FOR MULTIMODAL ANALYSIS OF SINGLE BIOMOLECULES

A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ELECTRICAL ENGINEERING

by

Shuo Liu

December 2014

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Vice Provost and Dean of Graduate Studies
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2014
# Table of Contents

List of Figures ........................................................................................................ viii

List of Tables ............................................................................................................ xix

Abstract ................................................................................................................ xx

Acknowledgement ................................................................................................... xxii

Chapter 1 Introduction .............................................................................................. 1

1.1 Emerging Optofluidics ..................................................................................... 1

1.1.1 PDMS (Polydimethylsiloxane) based optofluidic sensors ....................... 2

1.1.2 Photonic crystal based optofluidic sensors ............................................. 3

1.1.3 Fiber based optofluidic sensors ............................................................... 3

1.1.4 Optical ring resonator based optofluidic sensors .................................. 4

1.2 Theory of ARROWs ....................................................................................... 4

1.3 Fabrication of ARROWs ............................................................................... 8

1.4 Fluorescence Detection with ARROWs ...................................................... 11

1.5 Nanopore sensors ......................................................................................... 14

1.5.1 Biological nanopore ............................................................................... 16

1.5.2 Solid state nanopore .............................................................................. 16

1.5.3 Combination of optical detection and solid state nanopore ............... 18
Chapter 2 Solid-state Nanopore fabrication methods and their effects on single nanoparticle detection

2.1 Nanopore fabrication methods
   2.1.1 Direct ion beam drilling
   2.1.2 Direct electron beam drilling
   2.1.3 Etching
   2.1.4 Nanopore shrinking

2.2 Nanopore fabrication using different methods
   2.2.1 Nanopore fabrication using direct drilling, electron beam shrinking and ion beam shrinking
   2.2.2 Nanopore fabrication using deposition shrinking

2.3 Electrical nanopore characterization
   2.3.1 Cross section imaging of 50 × 100 nm nanopores
   2.3.2 Cross section imaging of 50 × 50 nm nanopores
   2.3.3 Cross section imaging of deposition shrunk nanopore
   2.3.4 Energy dispersive X-ray spectroscopy analysis of nanopores

2.5 Nanopore-shape-dependent blockade signals in single nanoparticle detection
   2.5.1 Setup and materials
   2.5.2 Results and discussion
2.5.2.1 I-V Curves................................................................................................. 44
2.5.2.2 Blockade Amplitude and Dwell Time...................................................... 48
2.5.2.3 Change of Blockade Amplitude and Dwell Time................................. 53
2.5.2.4 Capture Rate............................................................................................. 56

2.6 Summary........................................................................................................ 60

Chapter 3 Electrical and optical detection of single nanoparticles with nanopore-ARROWs ............................................................................................................. 61

3.1 Advantages, development and challenges of multi-mode detection with nanopore............................................................................................................. 61

3.1.1 Embedded electrodes.................................................................................. 62
3.1.2 Optical detection.......................................................................................... 64

3.2 Integration of nanopore on ARROWs.............................................................. 65

3.2.1 Integration using Cr etch stop layer........................................................... 66
3.2.2 FIB fabrication............................................................................................ 67

3.3 Experimental setup......................................................................................... 69

3.4 Correlated electro-optical detection of single nanoparticles....................... 71

3.4.1 Correlated electro-optical detection of 500, 200, and 100nm nanoparticles .................................................................................................................. 72

3.4.1.1 Experimental setup................................................................................. 72
3.4.1.2 Results and discussion ................................................................. 73
3.4.2 Detection of 100 nm and 200 nm nanoparticle mixture ..................... 79
  3.4.2.1 Experimental setup .................................................................... 79
  3.4.2.2 Results and discussion .............................................................. 80
3.5 Discussion .......................................................................................... 83
3.6 Summary ............................................................................................. 87

Chapter 4 Electrical and optical detection of single bio-nanoparticles with nanopore-ARROWs ........................................................................................................... 88
4.1 Virus detection ...................................................................................... 88
  4.1.1 Correlated electro-optical detection of virus .................................. 88
  4.1.2 Electro-optical discrimination of virus and nanoparticle ............... 90
4.2 Electro-optical detection of λ-DNA .................................................... 93
  4.2.1 Correlated electro-optical detection of λ-DNA ............................... 94
  4.2.2 Determination of optical mode location ....................................... 102
  4.2.3 Effects of nanopore shape on detection of λ-DNA configuration ...... 106
4.3 Electrical detection of polysome ...................................................... 108
  4.3.1 Electrical discrimination of monosome, disome and trisome .......... 111
  4.3.2 Analysis of step-like blockades .................................................... 113
4.4 Summary ........................................................................................... 116
Chapter 5 Summary and outlook .................................................................117

5.1 Summary ..............................................................................................117

5.2 Outlook .................................................................................................118

Appendix ......................................................................................................121

A. Setup for electro-optical measurement ..................................................121

B. COMSOL simulation of nanopore ..........................................................124

C. COMSOL simulation of particle distribution ........................................148

Bibliography ...............................................................................................158
List of Figures

Figure 1-1. Longitudinal section diagram of an ARROW waveguide. The liquid core with thickness $d_c$ and refractive index $n_c$ is surrounded by cladding layers with thickness $t_1/t_2$ and refractive index $n_1/n_2$. Light propagates along the $z$ direction with a propagation constant $\beta$. The cladding layers above and below the liquid core function as Fabry-Perot etalons to confine the light propagating in $y$ direction with a wave vector $k_T$. ................................................................. 5

Figure 1-2. The optical mode profiles in solid-core and liquid core waveguides. (a) Models of solid-core waveguide (up) and liquid-core waveguide (down) built in FIMMWA VE, and the simulated first order modes. (b) Optical mode profiles in solid-core (up) and liquid-core (down) waveguides. ................................................................. 7

Figure 1-3. Layout of a typical ARROW chip. The ARROW chip consists of liquid core (blue) and solid core (pink) waveguides. Reservoirs are fixed around the openings of the liquid core waveguide for liquid loading. ....................................................... 8

Figure 1-4. The fabrication process of ARROW. (a) A SU-8 sacrificial core is patterned on bottom ARROW layers. (b) A layer of photoresist (AZ P4620) is spun coated over the whole wafer. (c) The photoresist over the core is gradually developed before the photoresist on other parts of the wafer. (d) A thin nickel film (75~80nm) is then deposited using e-beam evaporation. (e) After lift-off, the nickel film is left at desired locations to protect the core and solid core waveguides during the following dry etching process. (f) Self-aligned pedestals are etched using anisotropic plasma
etching. (g) Top thick SiO\textsubscript{2} layer is deposited. (h) Ridge waveguides are then formed by dry etching the topmost SiO\textsubscript{2} layer. (i) The ends of the sacrificial core material are exposed by plasma etching through the top layers, and the sacrificial material is removed by acid etching.

Figure 1-5. The diagram of fluorescence detection on ARROWS.[43] .......... 12
Figure 1-6. Commonly used (a) biological nanopore and (b) solid state nanopore.[62], [63].......................................................................................................................... 15
Figure 1-7. A typical setup for the combined nanopore sensing and optical detection.[88] ........................................................................................................ 19
Figure 2-1. Definition of the diameter of a nanopore........................................ 21
Figure 2-2. Diagram of the setup for ion beam milling.[79] ......................... 23
Figure 2-3. Change of nanopore diameter versus electron beam scanning time. 3 kV, 5 kV and 10 kV accelerating voltages were used. ........................................ 28
Figure 2-4. Top view of nanopores fabricated with different methods. (a) Directly drilled 50 nm nanopore. (b) 50 nm nanopore (right) shrunk from 114 nm nanopore (left). (c) 50 nm nanopore (right) shrunk from 70 nm nanopore. ................... 30
Figure 2-5. Scanning electron micrographs of 50nm nanopores made on 100nm membranes. The left column shows top-down images of (a) directly drilled (DD), (c) electron beam shrunk (EBS), and (e) ion beam shrunk (IBS) nanopores. Images (a) and (g) were taken with 20kV electron beam, and image (d) was taken using 5kV electron beam. The right column shows side views of the same nanopores after deposition of a protective top layer of platinum and cross-sectioning. .................. 31
Figure 2-6. Scanning electron micrographs of 50nm nanopores made on 50nm membranes. The left column shows the cross section images of (a) directly drilled, (b) electron beam shrunken and (c) ion beam shrunk nanopore. After a thin layer of platinum is deposited as the protective layer, the nanopore is cleaved in the middle and imaged. In (d-f), the shape of nanopore is outlined. ..................................................... 32

Figure 2-7. Scanning electron micrographs of 150nm nanopores made on an ARROW chip.......................................................... 34

Figure 2-8. Pattern fabricated for the Energy Dispersive X-ray Spectroscopy. The small nanopore in the middle of four bigger pores is the nanopore to test, and the big square and four pores are marks. .......................................................... 37

Figure 2-9. The Energy Dispersive X-ray Spectra over a 500 nm square centered at (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores. The elements for each peak are marked. The green area is the control spectrum, which was taken over a 500 nm square far away from the nanopore.............................. 37

Figure 2-10. Element maps of a larger region around the (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores. The rectangles in (a) and (b) show the regions where element maps were plotted, and the element map was plotted for the whole image of (c). The distribution of C, O and Ga is shown in a1-a3, b1-b3 and c1-c3, respectively. The bright spot in (b) is an iron deposition, which could be induced by the contaminations in the SEM chamber or on the sample holder. ..... 38

Figure 2-11. The Energy Dispersive X-ray Spectra of points (a) 1, (b) 2 and (c) 3 on the chip with an electron beam shrunk nanopore. Inset: Spectra are taken at point 1:
nanopore, point 2: Si3N4 membrane and point 3: supporting substrate.

Figure 2-12. (a) Schematic diagram of the 2D axial symmetric model for finite element calculation. Dotted line is the symmetric axis. The area of the red rectangle is partially enlarged in (b).

Figure 2-13. Schematic diagram of the experimental setup. The thin membrane is fabricated on a Si substrate. cis chamber is above and trans chamber is below the membrane. After the nanoparticles are added into the cis chamber, a command voltage is applied across the nanopore. Since the nanoparticles are negatively charged, they will be moved through the nanopore by the electrical force.

Figure 2-14. Ceramic nanopore holder.

Figure 2-15. (a) Experimental I-V curves (dots) of directly drilled, electron beam shrunk and ion beam shrunk nanopores. The colorized lines are the simulation results for three kinds of nanopores. The black line stands for the I-V curve of a nanopore with an ideal cylinder cross section. (b) The shapes of nanopores built in our model. From top to bottom: directly drilled nanopore, electron beam shrunk nanopore and ion beam shrunk nanopore.

Figure 2-16. I-V curves of (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores in salt solutions of different concentrations.

Figure 2-17. Nanopore conductance versus KCl concentration. Our operating concentration is 0.2M.

Figure 2-18. Signal traces of (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores. (d-f) are enlarged view of typical signals. The red dotted
lines mark where the blockade starts and stops. .......................... 48

Figure 2-19. (a) Scatter plot of events detected by three kinds of nanopores when the voltage is 100 mV. (b) The histogram of relative conductance change, $\Delta G/G$, of three nanopores at 100 mV. (c) The histogram of dwell time of three nanopores at 100mV. (d) Simulated electric field strength along z axis of three kinds of nanopores. ................................................................................................................. 49

Figure 2-20. (a) The conductance change is calculated by moving a particle from top to bottom along z axis. Here we take the electron beam shrunken nanopore as an example. The calculated current versus location is plotted in (b). (c) The comparison between relative conductance changes predicted by numerical and analytical solutions, and experimental results. ................................................................................................................. 51

Figure 2-21. The change of blockade amplitude versus the voltage. Dots are experimental results and lines are simulation results......................................................... 54

Figure 2-22. Simulation of time dependent blockade for (a) directly drilled, (b) electron beam shrunken and (c) ion beam shrunken nanopores. (d) Normalized experimental and simulated dwell time versus voltage. Dots are experimental results and lines are simulation results. The actual values of experimental results are marked on the right-hand y-axis. .......................................................... 56

Figure 2-23. (a) Capture rate changes as the voltage changes. The data is from the directly drilled nanopore. (b) Capture rate versus voltage. (c-e) Histograms of time-delay between successive events from directly drilled, electron beam shrunk and ion beam shrunk nanopores. Blue lines are the fitted curve. ......................................................... 58
Figure 3-1. Electrode embedded nanopores. (a) A nanopore in a capacitor membrane. Insets: Left: A TEM micrograph of the capacitor-membrane. Right: A lattice image of a nanopore sputtered into the capacitor membrane.[153] (b) A single DNA molecule is translocating through a nanopore fabricated in a SiN₃ membrane. Schematic drawing shows location of the nanogap electrodes made in Pt/Ti layers. 5nm and 150nm thick Al₂O₃ layers were deposited on the nanogap and the microelectrodes.[156]

Figure 3-2. The fabrication flow diagram of the process of creating a micropore in a hollow ARROW waveguide.[161]

Figure 3-3. Nanopore fabrication on the thick top SiO₂ layer. (a) Milling of 2 × 2μm square micropore. A hole at the corner of the micropore indicates the micropore milling should be stopped. (b) The hole is then closed by FIB-assisted SiO₂ deposition. (c) A nanopore is milled using a focused ion beam. No further post-processing such as size sculpting is applied.

Figure 3-4. Principle of electro-optical single nanoparticle detection and two different configurations. (a) Each nanoparticle produces two characteristic signals – a transient current decrease and a fluorescence spike - separated by a characteristic time Δt. (b) Different nanopore locations on ARROWs, and the expected signals.

Figure 3-5. Implementation of delay-free, correlated electro-optical detection. (a) Layout of the nanopore-optofluidic chip. The nanopore is located right on top of the optical excitation area where liquid-core (horizontal) and solid-core (vertical) waveguides intersect. (b) Schematic view of the cross section of the detection area,
showing a microscale opening in the waveguide cladding and a nanopore drilled through the membrane. (c) Top-down view of the detection area. (d) Tilted view of the detection area.

Figure 3-6. Correlated electro-optical detection of 500nm nanoparticles. (a) Experimental and artificial electrical (black) and optical (blue) signals. Inset: Zoomed-in view of an artificial signal. (b) Cross correlation function between artificial electrical and optical signals.

Figure 3-7. Correlated electro-optical detection of (a) 200nm and (b) 100nm nanoparticles.

Figure 3-8. (a) Scatter plot of relative conductance versus blockade duration of electrical blockades of 500nm, 200nm, and 100nm beads. (b) Histograms of fluorescence signal brightness of 500nm, 200nm, and 100nm beads.

Figure 3-9. Nanopore gate optofluidic device. Schematic view of intersecting solid-core (orange) and liquid-core (blue) optical waveguides on a silicon chip with particles and electrodes in reservoirs (inset: photograph of chip).

Figure 3-10. Gated electro-optical detection of single nanoparticles. (a) Fluorescent nanoparticles of two different diameters (100/200nm) are translocated through a 250nm nanopore. (b) Electrical blockade (top) and optical fluorescence (bottom) signals showing correlated single-particle detection events (4 examples highlighted with dashed lines). (c) Cross-correlation of electrical and optical signals showing single peak that enables accurate determination of flow velocity in the waveguide channel. The particle translocation rate was 35.6/min, but rates in excess of 1,000/sec
are possible.[163]........................................................................................................ 81

Figure 3-11. Identification of nanoparticle subpopulations. (a) Fluorescence intensity histogram. (b) Scatter plot of electrical blockades revealing the two subpopulations by current blockade depth. (c) Multiparameter analysis enabling assignment of optical properties to particle subpopulations; dashed lines: optical signal range with ambiguous particle size assignment........................................................................... 82

Figure 3-12. Optical excitation waveguide characterization. (a) Facet of the solid-core waveguide used to couple light into the optofluidic chip; (b) Optical mode (at 632.8 nm) observed at the output end of the solid-core excitation waveguide...................... 84

Figure 3-13. Electric potential (a) and electric field (b) distributions around nanopore. ........................................................................................................................................... 86

Figure 4-1. Electro-optical detection of single H1N1 influenza A viruses. (a) Schematic view of 120nm virus particles and 157nm nanopore. (b) Electrical blockade (top) and optical fluorescence (bottom) signals showing correlated single-virus detection events. (c) Scatter plot of electrical signals showing narrowly distributed blockade depths and dwell times. (d) Histogram of fluorescence signal. (e) Cross-correlation of optical and electrical virus detection signals................................. 89

Figure 4-2. Identification of influenza viruses within heterogeneous particle mixture. (a) Schematic view of the virus/nanoparticle mixture and nanopore; (b) electrical blockade (top) and spectrally resolved optical fluorescence (bottom) signals from viruses (red fluorescence) and nanoparticles (blue); (c) scatter plot of electrical signal suggesting nearly identical blockade depths but particle-dependent dwell times. ..... 91
Figure 4-3. Cross-correlation of optical and electrical virus detection signals for various combinations of dwell-time/spectral subpopulations, enabling unambiguous identification and assignment of viruses to long (> 4ms) dwell times. 

Figure 4-4. (a) Layout of the experimental setup. An Argon ion laser was used as the excitation light source (dark blue arrow). The syringe pump is connected with the optofluidic chip using a PDMS adapter.[23] (b) Cross section diagram of ARROW and the detection process. (c) The diameter of the nanopore is shrunk from 80 nm (left) to 20 nm (right). The length of the scale bar is 20 nm.

Figure 4-5. (a) Typical electrical signal traces at 5V, 6V, and 7V. (b) Scatter plot of blockades depth versus duration. (c) Blockades depth versus voltage, and the linear fitting line. (d) Capture rate versus voltage, and the linear fitting line.

Figure 4-6. (a) Scatter plot of blockade depth versus duration. Data were from a 20nm nanopore fabricated on another device. Voltages across the nanopore were 3.6V, 6.3V, and 9V. (b) Comparison between two devices. Presented are the plots of blockade depth versus voltage, and the linear fit lines. (c) Comparison between two devices. Presented are the plots of capture rate versus voltage, and the linear fit lines.

Figure 4-7. (a) Typical electrical blockades (black) and optical signals (red). (b) The cross correlation function between electrical and optical signals. (c) The autocorrelation function of the optical signal (black dots), and the fitting curve (red line).

Figure 4-8. (a) Blue lines present the trajectories of 100 particles in the liquid core channel. The dark red cylinder is a diagram of laser beam. (b) Top: A cross section
view of the particle distribution. Bottom: The optical mode in the solid-core waveguide. The dotted lines show the location of the center of the optical mode. The length of the scale bar is 2 μm. ................................................................. 102

Figure 4-9. Comparison between experimental and simulated intensity distributions. (a) Experimental intensity distribution and the Poisson fit curve. (b-j) Simulated intensity distributions with optical modes at different vertical locations (3.1 – 3.9 μm above the bottom of the liquid core channel), and the Poisson fit curves. .............. 106

Figure 4-10. (a) The cross section image of a nanopore shrunk by SiO2 deposition. (b) A simulated blockade. Inset: the structures of nanopore and particle. (c) An experimentally observed blockade........................................................................... 106

Figure 4-11. An electron micrograph of a polysome.[179]................................. 109

Figure 4-12. The result of the polysome gel analysis (courtesy: Dr. Harry F. Noller). 1PS: Fairly pure monosomes; 2PS: Almost equal amounts of mono- and disomes, with smaller amounts of tri- and tetrasomes; 3PS: Almost equal amounts of disomes and trisomes, and nearly as much monosomes, and some tetrasones; 4PS: Nearly equal amounts of tri- and tetrasomes, with lesser amounts of mono- and disomes and some pentasomes. 5PS: Nearly equal amounts of mono-, di-, tri-, with less tetra and even less pentasomes. .................................................................................. 110

Figure 4-13. (a) Scatter plot of blockade amplitude versus duration of electrical signals got from monosome, disome and trisome measurements. (b)-(d) Blockade amplitude histograms of monosome, disome, and trisome.....................................................111

Figure 4-14. Step-like blockades found in the trisome measurement. They are
possibly caused by (a) trisome, pentasome, (b) tetrasome, (c) hexasome, and (d) heptasome.
List of Tables

Table 2-1. Comparison between different fabrication methods. ............................... 26

Table 3-1. A summary of recently published papers about electro-optical nanoparticle
detection with solid state nanopores.[157], [88], [158]–[160]................................. 65
Abstract

ELECTRO-OPTO-FLOWIDICS: NANOPORE-GATED DEVICES FOR MULTI-MODAL ANALYSIS OF SINGLE BIOMOLECULES

by

Shuo Liu

Biological and solid-state nanopore sensors have proven their capability of high-throughput electrical single molecule sensing and potential in DNA/RNA sequencing. On the other hand, optofluidics, the combination of integrated optics and microfluidics, has drawn extensive attention over the past decade. The combination of nanopore technology and optofluidics is bound to bring impressive features and superiority. In this work, we innovatively integrated the solid-state nanopore into an optofluidic device, anti-resonant reflecting optical waveguides (ARROWs), to form an electro-opto-fluidic sensing platform, and demonstrate for the first time correlated electro-optical detection of single biological nanoparticles.

In order to successfully fabricate the solid-state nanopore in the semiconductor based ARROWs, we developed different fabrication methods, which are either based on standard semiconductor processes, or require the help of a focused ion beam milling. Solid-state nanopores fabricated with these methods were thoroughly characterized, analyzed, and compared using cross section analysis, energy dispersive X-ray analysis, and finite element simulation. We demonstrated that the fabrication
dependent geometric shape of the pore determines the electrical blockade signal. Afterwards, our nanopore-optofluidic device was used for the correlated electro-optical detection of single synthesized nanoparticles, single viruses, and single DNA molecules. The nanopore functions as a smart gate for sequential introduction of single nanoparticles for optical analysis. We proved that the electrical signals and the optical signals of individual nanoparticles can be clearly detected and cross correlated with a fidelity of up to 100%. On top of that, the electrical signal and the optical signal were used together to distinguish and identify different particles which could not be differentiated using either one of the signals. Moreover, information about the flow velocity, the particle-nanopore interaction, and the particle’s fine structure could be extracted by analyzing electrical and optical signals. Furthermore, the combined analysis of optical signal intensity distribution and the simulated particle locations in the flow assisted us to find out the optical mode location. Both the results and the theoretical analysis show that our novel electro-opto-fluidic platform is promising for further scientific research and clinical applications.
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Chapter 1 Introduction

In this chapter, firstly we are going to make a brief introduction about optofluidics, especially optofluidic sensors. Then, the principle of anti-resonant reflecting optical waveguides (ARROWs), ARROW fabrication methods, applications of optofluidic sensors based on ARROWs, and nanopore sensors will be introduced with details.

1.1 Emerging Optofluidics

As a sensitive and reliable method, optical detection is being used and under development all around the world. Numerous applications of optical detection, like microscopy, spectroscopy, and optical imaging, are being reported for their contributions to the advancement of science and technology.[1]–[3] Compared to optics, microfluidics is a rather young field in science; however, it has impressed us with its broad applications in fluidic control and biological research, such as microfluidic mixer, sorter, and sensor etc.[4]–[8] As known as a sophisticated combination of optics and microfluidic systems, optofluidics attracted a lot of attentions in the past decade. By manipulating the speed, direction, and refractive index of fluids, one can easily control the flow movements and the light behaviors in the liquid channel. On the other hand, the integration of optical components hugely increases the functionality of the device. The capability of controlling fluid and light at the same time within a tiny dimension finds optofluidic devices a wide range of
applications, such as on chip dye lasers, sensors, optofluidic microscopes or even photobioreactors.[9]–[14]

Among all the applications of optofluidics, the optofluidic sensor is a research hot spot nowadays, in terms of its flexible design, small device dimension, low sample consumption, and high sensitivity. The growth of microfluidics, nanophotonics and fabrication technology enables people to continuously reduce the detection limit of the optofluidic sensor.[15] From synthetic particles to biological samples, from submicron- to nano-particles, from highly concentrated solutions to single particles, the remarkable evolution of objects of study reveals the powerful detection capability and great potential of the optofluidic sensor. The rapid development of optofluidic sensor also makes people see the promise of it as a replacement of conventional bulky equipment, especially the complicated biomedical examination equipment, considering the rising interest in point-of-care devices.

Different types of optofluidic sensors have been well developed and are widely used, and we are going to briefly introduce several typical platforms below.

1.1.1 PDMS (Polydimethylsiloxane) based optofluidic sensors

Compared to silicon based devices, PDMS, a transparent silicone elastomer can be easily cast without the need of expensive equipment. Thanks to its easy fabrication process, good optical transparency, low autofluorescence background, and deformability, a lot of PDMS sensors with different designs and functions teem in news at present.[16]–[19] The elasticity of PDMS enables the integration of a lot of
valves and pumps to finish the tasks of complicated fluids control.[20] Because of PDMS’s optical transparency, optical signals can be collected by directly imaging the detection region.[21] Optical fibers can also be inserted into the device to excite or collect optical signals, which makes the setup even more compact.[22] In addition, PDMS components are able to be integrated into other optofluidic platforms, in order to build more complex systems, as well.[23], [24]

1.1.2 Photonic crystal based optofluidic sensors

By carefully arranging the periodic nanostructures with different refractive indices in one or two directions, the motion of photons can be affected by the photonic band gap defined by the structure of photonic crystal. Photonic crystal structures have potential good optofluidic platforms, since the air holes can be filled with fluids, leading to the significant changes of the optical properties (usually the shift of transmission spectrum).[25]–[28] Besides planar photonic crystal structures, photonic crystal fibers can even realize optical sensing and fluids confinement at the same time. [29]–[31]

1.1.3 Fiber based optofluidic sensors

All-fiber optofluidic sensors are always based on photonic crystal fibers, because they can handle fluids and light simultaneously. Though traditional optical fibers cannot confine fluids without hollow cores, they can be easily buried or inserted into microfluidic devices as high quality optical waveguides.[32], [33] This makes the design of optofluidic platforms more flexible by saving the time adapting the waveguide structures.
1.1.4 Optical ring resonator based optofluidic sensors

This circular waveguide supports an optical resonant mode, which has an evanescent field reaches into the surrounding medium. Interactions between the analytes near the surface and the evanescent field will lead to a spectral shift in the resonant mode. Because the resonant mode can travel along the ring resonator for a lot of times, sensors based on optical ring resonator are sensitive to small changes of analytes. Planar ring resonators can interact with fluids in a liquid channel on top of the ring resonator.[34] Advanced liquid core ring resonators can finish the light-liquid interaction by confining fluids in the liquid channel.[35]

The optofluidic sensor discussed in present work is based on anti-resonant reflecting optical waveguides (ARROWs). ARROW based sensor is one of the most developed and powerful optofluidic platforms for single particle detection nowadays. Besides sensing, ARROWs’ applications include optofluidic filter, particle manipulation, on-chip slow light etc.[36]–[38] The principle, development and fabrication method of ARROWs will be talked about in the following sections.

1.2 Theory of ARROWs

The essence of optofluidics is the interaction between light and liquid within one device. Therefore it is reasonable and important to develop some kind of hollow optical waveguide which also allows liquid to flow inside. However, liquid, usually water or salt solutions here, has a lower refractive index than those of materials used in semiconductor fabrication. Thus the light definitely won’t be confined in the liquid
core of a waveguide designed with traditional concept (total internal reflection) and materials. People have found some ways around, such as Teflon AF waveguides, liquid core liquid cladding waveguides, nanoporous cladding waveguides, slot waveguides, and photonic crystal fibers. However, these solutions still have some limitations in the aspect of fabrication or integration.[39]

Different from liquid core waveguides mentioned above, ARROWs rely on anti-resonant reflection for wave guidance. Multiple SiO$_2$ and SiN/TaO$_2$ cladding layers form a Fabry-Perot in the transverse direction to confine the leaky mode in the hollow core.

![Figure 1-1. Longitudinal section diagram of an ARROW waveguide. The liquid core with thickness $d_c$ and refractive index $n_c$ is surrounded by cladding layers with thickness $t_1/t_2$ and refractive index $n_1/n_2$. Light propagates along the $z$ direction with a](image)

propagation constant $\beta$. The cladding layers above and below the liquid core function as Fabry-Perot etalons to confine the light propagating in $y$ direction with a wave vector $k_T$.

The longitudinal section diagram of a typical ARROW is shown in Fig. 1-1. The cladding layers function as Fabry-Perot etalons for propagation with the transverse wave vector $k_T$ in the $y$ direction. When the thickness of each layer is designed to meet the anti-resonant condition for $k_T$, there will be no transmission through the etalons (cladding layers). Thus, the high reflectivity in the transverse direction will confine the light to the core. The anti-resonant condition for the round trip phase shift in the $i^{th}$ cladding layer is described as:

$$\Phi = 2t_i k_T + \Phi_r = (2N - 1)\pi; N = 1, 2, \ldots \quad (1.1)$$

where $\Phi_r$ is the total phase shift from reflection at the two interfaces with the adjacent layers. The thickness $t_i$ of each high-index cladding layer should satisfy the antiresonant condition for a design wavelength $\lambda$ by the equation:[40], [41]

$$t_i = \frac{\lambda}{4n_i} (2N - 1) \left( 1 - \frac{n_i^2}{n_l^2} + \frac{\lambda^2}{4n_i^2 d_c^2} \right)^{-\frac{1}{2}} ; N = 1, 2, \ldots \quad (1.2)$$

where $n_i$ is the refractive index of the $i^{th}$ cladding layer. When $(\lambda/2d_c n_i)^2 \ll 1$, the above equation can be simplified to:

$$t_i \approx \frac{\lambda}{4} (2N - 1) (n_i^2 - n_l^2)^{-\frac{1}{2}} ; N = 1, 2, \ldots \quad (1.3)$$

Carefully optimized liquid core ARROW can have a loss as low as 0.26 cm$^{-1}$ at 633 nm.[42]
Figure 1-2. The optical mode profiles in solid-core and liquid core waveguides. (a) Models of solid-core waveguide (up) and liquid-core waveguide (down) built in FIMMWA VE, and the simulated first order modes. (b) Optical mode profiles in solid-core (up) and liquid-core (down) waveguides.

The models built in FIMMWA (Fig. 1-2) display the cross section structures of the solid-core and liquid-core waveguides. The solid-core waveguide is formed by etching in a ~ 5.2 µm thick SiO₂ layer. The ridge is ~ 1.8 µm wide on the top, and ~
4.2 µm wide on the bottom. The liquid-core waveguide is 5 µm high and 12 µm wide. The simulated first-order modes in both waveguides are also presented. As we can see, the optical modes locate approximately in the center of the waveguides in the vertical direction. By measuring the FWHM of the optical modes in both horizontal and vertical directions, the excitation volume can be calculated to be ~ 93 fL.

1.3 Fabrication of ARROWs

The layout of a typical ARROW chip is shown in Fig. 1-3. This platform consists of an inverse s-shaped liquid core waveguide (blue) and several solid core waveguides (pink). The function of solid core waveguides is to couple the light into and out of the liquid core waveguide.

Figure 1-3. Layout of a typical ARROW chip. The ARROW chip consists of liquid core (blue) and solid core (pink) waveguides. Reservoirs are fixed around the openings of the liquid core waveguide for liquid loading.
Standard semiconductor processing techniques were chosen to fabricate ARROWs, which is an advantage for mass production. ARROWs were fabricated on <100> crystal orientation Si wafers, which are convenient for cleaving. Before the cladding layers deposition, a thick SiO$_2$ layer was grown thermally on the Si wafer, in order to electrically isolate the ARROWs from the substrate. After that, alternate SiO$_2$ and Ta$_2$O$_5$ layers were then deposited onto the wafer using the methods of plasma-enhanced chemical vapor deposition (PECVD) and sputtering, respectively.[43] Following that, a SU8 sacrificial core was spun on, photolithography patterned and developed. Self-aligned pedestals were then etched using anisotropic plasma etching, for the purpose of reducing the loss of ARROWs.[44] Next, a thick SiO$_2$ layer was deposited as a mechanical support for the liquid core waveguide and a etch layer for the solid core waveguides.[45] Solid core waveguides and openings at two ends of the liquid core waveguide were then created by plasma etching. Finally, the sacrificial core was removed in NanoStrip or Piranha solution.
Figure 1-4. The fabrication process of ARROW. (a) A SU-8 sacrificial core is patterned on bottom ARROW layers. (b) A layer of photoresist (AZ P4620) is spun coated over the whole wafer. (c) The photoresist over the core is gradually developed before the photoresist on other parts of the wafer. (d) A thin nickel film (75~80nm) is then deposited using e-beam evaporation. (e) After lift-off, the nickel film is left at desired locations to protect the core and solid core waveguides during the following
dry etching process. (f) Self-aligned pedestals are etched using anisotropic plasma etching. (g) Top thick SiO$_2$ layer is deposited. (h) Ridge waveguides are then formed by dry etching the topmost SiO$_2$ layer. (i) The ends of the sacrificial core material are exposed by plasma etching through the top layers, and the sacrificial material is removed by acid etching.

1.4 Fluorescence Detection with ARROWs

Before the two dimensional waveguide array was designed, efficient fluorescence detection with ARROWs was first demonstrated by detecting Alexa 647 molecules dissolved in ethylene glycol solution. As low as $10^{-11}$ M dye solution could be detected within a sample volume of 57 pL.[46] However, this proof-of-principle experiment was done with an open-ended liquid core ARROW. Later, after the intersection geometry was successfully designed and fabricated, Alexa 647 molecules with a concentration as low as 500pM, corresponding to only 40 molecules in the excitation mode volume was detected. Parallel 2D waveguide arrays was also demonstrated by exciting at multiple locations.[47], [48]
Single molecule detection with ARROWs started with on-chip fluorescence correlation spectroscopy (FCS) measurements of Alexa 647 dye molecules. Dye solutions with concentrations as low as 10pM were measured. Fitted concentration revealed that less than one (0.35) molecule on average was detected within the excitation volume. In the meantime, a modified autocorrelation function was proposed, which was later used for lots of our on-chip single molecule detections.[49] Within the same year, a fully planar ARROW chip with both ends of the liquid core channel surrounded by reservoirs was used for detection and manipulation of single liposomes.[50] The experiment not only found a mean concentration of 0.64 liposomes in the excitation volume, but also demonstrated our optofluidic chip has the ability to electrically manipulate bioparticles. By that point, the optofluidic chip, experimental setup and analysis methods had all been developed for later various applications.
Afterwards ARROW design and fabrication process were further improved. Our optofluidic chip got higher optical throughput by depositing single over-coating instead of top cladding layers,[45] fabricating hybrid overcoat,[51] or fabricating ARROWS on a self-aligned pedestal.[44] $\text{Si}_3\text{N}_4$ was also replaced by low-temperature $\text{Si}_3\text{N}_4$ or $\text{Ta}_2\text{O}_5$,[43], [52] leading to lower photoluminescence background. We turned our attention to the single molecule detection of more interesting and important bioparticles. The Qβ bacteriophage on the single virus level was detected and analyzed using our optofluidic chip.[53] Diffusion coefficients, flow velocities and concentrations of viruses and their aggregates or structural parts were also extracted using FCS. Next, on the basis of traditional FCS, more analysis and detection methods were applied in our single molecule detections. For instance, dissociation of double-stranded DNA into Cy3 and Cy5 labeled oligonucleotides was observed with the help of fluorescence cross-correlation spectroscopy (FCCS) and fluorescence resonance energy transfer (FRET).[54]

The attempt to enrich the functionality of our optofluidic chip was never ceased. A dual-core configuration consisting of a normal ARROW and a tunable filter sections demonstrated its spectral tunability by tuning core index and pH.[36] In addition, vertical integration of other microfluidic devices brought the advantage of fluids control to our platform. Recently, a PDMS mixer-ARROW successfully labeled and detected single $\lambda$–DNA molecules. The integration of PDMS based splitter and filter showed their promising applications in specific on-chip particle detections as well.[23]
Above is just a brief introduction about part of our achievements in fluorescence detection with ARROWs. However, the trend of development is pretty obvious: future applications will focus on the integration of functional components and the detection of tiny bioparticles. As the demand of high single particle sensitivity increasing, we also seek a method to ensure that only one particle is in the excitation volume. In the following sections of the present work, we are going to talk about the motivation, method, and accomplishment of ARROWs integrated with a highly sensitive electrical component, nanopore sensor.

1.5 Nanopore sensors

A nanopore is a tiny hole in a thin membrane. Both sides of this membrane is filled with electrolyte. When a voltage is applied across this nanopore, an ionic current will pass through the nanopore. As a particle goes from one side of the membrane to the other side, the nanopore will be blocked for a short period, during which a current drop will be recorded by a sensitive amplifier. Theoretically, particles with different sizes, shapes, and electro chemical properties will generate current blockades with different amplitudes and dwell times. Therefore, by analyzing the blockades, we should be able to identify and distinguish particles. Nanopore sensors can be considered as an extension of the Coulter counter; however, compared to conventional Coulter counters with large openings,[55] nanopore’s diameter could be as small as several nanometers and the thickness of the membrane could be less than tens of nanometers, so it is much more sensitive to use a nanopore sensor to detect
tiny differences between particles. Thus, the nanopore is considered to be a very potential replacement tool for single particle detection and nucleic acids sequencing. Nanopore’s applications in the field of particle detection are successful. Efficient and precise detections of synthetic nanoparticles, viruses, DNA and RNA molecules have all been demonstrated using nanopore sensors.[56]–[61]

Particle detection is a rather simple task for nanopore, since nanopore originally aimed at becoming the next generation nucleic acids sequencing tool. Though until now nucleic acids sequence still cannot be directly read by letting a single nanopore read along a single nucleic acids molecule, nanopore array or functional nanopore devices have already been able to sequence largely duplicated, simplified or modified nucleic acids molecules.[62]

![Figure 1-6. Commonly used (a) biological nanopore and (b) solid state nanopore.[62], [63]](image)

Two kinds of nanopores are commonly used:
1.5.1 Biological nanopore

α-haemolysin and Mycobacterium smegmatis porin A (MspA) are two commonly used biological nanopores.[64], [65] After being inserted into lipid bilayer, biological nanopore’s ~1nm minimum inner diameter allows single stranded DNA or single stranded RNA to pass through. A major issue of nanopore sequencing is that if a DNA or RNA molecule goes through the nanopore too quickly, it won’t be possible for any detector to sample such high frequency data. Fortunately, one advantage of biological nanopore is the structure of the nanopore can be genetically engineered so that either the analyte translocation can be affected by the nanopore structure, or the analyte can interact with a functional adapter bound to the nanopore, resulting in slower translocation.[66]–[68] However, some disadvantages of biological nanopore are also apparent. For example: biological nanopore can’t be reused; the chemical properties of the sample solution must be strictly controlled; the minimum inner diameter can’t be greatly changed for larger particle detection, etc.

1.5.2 Solid state nanopore

Solid state nanopores are fabricated on Si₃N₄, SiO₂, metal, Al₂O₃, or graphene membranes.[63], [69]–[71] The research on solid state nanopore has become quite popular in the past decade, since solid state nanopore is mass producible, robust, environmentally insensitive, and tunable. Like biological nanopore, solid state nanopore has been successfully used in detections of nanoparticles, proteins, viruses, DNA/RNA molecules, and in nucleic acids sequencing as well. The similar problem
that solid state nanopore faces is also the fast translocation velocity, and lots of efforts have been taken to solve this issue. For example, it has been proven that by using the methods of modifying surface properties, tuning nanopore size, or adjusting salt concentration etc., the translocation can be slowed down effectively.[72]–[77]

Fabrication-wise, there are a lot of methods to make a nanoscale opening, such as etching, electron beam milling, ion beam sputtering, or laser beam burning.[78]–[82] Electron beam and ion beam have been mostly used, since they can fabricate precise tiny pores, as well as fine tune the nanopore size. In Chapter 2, we are going to discuss the details of nanopore fabrication based on electron and ion beam techniques, and compare the fabrication induced differences in nanopore geography and single particle detection.

Compared to biological nanopore, an advantage of solid state nanopore is that it can be easily integrated into other devices, and other sensing components can be integrated into nanopore as well. In order to integrate nanopore into microfluidic devices, the easiest way is to sandwich a nanopore fabricated in a free-standing membrane with polymer-based channels. For example, solid state nanopore sandwiched by PDMS channels has been used for single DNA detection[83] and particle trapping.[84] Of course, nanopore can also be directly fabricated onto the microfluidic chip. Two nanopores in series were used for an on-chip viruses measurement.[85] On the other hand, some functional devices were also integrated into solid state nanopore to ensure more accurate single molecule measurements. The prime example is the embedded nanogaps which are used for tunneling current
measurement.[86] Ultimately, it has already been proven that nanopore, electrodes and amplifier can all be integrated into a single compact nanopore detection chip,[87] which makes it possible for mass production and commercial use.

1.5.3 Combination of optical detection and solid state nanopore

Besides the electrical measurements with solid state nanopores, the feasibility of simultaneous electrical and optical detection with solid state nanopores has already been demonstrated. A typical setup is shown in Fig. 1-7.[88] An objective lens is always placed underneath the nanopore to focus the excitation beam on the nanopore, so that the particles will be excited right after they translocate through the pore. The integration of two single-molecule measurement modalities visualizes the translocation process, and also makes this setup suitable for the measurements that cannot be done with either method alone. More details and discussion will be found in Chapter 3.
In the present work, a solid state nanopore is fabricated directly into an ARROW chip, leading to a series of successful electrical gating and fluorescence detections of single nanoparticles. We are going to talk about the details about nanopore fabrication and exciting applications of our nanopore gated ARROWS in the following chapters.
Chapter 2 Solid-state Nanopore fabrication methods and their effects on single nanoparticle detection

One important feature of solid state nanopore is that the nanopore size can be adjusted to fit different particle sizes and applications. In order to figure out the way to optimize the size of nanopore, nanopores with different diameters and thicknesses have been tested and compared,[56] aiming to find how the diameter and thickness are going to influence the electrical signal. Meanwhile, a lot of models have also been built to simulate the behavior of solid state nanopore.[89]–[93] However, in most experiments or simulations, solid state nanopore was approximated as a regular cylinder, not a lot of attention has been paid to the exact shape of solid state nanopore. Of course, one reason for that is it’s quite hard to characterize the shape of nanopore, due to its tiny size.

In this chapter, we acquired the cross section images of nanopores fabricated in different ways, and predicted the nanopore resistances and the shapes of electrical blockades using a full 3D modelling.[94] To this end, we first fabricated three nanopores with the same diameter using three different methods (direct ion beam drilling, electron beam shrinking, and ion beam shrinking). Please note that since nanopores have irregular shapes, the diameter here means the smallest aperture of a nanopore (Fig. 2-1). After single particle measurements were done with these
nanopores, they were cross sectioned using focused ion beam for the shape characterization. At last, simulated electrical behaviors of those three nanopores based on a COMSOL model were compared with the experimental results, followed by the discussion. Besides using the methods mentioned above, some of the nanopores used in the present thesis were also made by deposition shrinking. We also characterized the shape of deposition shrunk nanopore, and simulated its electrical behavior. However, deposition shrunk nanopore was not compared with the other three types of nanopores. Instead, it was used for on-chip detection of λ-DNA molecules, which will be discussed in Chapter 4.

Figure 2-1. Definition of the diameter of a nanopore.
2.1 Nanopore fabrication methods

2.1.1 Direct ion beam drilling

Nanopore fabrication with focused Ar ion beam was first proposed by Li et al.[79] The setup illustrated in Fig. 2-1 implements a feedback-controlled ion sputtering system that counts the ions transmitted through the opening pore and stops the milling process at the appropriate time. After that method was developed, focused ion beam has been broadly used for nanopore fabrication, because of its rapidness, flexibility and versatility.[80], [95], [96] However, frequently used Ga ion beam has two disadvantages. First, it is hard to directly drill a nanopore smaller than 20nm, and the pore size cannot be controlled very precisely. Second, Ga ions can be implanted into the membrane, leading to a change of surface charge of nanopore.[97] Fortunately, Ga ion is not our only option of ion source, and different ion beams have different effects in nanopore fabrication. For instance, He ion beam was recently proven to be able to avoid the issues of Ga ion beam.[98]
2.1.2 Direct electron beam drilling

Storm et al. proved a tightly focused electron beam can also be used for nanopore fabrication.[80] It was demonstrated that a focused electron beam with a spot size of a few nanometers inside the TEM could be used to drill holes in thin free-standing SiO$_2$ membranes, with an estimated thickness of about 10 nm. Holes as small as 6 nm could be obtained using an electron-beam intensity above $1 \times 10^8$ A m$^{-2}$, but this technique does not give full control at the nanometer scale because no images can be recorded during drilling. Though high energy transmission electron microscope is always expensive, and drilling process usually takes a much longer time than ion beam drilling does,[99] electron beam drill is still being used for fabricating lots of nanopores on different materials, due to its single nanometer precision. Nanopores
down to several nanometers can be easily drilled, and the pore size can be precisely controlled during the fabrication process.[100], [101] Fabrication methods using low energy scanning electron microscope were also proposed in some work.[102]–[104] These methods lower the requirement for equipment, but an assistant gas, like XeF$_2$, is always required for the purpose of electron beam induced etching.

### 2.1.3 Etching

Ion or electron beam drilling are convenient for fabricating one or several nanopores, but not for mass production. As a conventional step to form fine structures on semiconductor devices, etching is also successfully used for solid state nanopore fabrication. Different methods are used to define the nanopore size and shape, followed by either dry or wet etching. For example, by using the electron beam lithography, a nanopore as small as $\sim 10\text{nm}$ can be defined and finally etched through.[80] However, similar to all other lithography methods, lots of simulations and characterizations are needed to figure out the ideal parameters to use.[105] Track etching uses heavy ion irradiation of Au or U to form latent tracks, followed by chemical etching. This method usually creates asymmetric conical nanopores, and the nanopore size can be from several nanometers to several micrometers. By using different ions and controlling the etching process, the nanopore geometry can be controlled.[106] Another option is feedback chemical etching, whose principle is straightforward. First, an inverted pyramid with a sharp tip is fabricated on the front of a silicon wafer
and a truncated pyramid on the back. Then, the silicon chip is mounted in an electrochemical setup to separate two chambers, which are filled with KCl solution and KOH solution respectively. A voltage is finally applied between two chambers and no current will be measured until two pyramids are etched through, which means a nanopore is made. Feedback chemical etching is affordable, and there’s no requirement of ion beam or electron beam equipment. However, the outcome may be affected by the lithography errors, and the nanopore may not be circular.[107]

2.1.4 Nanopore shrinking

Experimentally, it’s difficult to fabricate a solid state nanopore with the exact size we want. So it becomes important to find a way to adjust the nanopore size. Interestingly, when the ion beam and electron beam drilling were first time demonstrated, the fact that nanopore could be fine-tuned by either beam was discovered at the same time. The nanopore was first sculpted/shrunk with focused ion beam by Li et al.[79]. They proved a nanopore in S₃N₄ membrane could be shrunk by a scanning diffuse ion beam. This phenomenon can be explained either by the diffusion of surface S₃N₄ layer[108] or the diffusion of surface adatom generated by gallium ions[109]. Besides ion beam, electron beam can also sculpt the solid-state nanopore[110]–[115]. Not only high energy transmission electron microscope (TEM), but also the conventional scanning electron microscope (SEM) with a weaker beam can finish the task. Under the electron beam irradiation, the nanopore will gradually close up. This process seems to be very similar to the ion beam shrinking, but the mechanism of shrinkage is different.
It has been explained by either electron-beam-induced deposition of a carbon compound[111] or joule-heat-assisted irradiation-induced diffusion of membrane material[112]. Besides the two commonly used methods above, nanopore can be shrunk by a straightforward means, direct deposition as well. Depending on the applications, either an atomic layer or a thicker layer of specific materials can be deposited to adjust the pore size.[116]

To some extent, the finding of solid state nanopore’s tunability directly leads to solid state nanopore’s wide applications today, because the fabrication of nanopore with precise initial diameter is not a limit any more.

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<th>DIRECT ION BEAM DRILLING</th>
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<tr>
<td>SPEED</td>
<td>Within seconds.</td>
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<tr>
<td>PRECISION</td>
<td>Usually hard to directly drill a nanopore smaller than 20nm. With dedicated FIB and specific material, nanopore can be down to sub-5nm.[98], [117]</td>
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<th>DIRECT ELECTRON BEAM DRILLING</th>
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<tr>
<td>SPEED</td>
<td>Within minutes.</td>
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<tr>
<td>PRECISION</td>
<td>Single nanometer precision.[80], [99]</td>
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<th>ETCHING</th>
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<tr>
<td>SPEED</td>
<td>It takes a long time to etch the wafer to form the thin film, or to etch nanopores on thick membranes. Typical KOH etching rate is on the order of about a micron per minute.[118]</td>
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<tr>
<td>PRECISION</td>
<td>Nanopore size can be down to several nanometers. Careful characterization is needed.[105], [106]</td>
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<tr>
<td>SPEED</td>
<td>Within minutes.</td>
</tr>
<tr>
<td>PRECISION</td>
<td>Sub-nanometer precision.[79], [80]</td>
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Table 2-1. Comparison between different fabrication methods.
2.2 Nanopore fabrication using different methods

2.2.1 Nanopore fabrication using direct drilling, electron beam shrinking and ion beam shrinking

A DualBeam SEM/FIB microscope (FEI Quanta 3D FEG DualBeam SEM/FIB microscope) enables us to directly drill nanopore with focused ion beam and tune the nanopore size with an electron beam or ion beam[119], [120]. In principle, direct focused ion beam (FIB) drilling is straightforward[121]–[123]. A gallium ion beam hits a thin membrane and sputters the material away, leaving a nanopore in the membrane. However, it’s not guaranteed that the diameter of the nanopore is as expected, since the condition of the ion beam and the property of the membrane can influence nanopore’s final diameter a lot, and it’s not preferred to characterize the beam condition on the membrane where the nanopore will be drilled. Fortunately, even if the nanopore is larger than our expectation, reducing its size is still possible by using the methods discussed above.

Fabricating and tuning a nanopore is feasible; however, we are also curious about whether nanopores fabricated by different ways have similar or totally different shapes. Although some theoretical models and estimations have well approximated the performance of solid-state nanopore[124]–[126], it is still helpful to investigate and compare solid-state nanopores made by different methods. In this chapter, in order to compare the performances of nanopore fabricated differently, we studied three $50 \times 50$nm (diameter × thickness) nanopores made by direct focused ion beam drilling,
electron beam shrinking after FIB drilling, and ion beam shrinking after FIB drilling. 30 nm diameter nanoparticles were then tested with three nanopores, and electrical signals were compared and analyzed. Besides those $50 \times 50\text{ nm}$ nanopores, we also fabricated some nanopores using the method of deposition shrinking. Their shape and properties will also be discussed later.

The nanopores for comparison were fabricated on commercial 50nm thick silicon nitride membranes (DTF-050523, DuraSiN Film for TEM) with a DualBeam microscope (FEI Quanta 3D FEG DualBeam SEM/FIB microscope). All the nanopores are originally drilled with focused ion beam. Since we intend to compare the differences of $50 \times 50\text{ nm}$ nanopores fabricated by three various methods, nanopores with diameters of 50nm (Fig. 2-3a) and larger than 50nm (Fig. 2-3b left and 2-3c left) were drilled. Then the larger nanopores were shrunk to 50nm by electron beam scanning and ion beam scanning.

Figure 2-3. Change of nanopore diameter versus electron beam scanning time. 3 kV, 5 kV and 10 kV accelerating voltages were used.
We first shrank the nanopore with 114 nm diameter using electron beam. The advantage of this method is we can monitor the size of nanopore while it’s being scanned, until the diameter reaches 50 nm. The time needed to finish this process depends on several factors.[127] We did a characterization of nanopore diameter vs. scanning time with different electron beam currents. The results are plotted in Fig. 2-2, which shows the nanopore shrinking rate increases with decreasing accelerate voltage, since stopping power in the membrane increases with decreasing electron energy. Studies of other groups also showed similar results.[127], [128] In our work, we chose the electron beam with a low accelerating voltage (5kV) and weak beam current (11-13pA).

Another 70 nm diameter nanopore was shrunk by ion beam scanning. The accelerating voltage of the ion beam was 30kV, and the beam current was either 1.6pA or 10 pA. Different from the electron beam shrinking, we are not able to monitor the nanopore size in real time. The reasons are: first, the resolution of ion beam imaging is not as good as that of electron beam, so we are not able to see the nanopore clearly; second, we need to zoom out to scan a larger area or diffuse the beam, in order to avoid destroying the nanopore. We need to be cautious while using ion beam shrinking, since the nanopore can be completely closed after tens of full screen scanning.[129] Once fabricated, nanopore usually needs to be immersed in Piranha solution for cleaning and creating a hydrophilic surface by the formation of hydroxyl groups.[130]–[132] But in our experiment, we found the electron beam
shrunk nanopore cannot survive in the Piranha solution, so we didn’t do any post-processing to the nanopores prior to the nanoparticle translocation measurements.

Figure 2-4. Top view of nanopores fabricated with different methods. (a) Directly drilled 50 nm nanopore. (b) 50 nm nanopore (right) shrunk from 114 nm nanopore (left). (c) 50 nm nanopore (right) shrunk from 70 nm nanopore.

2.2.2 Nanopore fabrication using deposition shrinking

Besides free standing nanopores mentioned above, a lot of nanopores used in our nanopore-optofluidic platform were fabricated using FIB assisted deposition shrinking. A 30kV 1.6pA ion beam was used to deposit SiO$_2$ within a 0.7 $\times$ 0.7 $\mu$m square over the nanopore, in order to reduce the pore size. For nanopores with different initial sizes, we have to deposit SiO$_2$ for a short while (e.g. 30s) and check the nanopore size, and repeat, until the desired pore size is reached.

2.3 Electrical nanopore characterization

2.3.1 Cross section imaging of 50 $\times$ 100 nm nanopores

In order to acquire the shapes of nanopores, we cross sectioned nanopores with a focused ion beam. We first cleaved 50 $\times$ 100 nm nanopores for a test. Before ion
beam cleaving, a thin platinum layer is deposited over the nanopore as a protective layer[133]. The left column in Fig. 2-4 shows the top views of 50nm nanopores made by different methods mentioned in last section, and the right column displays the cross section views. As we can see from the cross section images, the directly drilled nanopore has a shape similar to an hour glass. The electron beam shrunk nanopore has an obviously enlarged thickness, caused by the deposition of some unknown material. We figured out what that material was by taking energy dispersive X-ray spectra (EDS) of a 50 × 50 nm nanopore. More details will be provided later. Compared to the other two nanopores, ion beam shrunk nanopore has the widest opening and a pointy edge at the aperture, which may lead to bigger blockade amplitude.

Figure 2-5. Scanning electron micrographs of 50nm nanopores made on 100nm
membranes. The left column shows top-down images of (a) directly drilled (DD), (c) electron beam shrunk (EBS), and (e) ion beam shrunk (IBS) nanopores. Images (a) and (g) were taken with 20kV electron beam, and image (d) was taken using 5kV electron beam. The right column shows side views of the same nanopores after deposition of a protective top layer of platinum and cross-sectioning.

### 2.3.2 Cross section imaging of 50 × 50nm nanopores

![Figure 2-6](image)

Figure 2-6. Scanning electron micrographs of 50nm nanopores made on 50nm membranes. The left column shows the cross section images of (a) directly drilled, (b) electron beam shrunken and (c) ion beam shrunk nanopore. After a thin layer of platinum is deposited as the protective layer, the nanopore is cleaved in the middle and imaged. In (d-f), the shape of nanopore is outlined.
Similarly, after the nanoparticle detections were done, 50nm thick 50nm wide nanopores were cleaved with focused ion beam for the cross section imaging. After the cleaning cross-section cleaving, the cross-sections of three types of nanopores were imaged and shown in Fig. 2-5. The first thing we notice is the shape of the nanopore. Generally, the cross-section of nanopore is assumed as an ideal cylinder[134], [135]. From the figures it is easy to tell none of three nanopores is ideal cylinder. The shape of the directly drilled nanopore is close to a cylinder, except the rounded edges on the top and the bottom. The size of directly drilled nanopore is as expected, 50 nm wide and 50 nm long. Electron beam shrunk nanopore is like a cylinder, too. However, it’s very interesting to find the length of the nanopore (about 130 nm) is significantly longer than our expectation. Before electron beam scanning, the original nanopore is drilled on the 50 nm membrane. After the electron beam scanning, a deposition of an unknown material makes the nanopore longer. Ion beam shrunk nanopore is apparently not a cylinder at all. Being close to a cone, it has a wider opening on the top and a narrower opening on the bottom.

By comparing the nanopores shapes, we also find the final shape depends on the initial size of the nanopore. The cross section images of 50 × 100 nm nanopores look different from those of 50 × 50nm nanopores. This means, we cannot simply assume nanopores with different diameters and lengths will have similar shapes, even if they are made with the same method. Further study about the mechanism of nanopore’s formation under different fabrication methods is needed, but it’s out of the range of
discussion in the present work.

2.3.3 Cross section imaging of deposition shrunk nanopore

Figure 2-7. Scanning electron micrographs of 150nm nanopores made on an ARROW chip.

Except 50nm nanopores, we also checked the shape of a deposition shrunk nanopore. The nanopore in Fig. 2-6 was drilled on an ARROW chip, and then shrunk from ~210nm to ~150nm by SiO$_2$ deposition. As is shown clearly, the deposition shrunk nanopore has a protruding edge on the top, which was created by the deposition. However, since the depositing material is the same as that of nanopore, we cannot tell
from the photo where the boundary of original membrane and deposition layer is.

2.3.4 Energy dispersive X-ray spectroscopy analysis of nanopores

In order to analyze the deposited material around the electron beam shrunk nanopore and see if electron beam or ion beam can influence the composition of the nanopore, Aaron Drake and Sreenivas Bhattiprolu at Oxford Instruments and Mark Betts at Tescan assisted us to take the spectra of three nanopores using Energy Dispersive X-ray Spectroscopy. All three types of nanopores were drilled with markers around, as shown in Fig. 2-7. The small nanopore in the middle of four markers is the nanopore to test, and the big square is also a marker. In Fig. 2-8, spectra of three types of nanopores were taken over a 500 nm square centered at each nanopore, and the control spectrum was taken over another 500 nm square far away from the nanopore. Theoretically, there should be only Si and N peaks, and no or weak carbon and oxygen peaks in the spectra. However, in practice, hydrocarbon is always being deposited by the scanning beam while we collected the spectra, and that’s why there are carbon and oxygen peaks in every spectrum.

There are some obvious differences between the spectra of these nanopores. Compared to the other two nanopores, electron beam shrunk nanopore has higher carbon and oxygen peaks. The existence of larger amount of carbon and oxygen around electron beam shrunk nanopore is even more obvious if we check the element maps of three nanopores (Fig. 2-9). In order to further prove the electron beam shrunk nanopore is covered by hydrocarbon, we took the spectra over a point on the
nanopore and two points nearby for comparison (Fig. 2-10). It is very apparent the electron beam shrunk nanopore has more carbon and oxygen than other points do, which proves the electron beam shrunk nanopore consists of hydrocarbon. Another finding in the spectra is both electron beam shrunk and ion beam shrunk nanopores contain a small amount of gallium. The reason why ion beam shrunk nanopore contains gallium is straightforward. It was drilled by the gallium ion beam and it was even shrunk by ion beam scanning, so the gallium was implanted in these processes. The reason why electron beam shrunk nanopore has some gallium but directly drilled nanopore doesn’t, we believe, is because it took longer time for the ion beam to drill the original bigger pore for later electron beam shrinking, leading to the deposition of a small amount of gallium ions.
Figure 2-8. Pattern fabricated for the Energy Dispersive X-ray Spectroscopy. The small nanopore in the middle of four bigger pores is the nanopore to test, and the big square and four pores are marks.

Figure 2-9. The Energy Dispersive X-ray Spectra over a 500 nm square centered at (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores. The
elements for each peak are marked. The green area is the control spectrum, which was taken over a 500 nm square far away from the nanopore.

Figure 2-10. Element maps of a larger region around the (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores. The rectangles in (a) and (b) show the regions where element maps were plotted, and the element map was plotted for the whole image of (c). The distribution of C, O and Ga is shown in a1-a3, b1-b3 and c1-c3, respectively. The bright spot in (b) is an iron deposition, which could be induced by the contaminations in the SEM chamber or on the sample holder.
Figure 2-11. The Energy Dispersive X-ray Spectra of points (a) 1, (b) 2 and (c) 3 on the chip with an electron beam shrunk nanopore. Inset: Spectra are taken at point 1: nanopore, point 2: Si3N4 membrane and point 3: supporting substrate.

2.4 Simulation of the ionic current through nanopore

As has been used in several modeling studies[134], [136], [137], three equations were used in our model to simulate the electric characteristics of the nanopore and the current change when nanoparticle goes through the nanopore. First, Poisson Equation describes the electric potential decided by the distribution of ions:

$$\nabla^2 \Phi = -\frac{F}{\varepsilon} \sum_i z_i c_i \quad (2.1)$$

where $\Phi$ is the electric potential, $F$ is Faraday Constant, $\varepsilon$ is Relative permittivity of
the solution, \( z_i \) is the charge of specie \( i \) and \( c_i \) is the concentration of specie \( i \).

The flux of ions (\( \vec{J}_i \)) in the solution under the influence of concentration gradient, electric field and flow speed is described by Nernst-Planck Equation:

\[
\vec{J}_i = -D_i \nabla c_i - \frac{z_i F}{RT} D_i c_i \nabla \Phi + c_i \vec{u} \quad (2.2)
\]

where \( D_i \) is the diffusion constant of ion \( i \), \( R \) is the gas constant, \( T \) is the temperature and \( \vec{u} \) is the fluid velocity.

Finally, Navier-Stokes Equation dictates the velocity field, which is influenced by pressure gradient, viscosity and electric field:

\[
\vec{u} \nabla \vec{u} = \frac{1}{\rho} (-\nabla p + \eta \nabla^2 \vec{u} - F(\sum c_i) \nabla \Phi) \quad (2.3)
\]

where \( \rho, p \) and \( \eta \) are the density, the pressure and the viscosity, respectively.

In this model, the ionic current through a surface can be obtained by integrating the current density:

\[
I = \int (z_1 \vec{J}_1 + z_2 \vec{J}_2) \cdot \vec{n} dS \quad (2.4)
\]

It is hard to find an analytical solution for the above equations, so we built a 2D axisymmetric model (Fig. 2-11) to solve this problem with finite element method. All the three equations were coupled and solved numerically by COMSOL Multiphysics. The nanopore in Fig. 2-11 is 50 \( \times \) 50 nm. Here we take the directly drilled nanopore as an example. cis and trans chambers were set to be 4 \( \mu m \) wide and 2 \( \mu m \) long. The maximum mesh size on the nanopore surface is 1 nm. The constants used in our model are: \( T = 298K \), \( D_K = 1.957 \times 10^{-9} m^2/s \), \( D_{Cl} = 2.032 \times 10^{-9} m^2/s \), \( \varepsilon = 80 \),
\[ \eta = 10^{-3} \text{Pa s} \quad \rho = 10^3 \text{kg/m}^3 \quad z_k = 1 \quad z_{cl} = -1 \quad F = 96485.3365 \text{C/mol} \quad R = 8.31 \text{J/mol/K} \].

Figure 2-12. (a) Schematic diagram of the 2D axial symmetric model for finite element calculation. Dotted line is the symmetric axis. The area of the red rectangle is partially enlarged in (b).

2.5 Nanopore-shape-dependent blockade signals in single nanoparticle detection

2.5.1 Setup and materials

As shown in Fig. 2-12, the nanopore connects the cis and trans chambers. Negatively charged nanoparticles are only added into the cis chamber. Once a command voltage
is applied, nanoparticles will be moved by electric force through the nanopore to the trans chamber.

Figure 2-13. Schematic diagram of the experimental setup. The thin membrane is fabricated on a Si substrate. cis chamber is above and trans chamber is below the membrane. After the nanoparticles are added into the cis chamber, a command voltage is applied across the nanopore. Since the nanoparticles are negatively charged, they will be moved through the nanopore by the electrical force.

30 nm Carboxyl polymer nanoparticles were purchased from Bangs Laboratories, Inc (PC02N/9934). Before the experiments, nanoparticles were diluted to $5.4325 \times 10^{13}$ particles/mL in 0.2M Potassium Chloride solution (20mM CHES, pH=9, 0.01% v/v Triton X-100). All the aqueous solutions used in this work were filtered with 10nm diameter filter (Whatman Antop 10). Before the electrolyte and
nanoparticles were added into the chamber, the nanopore was flushed by isopropanol and DI water, which is helpful for wetting the nanopore. The nanopore was clamped by two ceramic holders (Fig. 2-13). The nanoparticle solution was then added into the cis chamber, and the electrolyte without nanoparticles was added into the trans chamber. An Axon Axopatch 200B patch clamp amplifier was used to apply the voltage across the nanopore. After filtered by an on board 10 kHz low-pass Bessel filter, the analog signal was digitized by an Axon Digidata 1440A digitizer at 250 kHz.

![Ceramic nanopore holder](image)

Figure 2-14. Ceramic nanopore holder.
2.5.2 Results and discussion

2.5.2.1 I-V Curves

Figure 2-15. (a) Experimental I-V curves (dots) of directly drilled, electron beam shrunk and ion beam shrunk nanopores. The colorized lines are the simulation results for three kinds of nanopores. The black line stands for the I-V curve of a nanopore with an ideal cylinder cross section. (b) The shapes of nanopores built in our model. From top to bottom: directly drilled nanopore, electron beam shrunk nanopore and ion beam shrunk nanopore.

The basic property of a nanopore is its electric conductance or resistance. By ramping up the voltage from -1000 mV to 1000 mV with an increment of 100 mV and recording the ionic currents, we got the I-V curves for three types of nanopores (Fig.
2-14a). Before we had the cross section images of these nanopores, we thought they should all be 50 nm wide and 50 nm deep, no matter how they were fabricated. Thus their I-V curves are supposed to look like the black line in Fig. 2-14a. However, due to the shape differences shown in the last section, three kinds of nanopores had three different I-V curves. It can be easily explained that the electron beam and ion beam shrunk nanopores have the smallest and largest conductance. According to $G = \sigma A/l$ ($G$ is the conductance, $\sigma$ is electrical conductivity, $A$ and $l$ are the cross-section area and the length of the conductor), the electron beam and ion beam shrunk nanopores should have the smallest and the largest conductance, since they have longest channel and widest upper opening, respectively. But in order to get a quantitative estimation of I-V curves, we numerically simulated I-V curves with our model. Being different from any models built before, the shape of the nanopore was also considered in our model and was drawn to be as similar as what we saw in the cross section images. As is shown, I-V curves calculated using these models (colorized lines in Fig. 2-14a) deviate from the ideal condition (black line in Fig. 2-14a), and meet the experimental results quite well. In our simulation, the surface charge density of the nanopore was ignored. Surface charge is important not only in rectifying pores, it also affects ionic concentrations in perfectly symmetric structures. In order to examine whether surface charges on the pore walls can indeed be ignored, I-V curves of different nanopores in salt solutions of different concentrations, and pore conductance versus KCl concentration were plotted. Fig. 2-15 shows that the ion current rectification is weak experimentally in salt solutions of different concentrations. The linear relationship
presented in Fig. 2-16 confirms that the influence of the surface charges can indeed be neglected. Thus, we think the error caused by the electrically neutral surface is neglctable.

Figure 2-16. I-V curves of (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores in salt solutions of different concentrations.
Figure 2-17. Nanopore conductance versus KCl concentration. Our operating concentration is 0.2M.
2.5.2.2 Blockade Amplitude and Dwell Time

Figure 2-18. Signal traces of (a) directly drilled, (b) electron beam shrunk and (c) ion beam shrunk nanopores. (d-f) are enlarged view of typical signals. The red dotted lines mark where the blockade starts and stops.

30 nm Carboxyl polymer nanoparticles (Bangs Laboratories, Inc, PC02N/9934) were
used for nanoparticle translocation measurements. When a 100 mV voltage was applied, particles were moved from the cis chamber to the trans chamber through the 50 nm nanopore by the electric force. In this process, the conductance of the nanopore, thus the ionic current, was influenced by the nanoparticle.

Figure 2-19. (a) Scatter plot of events detected by three kinds of nanopores when the voltage is 100 mV. (b) The histogram of relative conductance change, $\Delta G/G$, of three nanopores at 100 mV. (c) The histogram of dwell time of three nanopores at 100 mV. (d) Simulated electric field strength along z axis of three kinds of nanopores.
The relative conductance changes of three nanopores are calculated by \( \Delta G/G = \Delta I/I \), where \( \Delta I \) is the amplitude of the blockade, and \( I \) is the baseline current. As shown in Fig. 2-18a-c, three nanopores gave us different conductance change distributions and dwell time distributions, though we thought these nanopores should have the same performance. The mean \( \Delta G/G \) of directly drilled, electron beam shrunk and ion beam shrunk nanopores are 0.1042, 0.0610 and 0.0775, respectively. Qualitatively, if we assume the relative conductance change is approximately equal to the ratio of particle volume to the nanopore volume[138], then the directly drilled nanopore with the smallest volume will get the biggest conductance change, and the electron beam shrunk nanopore with the biggest volume will get the smallest conductance change, which agrees with the trend of experimental data. Numerical and analytical calculations will be carried out later to estimate the relative conductance change. The mean dwell times of directly drilled, electron beam shrunk and ion beam shrunk nanopores are 0.1942 ms, 0.2585 ms and 0.1714 ms, respectively. Dwell time is reversely proportional to the particle velocity, which is proportional to the electric field strength. As is shown in Fig. 2-18d, electron beam shrunk, directly drilled and ion beam shrunk nanopores have ascending peak values of electric field strength, leading to descending dwell times. Further study about the surface properties of nanopore and nanoparticle is needed to precisely estimate the absolute dwell time value, and this is out of the discussion of the present work.
Figure 2-20. (a) The conductance change is calculated by moving a particle from top to bottom along z axis. Here we take the electron beam shrunken nanopore as an example. The calculated current versus location is plotted in (b). (c) The comparison between relative conductance changes predicted by numerical and analytical solutions, and experimental results.

In order to estimate the conductance change caused by the introduction of nanoparticle in the nanopore, we add a particle into our model and move it from top
to bottom along z axis with a 5 nm spacing between each point (Fig. 2-19a). At each point, the ionic current through the nanopore was calculated and we can see how the current changes if we plot the currents versus particle locations (Fig. 2-19b). Before the numerical method for calculating the nanopore current was developed, lots of analytical methods were attempted. The most straightforward and simplest way is to calculate the resistance change by calculating the excluded volume when a particle is present in the nanopore, while assuming the nanopore has a regular shape. Here, we further compared experimental results and our simulation with a recently corrected analytical solution for the relative conductance change:

\[
\frac{\Delta G}{G} = \frac{d^3}{L + \frac{\pi D}{4}} D^2 \tag{2.5}
\]

where \(G\), \(d\), \(L\) and \(D\) are nanopore conductance, particle diameter, nanopore length and nanopore diameter, respectively.

Please notice in the analytical solution we use 50 nm diameter and 50 nm length for directly drilled and ion beam shrunk nanopores, 50 nm diameter and 130 nm length for the electron beam shrunk nanopore. Compared to the numerical simulation, the analytical solution deviates more from the experimental data. The directly drilled nanopore is the one that is closest to the ideal shape of a 50 nm wide and 50 nm long cylinder, thus the analytical solution can approximately predict the relative conductance change. For the case of electron beam shrunk nanopore, though it seems the analytical solution is close to the experiment value, we could get a much larger difference if we assume this nanopore is also 50 nm wide and 50 nm long. When the
shape of the nanopore deviates a lot from an ideal cylinder, the analytical solution gives a large difference between calculation and experimental data, which is the case of ion beam shrunken nanopore. So, the numerical method is more powerful when an accurate estimation is needed for a nanopore with an irregular shape.

2.5.2.3 Change of Blockade Amplitude and Dwell Time

Knowing what kind blockade we can get is important for deciding an appropriate voltage for specific applications. The first thing we characterized in our experiment was the blockade amplitude. In Fig. 2-20, the amplitudes under 100 mV, 200 mV, 300 mV and 400 mV voltages were recorded and plotted versus the voltage. As has been found in a couple of works,[73], [139], [140] the amplitude almost linearly increased as the voltage became higher. As mentioned in the last section, we can also calculate the blockade amplitude using our numerical simulation. Fig. 2-20 shows the simulation meets quite well with the experimental results.
Figure 2-21. The change of blockade amplitude versus the voltage. Dots are experimental results and lines are simulation results.

Though, at higher voltage, higher amplitude can potentially increase the signal noise ratio (if the noise level is still good when the baseline current is high), the dwell time is becoming shorter and shorter. Instead of fitting the dwell time as a function of voltage with several commonly used models, we numerically simulated the dwell time of particle going through three kinds of nanopores, and compared the results with our experimental data.

In order to get the nanoparticle’s dwell time of passing the nanopore, we need to calculate the local velocity of nanoparticle: \( v = \frac{\zeta}{\eta} \left( |\zeta_{\text{pore}}| - |\zeta_{\text{particle}}| \right) E \eta \), where \( \varepsilon, \eta, \zeta \) and \( E \) are permittivity, viscosity, zeta potential and electric field strength. Then this local velocity can be used to calculate the time interval between each location along z.
axis in the nanopore. The sum of the time intervals can well predict the dwell time a nanoparticle needs to go through the nanopore. When the zeta potential of nanopore and nanoparticles are unknown, the expression of velocity above can be simplified to \( v = CE \), where \( C \) is a constant. By summing up all the time intervals between each location where we locate the nanoparticle \( \Delta t = \Delta z/v \), the dwell time will be
\[
t_{\text{sum}} = \Delta t_1 + \Delta t_2 + \cdots = \left( \Delta z_1/E_1 + \Delta z_2/E_2 + \cdots \right)/C.
\]
In the same way, we can get all the dwell time expressions for three kinds of nanopores under different voltages. Though there is an unknown constant in all the expressions, we can still normalize and compare the trends of them. In order to compare the simulation results with experimental data, experimental data were also normalized and plotted in Fig. 2-21d.

We assume the zeta potentials of the nanopores are equal (and therefore the constants \( C \) are equal) and fit the simulation to the experimental data using a least squares fit (yielding \( C = 1.57 \times 10^{-9} \text{ m}^2/\text{Vs} \)), as shown in Fig. 2-21d. Simulated dwell times of the three nanopore types are inversely proportional to the applied voltage, and they match the experimental data well. While previous studies have focused on surface modification to predict and control dwell time, our work shows that the shape of nanopore plays a fundamental role in determining the speed of translocation.
Figure 2-22. Simulation of time dependent blockade for (a) directly drilled, (b) electron beam shrunken and (c) ion beam shrunken nanopores. (d) Normalized experimental and simulated dwell time versus voltage. Dots are experimental results and lines are simulation results. The actual values of experimental results are marked on the right-hand y-axis.

2.5.2.4 Capture Rate

The time between successive events follows a simple exponential distribution when the particles do not interact with each other[124], [141]:

$$ P(t) = Ce^{-\beta} \quad (2.6) $$
where $C$ is a constant and $f$ is the capture rate.

To determine the capture rate, the distribution of the time-delay between successive events for three kinds of nanopores at 100 mV is plotted in Fig. 2-22c-e. According to the equation above, by fitting the histogram with an exponential curve, we can get the capture rate $f$. The fitted capture rates from the histograms of time-delay between successive events from directly drilled, electron beam shrunk and ion beam shrunk nanopores are 7.4 s$^{-1}$, 5.2 s$^{-1}$ and 12.3 s$^{-1}$, respectively. If the capture is not a diffusion limited process, which means the particles that get close to the nanopore won’t be 100% captured and they have to overcome an energy barrier to go through the nanopore, the capture rate will increase exponentially with the voltage. This kind of process is governed by Van’s Hoff-Arrhenius law:[141]–[143] $f = f_0 \exp(V/V_0)$, where $f_0 \approx v \exp(-U*/k_BT)$, $|V|/V_0 = zeV/k_BT$, $v$ is a frequency factor, $U*$ is the activation barrier and $ze$ is the effective charge of the nanoparticle.
Figure 2-23. (a) Capture rate changes as the voltage changes. The data is from the directly drilled nanopore. (b) Capture rate versus voltage. (c-e) Histograms of time-delay between successive events from directly drilled, electron beam shrunk and ion beam shrunk nanopores. Blue lines are the fitted curve.

By fitting the curves in Fig. 2-22b, we get $f = 4.37212 e^{0.0063 \nu}$, $f = 2.93464 e^{0.0063 \nu}$ and $f = 6.44502 e^{0.0063 \nu}$ for directly drilled, electron beam shrunk and ion beam shrunk nanopores, respectively. According to $f_0 \approx \nu \exp(-U^*/k_B T)$, in order to get the activation barrier $U^*$, $\nu$ needs to be estimated first. $\nu$ is determined by $\nu = C D_{\text{diff}} A_{\text{pore}} / L_{\text{pore}}$, where $C$, $D_{\text{diff}}$, $A_{\text{pore}}$ and $L_{\text{pore}}$ are particle concentration, particle
diffusion coefficient, nanopore cross section area and nanopore length, respectively. \(C\), \(D_{\text{diff}}\) and \(A_{\text{pore}}\) are the same for three nanopores: \(C = 5.4325 \times 10^{19} \text{ particles/m}^3\), \(D_{\text{diff}} = k_B T / 6 \pi \eta r = 1.646 \times 10^{-11} \text{ m}^2/\text{s}\) and \(A_{\text{pore}} \approx 1.9625 \times 10^{-15} \text{ m}^2\). \(L_{\text{pore}}\) is 50 nm for directly drilled and ion beam shrunk nanopore, 130 nm for the electron beam shrunk nanopore. By substituting all the values into expression of \(f_0\), the activation barriers for directly drilled, electron beam shrunk and ion beam shrunk nanopores are calculated to be \(2.08 k_B T\), \(1.53 k_B T\) and \(1.69 k_B T\), respectively.

That the electron beam shrunk nanopore has a lower activation barrier is a little confusing, since it also has the lowest capture rate. Again, we try to assume the capture is a diffusion limited process, then the capture rate follows:

\[
f = 2 \pi D r^* \Delta V = \left(\frac{\pi d^2 \mu}{4l}\right) \cdot \Delta V\]

[144], where \(r^* = (d^2 \mu / 8lD) \cdot \Delta V\) is a characteristic length scale where the motion of particles changes from purely diffusive (particle-nanopore distance \(> r^*\)) to drift-dominated (particle-nanopore distance \(< r^*\)).

\(d, l, D, \mu, \Delta V\) are nanopore diameter, nanopore length, particle diffusion coefficient, particle electrophoretic mobility and the applied voltage, respectively. Due to the different shapes of three kinds of nanopore, \(f_D = f_I = (50^2/4 \cdot 50) \cdot \pi \mu \Delta V = 12.5 \pi \mu \Delta V\), \(f_E = (50^2/4 \cdot 130) \cdot \pi \mu \Delta V = 4.8 \pi \mu \Delta V\). We assume here directly drilled nanopore and ion beam shrunk nanopore have the same diameter and length, so the capture rates are the same. From the results above, we can find the electron beam shrunk nanopore has a lower capture rate compared to the other two pores, which is similar to the trend of our experimental data. However, if the capture is really a diffusion limited process,
the curves in Fig. 2-22b will all be linear, which doesn’t fit all the data as well as exponential curve. But if we only revisit the green dots in Fig. 2-22b and calculate the linear fitting, the fitted line looks fine, though the R-square is only 0.93.

2.6 Summary

In conclusion, we demonstrated the first comprehensive study of blockades based on 3D pore shapes. Three nanopores of 50 nm diameter were fabricated on 50 nm thick Si₃N₄ membranes using directly focused ion beam drilling, electron beam shrinking and ion beam shrinking. By characterizing the cross section shapes of three nanopores, we find directly drilled nanopore is close to the cylinder, electron beam shrunk nanopore is much longer caused by the deposition of hydrocarbon, and ion beam shrunk nanopore becomes conical. Because of different shapes, I-V curves of three types of nanopores are obviously different. Electron beam shrunk nanopore has the smallest conductance, and the ion beam shrunk nanopore has the largest. Our simulated I-V curves agree with the experimental results. 30 nm nanoparticles were tested with three nanopores, and the electrical events from three nanopores are different, because of different nanopore geometries. We compared, simulated and analyzed the performance of translocation events. Blockade amplitude and capture rate increase, and dwell time decreases as the voltage increases. Again, our simulation gives results close to the experimental data. Our study reveals the geometry differences induced by the various fabrication methods, and shows these geometry differences could influence the single nanoparticle detection with solid state nanopore.
Chapter 3 Electrical and optical detection of single nanoparticles with nanopore-ARROWs

3.1 Advantages, development and challenges of multi-mode detection with nanopore

Though solid state nanopore is successful in detecting and distinguishing nanoparticles, it still has a long way to go before the goal of nucleic acids sequencing can be achieved. Since the sampling rate of electrical detector is limited, currently the biggest issue of solid state nanopore sequencing is that many details during the fast translocation of molecules through nanopore cannot be recorded.\cite{62}, \cite{145} At the same time, the spatial resolution of solid state nanopore is also not ideal, because the thickness of solid state nanopore is always way larger than the distance between bases. In order to slow down the translocation, a lot of methods have been proposed and tested.\cite{146}–\cite{148} Extremely thin membrane, such as graphene membrane, has also been tested to increase the spatial resolution.\cite{139}, \cite{149}–\cite{151}

While some people are trying to slow down the translocation velocity or to fabricate thinner and thinner membranes, others are also thinking whether it is feasible to sequence nucleic acids with the help of other nanoscale devices or other detection methods.
3.1.1 Embedded electrodes

Nanopore detection is based on the measurement of the ionic current change caused by the translocation of nanoparticle through the pore. This kind of measurement is limited by the structure of nanopore, i.e. diameter, thickness, and shape. If we can by some means embed some electrodes into or around the nanopore, then nanoparticles can be directly detected by the electrodes when nanoparticles go through the pore, without the limitation of nanopore structure. Also, if we combine the nanopore detection with the electrode detection, more information of nanoparticles may be revealed.

Two examples of electrodes integration into solid state nanopore are either building the solid state nanopore as a metal oxide semiconductor field effect transistor,[152], [153] or fabricating a tiny electrode gap inside or on top of the solid state nanopore to measure the tunneling current.[154]–[156]
Figure 3-1. Electrode embedded nanopores. (a) A nanopore in a capacitor membrane. Insets: Left: A TEM micrograph of the capacitor-membrane. Right: A lattice image of a nanopore sputtered into the capacitor membrane.[153] (b) A single DNA molecule is translocating through a nanopore fabricated in a SiN$_x$ membrane. Schematic drawing shows location of the nanogap electrodes made in Pt/Ti layers. 5nm and 150nm thick Al$_2$O$_3$ layers were deposited on the nanogap and the microelectrodes.[156]

Fig. 3-1a shows the structure of a solid state nanopore MOS-capacitor, which consists of a thin silicon dioxide layer sandwiched between two heavily doped electrodes: one made from polysilicon and another from crystalline silicon. The operation principle is that once a charged nanoparticle passes the nanopore, the change of the charge inside the nanopore will lead to the change of the gate voltage between the polysilicon layer
and the crystalline silicon layer. Though theoretical simulations predicted the possibility of detection at the single-base molecular level, it hasn’t been demonstrated yet.

Another popular approach is to measure the tunneling current of nanoparticles with a nanogap inside the nanopore. Fig. 3-1b is a typical structure diagram. When the nanogap is small enough, tunneling current through nanoparticles can be detected when the nanoparticle is moving through the nanopore. Theoretically, different nucleotides have different intrinsic tunneling currents, so the spatial resolution of nanopore detection is greatly improved without using very thin membrane. Though the fabrication of nanogap is still challenging, the successful experimental identification of single nucleobase molecules and oligomers lets us see the feasibility of electrical solid state nanopore sequencing.

3.1.2 Optical detection

Label-free detection is one of the original intentions of developing nanopore sequencing. However, after people see the issues of nanopore detection, more and more attention is paid to the combination of the powerful optical detection with the sensitive nanopore detection. The following table summarizes some recently published work on the electro-optical detection using solid state nanopores.
Table 3-1. A summary of recently published papers about electro-optical nanoparticle detection with solid state nanopores.[157], [88], [158]–[160]

<table>
<thead>
<tr>
<th>Year (Journal)</th>
<th>Pore Size</th>
<th>Particle</th>
<th>Particle Concentration</th>
<th>Fully Planar?</th>
<th>Simultaneous detection?</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010 (Rev. Sci. Instrum.)</td>
<td>7 nm</td>
<td>ds-DNA, DNA-protein complexes</td>
<td>0.1-0.2 nM</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2013 (Sci. Rep.)</td>
<td>1.2 × 4 µm</td>
<td>1.1 µm bead</td>
<td>NA</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2013 (ACS Nano)</td>
<td>3.8 nm</td>
<td>ds-DNA</td>
<td>10 pM</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2013 (Appl. Phys. A)</td>
<td>7 nm</td>
<td>ds-DNA</td>
<td>NA</td>
<td>No</td>
<td>Maybe?</td>
</tr>
<tr>
<td>2014 (Nanoscale)</td>
<td>5 nm</td>
<td>ds-DNA</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

As we can see from the table, though most of the platforms are able to detect DNA molecules and have the capability of simultaneous electro-optical detection, none of them is fully planar, which may lead to some problems, such as the tricky alignment, the detection of false optical signals, photobleaching, and the difficulty to add more functions.

### 3.2 Integration of nanopore on ARROWs

As discussed in Chapter 1, we have demonstrated ARROW chip is a perfect platform for fluorescence detection. Beyond that, ARROW chip is also a good choice for nanopore integration. First of all, the thick top layer is a good location for nanopore
fabrication. Nanopore needs to be fabricated into a thin membrane. The flat and wide top layer enables us to develop an etching or milling method to remove the material until a uniform and thin layer is left. Second, the ARROW chip integrates the excitation waveguide, liquid channel, and detection waveguide on a single compact chip, relieving the hassle of optical alignment. Also, the planar configuration assures that there will be no false fluorescence signal, that is to say, the particles that haven’t got into the liquid channel won’t be excited accidentally. Last but not least, nanopore’s location on the liquid channel is flexible, allowing us to fulfill different detection purposes.

3.2.1 Integration using Cr etch stop layer

![Fabrication flow diagram](image)

Figure 3-2. The fabrication flow diagram of the process of creating a micropore in a hollow ARROW waveguide.[161]

Initially, nanopore fabrication was done by adding several steps into the established
ARROW fabrication process.[161] After the first top ARROW layer was grown, an 8 μm × 8 μm, 200 nm thick chrome square was fabricated using a liftoff technique. The chrome etch stop layer kept the SiN layer below it intact during a subsequent reactive ion etching (RIE) that formed the micropore. After the remaining layers were deposited, a micropore etch mask was patterned and aligned directly over the chrome etch stop layer. After the micropore etch was finished, the chrome etch stop layer was removed using a wet chrome etchant, leaving a thin membrane between the micropore and the liquid channel. The nanopore was finally drilled with FIB, as discussed in Chapter 2.

The merit of this method is that the standardized protocol assures its reproducibility. But it lacks the flexibility. If we need to make a nanopore at a different location, we have to make a new mask, and also have to make a new wafer of ARROWS.

3.2.2 FIB fabrication

According to the experience in experiments, it is very normal to come across an optically good chip without a micropore. In this case, we can fabricate the micropore and the nanopore with FIB milling.
As is shown in Fig. 3-3, first a $2 \times 2 \mu m$ square micropore was milled into the top thick layer using 30kV, 0.3nA ion beam. After about 6 min, up to four cracks showed up suddenly on one or up to four corners of the micropore, which meant the corners of the micropore were already drilled through. As soon as the cracks were seen, the ion beam patterning must be stopped immediately, otherwise the whole membrane would be gone very soon. Next, the cracks on the corners needed to be closed by gas-assisted deposition of SiO$_2$. Since the size of the crack was uncertain, we had to deposit a $0.7 \times 0.7 \mu m$ square of SiO$_2$ using 30kV, 1.6pA ion beam for 30 s, and then checked if the crack was closed. If not, deposition had to be done again. After all the cracks were closed, a nanopore was drilled on the membrane. This method guarantees
the maximum flexibility, but doesn’t define the membrane thickness well.

### 3.3 Experimental setup

Figure 3-4. Principle of electro-optical single nanoparticle detection and two different configurations. (a) Each nanoparticle produces two characteristic signals – a transient current decrease and a fluorescence spike - separated by a characteristic time $\Delta t$. (b) Different nanopore locations on ARROWs, and the expected signals.
Fig. 3-4a illustrates the operational principle of the electro-optical single particle analysis device. Upon translocation through the nanopore into the ARROW channel, nanoparticles generate a particle-dependent, characteristic current blockade (dip in ionic current). They then pass through the optical excitation spot and generate a second specific signal - a spike in the optical signal $P$ that is collected at the chip edge with a photodetector. Ideally, both signals originate from single particles and should be highly correlated, providing both optical and electrical information.

As mentioned above, one advantage of our platform is that the nanopore location is flexible. Fig. 3-4b shows two typical nanopore positions used in this work. When the nanopore is fabricated directly on top of the waveguides intersection (Fig. 3-4b left), the fluorescent nanoparticle will be optically excited as soon as it gets into the channel through nanopore, and thus there will be no delay between electrical signal and optical signal. The nanopore could also be fabricated far away from the waveguides intersection (Fig. 3-4b right). In this case, when the particle passes the nanopore, an electrical signal will be detected. After a short while, an optical signal will be detected when the particle is moved to the optical excitation area by the flow. Correlated electro-optical detection can be done with nanopores drilled at both locations, but the latter location will enable us to extract the flow velocity in the liquid channel.
3.4 Correlated electro-optical detection of single nanoparticles

The final goal is to use our platform to realize the electro-optical detection of tiny biological particles, such as viruses, ribosomes, DNA/RNA molecules, etc. But as usual, we started prototyping with inorganic polystyrene particles, since they have accurate size, stable chemical property, and simple preparation process. Nanoparticles used in the following sections were either FluoSpheres or TetraSpeck, which contain fluorescent dyes inside for fluorescence detection.
3.4.1 Correlated electro-optical detection of 500, 200, and 100nm nanoparticles

3.4.1.1 Experimental setup

Figure 3-5. Implementation of delay-free, correlated electro-optical detection. (a) Layout of the nanopore-optofluidic chip. The nanopore is located right on top of the optical excitation area where liquid-core (horizontal) and solid-core (vertical) waveguides intersect. (b) Schematic view of the cross section of the detection area, showing a microscale opening in the waveguide cladding and a nanopore drilled through the membrane. (c) Top-down view of the detection area. (d) Tilted view of the detection area.
As mentioned above, it is flexible to choose the location to fabricate the nanopore on the ARROW chip, so we decided to drill the nanopore right on top of the intersection of solid core and liquid core waveguides for the proof of principle single nanoparticle detection. In this way, there is supposed to be no delay between the electrical signal and optical signal. Fig. 3-5 presents the experimental setup. The micropores used in the following sections were all fabricated by FIB milling as discussed in section 3.2.2. The nanopores were either directly drilled by the focused ion beam or shrunk by SiO$_2$ deposition. Reservoirs were fixed around the micropore and two ends of the liquid core waveguide. Please notice that reservoir 1 was glued on the chip using PDMS, since the lower refractive index of PDMS won’t affect the light propagation in both solid core and liquid core waveguides. Reservoirs 2 and 3 were glued using wax (Crystalbond 509-3). After the liquid core channel and the reservoir 1 were filled with buffer solution and nanoparticles, respectively, an Axopatch 200B Clamp patch amplifier was applied between reservoir 1 and 3. The amplifier supplied the voltage across the nanopore, and recorded the ionic current at the same time.

3.4.1.2 Results and discussion

Correlated electro-optical detection of single 500nm, 200nm, and 100nm nanoparticles were achieved, respectively. A nanopore with the size of 520 nm was used for measuring 500 nm nanoparticles (TetraSpeck, final concentration: $1.5 \times 10^8$ particles/mL, buffer: 0.01M KCl, 20nM BICINE, 0.01% v/v TritonX, pH 7.6). When
4V voltage was applied between reservoir 1 and 3, 500 nm nanoparticles were driven through the nanopore. When the nanoparticles got into the channel, they were immediately excited by the laser, resulting in clear fluorescence signals. Totally 113 particles were detected both electrically and optically. As can be seen in Fig. 3-6a, both the electrical signal and the optical signal are very clear. From the figure, we can even directly tell by eye that the electrical signal and the optical signal are well correlated. However, we cannot directly calculate the cross correlation function between these two signals. First, the electrical signal and the optical signal have different resolutions. The electrical signal have a 4 µs resolution, but the optical signal only has at best 0.1 ms resolution. And, the individual spikes in two signal traces have different width. Normally, an electrical blockade has a duration of about 0.1~10 ms, and each optical spike is about 100 ms wide. Thus we won’t get a good cross correlation function if we calculate directly. In order to solve this issue, we extracted the time of each peak in two signal traces, and constructed normalized artificial signal traces for cross correlation calculation. Normalized artificial signals are shown in Fig. 3-6a. The resolution of the artificial signal is 10ms, and each pulse is set to be 80ms wide. C(τ) was then calculated using the normalized signals with the crosscorr function in MATLAB.
Figure 3-6. Correlated electro-optical detection of 500nm nanoparticles. (a) Experimental and artificial electrical (black) and optical (blue) signals. Inset: Zoomed-in view of an artificial signal. (b) Cross correlation function between artificial electrical and optical signals.
Similarly, 200nm and 100nm nanoparticles were measured with 260nm and 160nm nanopores as well. 57 and 99 particles were detected electrically and optically for the 200nm and 100nm nanoparticles, respectively. The results are shown in Fig. 3-7.
Though the electrical signals are a little noisier for these two experiments, we can still get a very strong cross correlation between electrical and optical signals. And we also need to notice that for all kinds of nanoparticles, we identified the nanoparticles electrically and optically with a 100% fidelity.

Figure 3-8. (a) Scatter plot of relative conductance versus blockade duration of electrical blockades of 500nm, 200nm, and 100nm beads. (b) Histograms of fluorescence signal brightness of 500nm, 200nm, and 100nm beads.

The scatter plot of electrical signals of 500nm, 200nm, and 100nm nanobeads is shown in Fig. 3-8a. Data points of 500nm beads show a tight distribution. Data points of 200nm beads have a wider distribution of blockade duration, and 100nm beads have a wider distribution of blockade amplitude. This observation can be possibly explained by considering the nanopore size and the noise level of electrical signal. For 500nm beads, a 520nm nanopore will limit the pathway of beads strictly, leading to more uniform amplitude and duration distributions. The quiet electrical signal
indicates that there are no or few bubbles inside the nanopore,[162] so the translocation of beads is not affected. However, 260nm/160nm nanopores enable 200nm/100nm beads to go through the nanopore with different routes, leading to wider distributions of amplitude and duration. The noisy electrical signals shown in Fig. 3-7 could be an evidence of bubbles in the nanopore, which could affect the translocation.

Fig. 3-8b presents the histograms of the fluorescence signal brightness of 500nm/200nm/100nm nanobeads. Theoretically the brightness should scale quadratically (surface-labeled bead) or cubically (volume-labeled bead) with the bead size. However, though the brightness increased with the incremental bead size in our experiments, we didn’t find the cubic relationship between them. Since the data was obtained using different chips and the nanopore sizes were different, nanobeads’ locations in the channel, excitation beam intensities at the excitation spot, and chips’ throughputs could vary. This could possibly explain why the cubic relationship was not found. In addition, an overlap between histograms of 100nm/200nm or 200/500nm beads can always be found. That is to say, it is questionable whether we can tell two kinds of beads apart by using the fluorescence signals alone. In the following section, the study on the mixture of 100nm/200nm beads will be discussed.
3.4.2 Detection of 100 nm and 200 nm nanoparticle mixture

3.4.2.1 Experimental setup

Figure 3-9. Nanopore gate optofluidic device. Schematic view of intersecting solid-core (orange) and liquid-core (blue) optical waveguides on a silicon chip with particles and electrodes in reservoirs (inset: photograph of chip).

Now that we have proven it is feasible to achieve the correlated electro-optical detection of single nanoparticles with our platform, we are wondering if it is possible to distinguish nanoparticles with different sizes in a mixture. We are also curious about if the correlated electro-optical detection can still be done when the nanopore is fabricated far away from the intersection of waveguides.

Fig. 3-9 shows a schematic view of the electro-optical sensing device. This time the micropore and the nanopore were fabricated far away from the waveguides intersection. Fluid reservoirs were attached over the ends of the liquid-core channel.
and over the nanopore. Solutions containing nanoparticles were introduced into reservoir 1 and individual particles were drawn through the nanopore into the waveguide channel with an applied voltage. Once inside the channel, particles were moved toward the optical excitation spot with pressure applied between reservoirs 2 and 3. The inset to Fig. 3-9 shows a photograph of the entire ~1cm² chip.

3.4.2.2 Results and discussion

In order to demonstrate the ability of the nanopore device to act as a smart gate with optical and electrical single particle resolution, we introduced a mixture of fluorescent nanoparticles with different diameters (100 and 200nm) to a 250nm pore as shown in Fig. 3-10a. Electrical current (I(t), top) and optical fluorescence (P(t), bottom) were then recorded for an applied voltage of 3V and are displayed in Fig. 3-10b. Clear signals can be observed in both traces, and the signals are highly correlated in time. This correlation is verified by computing the cross correlation C(τ) between I(t) and P(t) (Fig. 3-10c).

For data pairs \((x_1, y_1), (x_2, y_2), \ldots, (x_n, y_n)\), an estimate of the lag \(\tau\) cross correlation is

\[
C_{xy}(\tau) = \frac{1}{n} \sum_{t=1}^{n-\tau}(x_t - \bar{x})(y_{t+\tau} - \bar{y}) \quad \tau = 0, 1, 2\ldots \tag{3.1}
\]

where \(\bar{x}\) and \(\bar{y}\) are the sample means of the series.
Figure 3-10. Gated electro-optical detection of single nanoparticles. (a) Fluorescent nanoparticles of two different diameters (100/200nm) are translocated through a 250nm nanopore. (b) Electrical blockade (top) and optical fluorescence (bottom) signals showing correlated single-particle detection events (4 examples highlighted with dashed lines). (c) Cross-correlation of electrical and optical signals showing single peak that enables accurate determination of flow velocity in the waveguide channel. The particle translocation rate was 35.6/min, but rates in excess of 1,000/sec are possible.[163]
Fig. 3-10c shows that $C(\tau)$ exhibits a single, well defined peak at $\tau=5.8s$ which corresponds to the time the particles take to travel from the nanopore to the optical excitation spot under an applied negative pressure. Since all physical dimensions are known, we can immediately extract the velocity as 270 $\mu$m/s. No spurious optical peaks without corresponding electrical current blockades were observed, confirming the absence of simultaneous translocations of multiple particles through the nanopore.

Figure 3-11. Identification of nanoparticle subpopulations. (a) Fluorescence intensity histogram. (b) Scatter plot of electrical blockades revealing the two subpopulations by current blockade depth. (c) Multiparameter analysis enabling assignment of optical properties to particle subpopulations; dashed lines: optical signal range with ambiguous particle size assignment.

Fig. 3-11a shows the distribution of the optically detected signal amplitudes. Ideally, one would expect two subpopulations corresponding to the larger/brighter and smaller/darker nanoparticles, respectively. However, this information is almost completely lost due to statistical variations of the particle brightness and the exact
location within the optical excitation volume. The electrical signal depicted in Fig. 3-11b, on the other hand, shows two well-separated subpopulations, allowing for identification of particle size by the depth of the current blockade. Due to their 1:1 correspondence, optical and electrical signals can be combined as shown in Fig. 3-11c which depicts optical brightness versus blockade amplitude for each detected particle. Now the distribution of optical signals becomes clear and the optical properties can be analyzed for each particle size. While larger particles are indeed generally brighter ($P_{\text{ave}}=591 \text{ cts/s, } \sigma=18.9 \text{ cts/s}$) compared to the smaller ones ($P_{\text{ave}}=238 \text{ cts/s, } \sigma=9.9 \text{ cts/s}$), there is a brightness region (indicated by dashed lines) in which the particle subpopulations cannot be resolved using the optical signal alone. Because single nanoparticles can be detected both electrically and optically, the nanopore gate enabled direct extraction of the flow speed as well as unambiguous particle discrimination and resolution of the optical fluorescence statistics.

3.5 Discussion

The principle of correlated electro-optical single particle detection is actually very straightforward. However, there are still several tricky things which need to be paid attention to.
Figure 3-12. Optical excitation waveguide characterization. (a) Facet of the solid-core waveguide used to couple light into the optofluidic chip; (b) Optical mode (at 632.8 nm) observed at the output end of the solid-core excitation waveguide.

We didn’t realize that the optical mode location in the solid core waveguide plays such an important role until we found all the successful correlated electro-optical detections of single nanoparticles were done with chips that have optical modes located above the theoretical position (vertically in the center of the waveguide, see Fig. 1-2). When the optical mode location in the solid-core waveguide is higher (as shown in Fig. 3-12b), it’s possible for us to achieve the correlated electro-optical detection. When the mode is at the theoretical position, we haven’t done the correlated electro-optical detection successfully. However, even if the mode location is high, it’s not guaranteed that every chip will work. We have to try all the chips one by one.
The amount of particles detected, and the quality of optical signals, don’t depend on the mode location. The nanopore itself decides how many particles can go through. Normally, nanopore will let tens of or more than 100 particles in. After that, it becomes hard to get more blockades during an experiment. Probably the pore is already clogged, or a lot of particles stick to the wall of the reservoir.

The intensity of the fluorescence signal depends on the optical throughput of the liquid-core channel. Though part of the fluorescence intensity is contributed by higher order mode in the liquid-core channel, it is hard to measure the throughput of them. Usually when the throughput of the first order mode is good, the chip has a big chance to work. However, if the first order mode in the liquid core waveguide is not very strong, sometimes the chip will also work. An easy way to test the chip is to load particles or dyes into the channel and check whether any good optical signal can be detected.
The details of the electric field distribution for a given applied voltage are always of interest. In order to study this issue, a 3D axial symmetric simulation of the field distribution around a nanopore was carried out with COMSOL. Due to the cylindrical symmetry of the problem, the calculation can be restricted to the r-z plane. Results are shown for $r \geq 0$ where $r=0$ is the central symmetry axis of the pore. The nanopore is 150nm wide and 170nm deep. The chambers on both sides of the nanopore are 4µm wide and 2µm deep. A 1V voltage is applied between the top and the bottom of this structure. The electric potential distribution is shown as surface plot (Fig. 3-13a) and the streamlines (Fig. 3-13b) stand for the electric field distribution. Fig. 3-13a shows that the potential drop occurs across the nanopore, and Fig. 3-13b shows that the
corresponding electric field is therefore concentrated within the pore.

3.6 Summary

In conclusion, we found methods to fabricate micropore and nanopore directly on ARROW chips, and we showed that nanopore-gate based single nanoparticle analysis is a new approach to the study of single nanoparticles that is more powerful than each individual technique.[164] For example, the 1:1 correspondence between electrical and optical signals proved the single particle nature of each method and allows one to rule out spurious events such as transient blockades of the nanopore or spurious optical signals. Inorganic nanobeads were identified using different combinations of optical and electrical parameters (fluorescence intensity, wavelength, current blockade depth and dwell time). Based on the successful correlated electro-optical detection of signal 500nm, 200nm, and 100nm nanoparticles, we demonstrated that this method allows for identification of a specific subpopulation from a heterogeneous mixture.
Chapter 4 Electrical and optical detection of single bio-nanoparticles with nanopore-ARROWs

In the very beginning of the thesis, we stated the ultimate goal is to use ARROW chips to detect and analyze single nanoscale biological particles. The integration of nanopore and successful correlated electro-optical detection of single inorganic nanoparticles enable us to go a step further to our final goal. In this chapter, we are going to discuss the detection and analysis of single viruses, DNA molecules, and polysomes using our nanopore-optofluidic chip.

4.1 Virus detection

4.1.1 Correlated electro-optical detection of virus

Influenza A (H1N1) virus is the subtype of influenza A virus that was the most common cause of human influenza (flu) in 2009. Until March 21, 2010, 213 countries and overseas territories/communities had reported laboratory confirmed cases of pandemic influenza H1N1 2009, including at least 16,931 deaths, according to a WHO report. After that, the large scale outbreak was controlled, and only tens of infections/deaths were still reported in 2013 and 2014.[165] However, the emergence of influenza A (H1N1) virus in 2009 highlighted the importance of having a fast test
facilities in place to react to emerging viruses.

In order to develop a compact H1N1 virus sensor and demonstrate that electro-optical single molecule analysis is applicable to biologically relevant nanoparticles, we fluorescently labeled influenza A H1N1 viruses (diameter ~104nm[166]) and introduced them to a d=157nm pore (see Fig. 4-1a). Purified deactivated Human Influenza A/PR/8/34 (H1N1) was obtained from Advanced Biotechnologies. Viral concentration was specified at $5.3 \times 10^{11}$ virus particles/mL prior to inactivation. The viral capsids were labeled using mono-reactive Cy5 dye (Amersham) according to manufacturer instructions. The labeled virus was separated from unreacted dye using a PD midiTrapTM G-25 column (GE Healthcare). The first eluted fraction (flow through) was used for subsequent testing.

Figure 4-1. Electro-optical detection of single H1N1 influenza A viruses. (a) Schematic view of 120nm virus particles and 157nm nanopore. (b) Electrical blockade (top) and optical fluorescence (bottom) signals showing correlated single-virus detection events. (c) Scatter plot of electrical signals showing narrowly distributed blockade depths and dwell times. (d) Histogram of fluorescence signal. (e)
Cross-correlation of optical and electrical virus detection signals.

Fig. 4-1b shows a subset of the electrical and optical signals obtained at an applied voltage of 4V. The apparent correlation between the signals is obvious from the raw data traces. Fig. 4-1c shows very uniform blockade depths and durations, suggesting that individual virus particles were detected during translocation. Since the nanopore limits the entry of single viruses, the histogram of fluorescence signal in Fig. 4-1d also shows a uniform distribution. The average intensity of fluorescence signals is about 29 cts/10ms. The cross correlation (Fig. 4-1e) was calculated using the method mentioned in the last chapter, and showed a single peak with almost no delay since the nanopore was placed directly on top of the optical excitation area. All viruses were detected with 100% fidelity (each current blockade has a corresponding optical peak and vice versa). This result represents the first unambiguous optical detection of single virus particles on a chip and the simultaneous characterization of their electrical blockade properties.

4.1.2 Electro-optical discrimination of virus and nanoparticle

We also introduced a mixture of almost equally sized nanoparticles (100nm, fluorescing at 515nm), and labeled virus particles (80-120nm, fluorescing at 670nm) to the same nanopore (Fig. 4-2a), in order to test our platform’s sensitivity of distinguishing similar particles.
Figure 4-2. Identification of influenza viruses within heterogeneous particle mixture. (a) Schematic view of the virus/nanoparticle mixture and nanopore; (b) electrical blockade (top) and spectrally resolved optical fluorescence (bottom) signals from viruses (red fluorescence) and nanoparticles (blue); (c) scatter plot of electrical signal suggesting nearly identical blockade depths but particle-dependent dwell times.

Here, fluorescence was routed through a dichroic mirror, then spectrally filtered for the two colors used. The electrical signal trace in Fig. 4-2b shows clear blockade events of the same magnitude as in Fig. 4-1, albeit somewhat noisier, and a detection rate of 22.4/min. The particles can be identified cleanly with the help of the optical signal which is shown for both red (virus) and blue (nanoparticles) channels. Again, a 1:1 correspondence with 100% detection fidelity is observed for a total of 144
particles. The scatter plot for the electrical signal (Fig. 4-2c) shows very uniform blockade depths which would be expected given the almost identical particle sizes, and a relatively continuous distribution of the dwell times.

As we can see from Fig. 4-2c, two subpopulations formed in the scatter plot, which probably means nanoparticles and viruses have distinct electrical signal properties. If the electrical signal is separated into two dwell time subpopulations (short and long) by the vertical dashed line, and cross-correlated with the optical channels (red and blue), the four cross correlation signals shown in Fig. 4-3 are obtained. These immediately identify all particles, showing that viruses (red) have long dwell times.

Figure 4-3. Cross-correlation of optical and electrical virus detection signals for various combinations of dwell-time/spectral subpopulations, enabling unambiguous identification and assignment of viruses to long (> 4ms) dwell times.
(>4ms) while the nanoparticles (blue) have shorter (<4ms) dwell times due to their different physical properties such as surface charge and mass. We note that this assignment is error-free as is evident from the complete absence of correlations in (red, short) and (blue, long) cases. These results show that we can both count and identify labeled pathogens unambiguously from a particle mixture using the combination of electrical and optical signal channels.

4.2 Electro-optical detection of λ-DNA

The most important and powerful application of nanopore is nucleic acids sequencing. Though direct sequencing with either biological or solid state nanopore is still challenging, it is promising to sequence the nucleic acids using nanopore with the help of optical methods. For instance, with the help of fluorescence detection, the feasibility of two color converted DNA readout based on a binary code has been demonstrated,[167] while direct DNA sequencing using solid state nanopore is still challenging. In this work, we focus on the proof of principle demonstration of DNA detection with our chip.

Here, we propose and demonstrate a nanopore-optofluidic platform, where a solid state nanopore is fabricated into an optofluidic chip, aiming at dual-modality on-chip λ-DNA detection. The analysis of electrical and optical signals provides us detailed information about the λ-DNA translocation dynamics, fluidic velocity in the liquid core channel, and precise optical excitation region in the liquid core waveguide. Especially, the correlated measurement of electrical and optical signals is promising
for the future specific detection of single particles or even on-chip electro-optical sequencing. At last, we also find that the blockade shape depends on the configuration of λ-DNA molecules, with the help of a COMSOL simulation.

4.2.1 Correlated electro-optical detection of λ-DNA

Figure 4-4. (a) Layout of the experimental setup. An Argon ion laser was used as the excitation light source (dark blue arrow). The syringe pump is connected with the optofluidic chip using a PDMS adapter[23] (b) Cross section diagram of ARROW and the detection process. (c) The diameter of the nanopore is shrunk from 80 nm (left) to 20 nm (right). The length of the scale bar is 20 nm.

Shown in Fig. 4-4a is the layout of our optofluidic chip. There is a distance between the nanopore and the intersection of waveguides, so that the velocity information can also be extracted by analysing the cross correlation function between electrical and
optical data. Fig. 4-4b presents the cross section view of the liquid core waveguide. A 2 × 2 µm opening was first milled on the top layer using focused gallium ion beam (FIB), leaving a ~170 nm thick membrane. An 80 nm nanopore was then drilled on the membrane, followed by a local SiO₂ deposition with FIB to shrink down the nanopore size to 20 nm (Fig. 4-4c). Three reservoirs were glued over the channel ends and over the nanopore for sample loading. λ-DNA was labelled before the experiment with SYBR Gold (Invitrogen) for optical excitation. After the channel was filled with 1× T50 buffer and λ-DNA solution was added into the reservoir over the nanopore, a patch clamp amplifier (Axopatch 200B) was applied to the chip as a voltage source and an amp meter. Meanwhile, a syringe pump was maintaining a continuous flow in the channel with a pump rate of 50 nL/min. As soon as the DNA molecule was electrically driven into the liquid-core waveguide through the nanopore, it would be moved by the flow to the excitation area and optically detected (Fig. 4-4b). During this process, an electrical signal was recorded by the patch clamp amplifier while the λ-DNA was passing the nanopore, and an optical signal was collected by an APD when the λ-DNA was excited.
Figure 4-5. (a) Typical electrical signal traces at 5V, 6V and 7V. (b) Scatter plot of blockades depth versus duration. (c) Blockades depth versus voltage, and the linear fitting line. (d) Capture rate versus voltage, and the linear fitting line.

In the experiment, voltages of 5V, 6V and 7V were applied between reservoirs. Since the resistances of the nanopore and the liquid core channel are dominant between reservoirs, we consider the voltage drop across the nanopore as:

\[
V_{\text{nanopore}} = V_{\text{applied}} \frac{R_{\text{nanopore}}}{R_{\text{nanopore}} + R_{\text{channel}}} \quad (4.1)
\]
$R_{\text{nanopore}}$ can be obtained from COMSOL simulation. The channel resistance can be calculated using the equation $R_{\text{channel}} = \rho L_{\text{channel}}/S_{\text{channel}}$, where $\rho$, $L_{\text{channel}}$, and $S_{\text{channel}}$ are the electrolyte resistivity, channel length and channel cross section area, respectively. By comparing the simulated resistances of a 20nm wide 170nm deep nanopore and a 2.5mm long liquid-core channel, the actual voltage drop across the nanopore was calculated to be 4.5V, 5.4V, and 6.3V. Typical electrical signal traces at each voltage are shown in Fig. 4-5a. It clearly shows the electrical signal has different blockade depths at different voltages. The scatter plot of blockade depths versus durations (Fig. 4-5b) not only confirms that observation, but also suggests the voltage is influencing the distribution of blockade durations as well. The blockade duration has a wider distribution when lower voltage is applied. A possible explanation is that the variation in $\lambda$-DNA shape creates a wide distribution in blockade durations when the applied voltage is lower, and there is a more uniformly stretched DNA population at higher voltages. The uniform distributions of blockade depths at different voltages indicate that only single $\lambda$-DNA molecules go through the nanopore. The average blockade depth increases linearly with the incremental voltage, following the linear fitting function at a rate of 540 pA/V. The $x$-intercept of 2.8 V suggests there is a diffusion limited capture region above a threshold voltage of 2.8 V. Compared with lower threshold voltages found in other work,[168], [169] the higher value in our experiment is possibly caused by the large membrane thickness, lower buffer concentration, and lower molecular concentration. We also notice a linear dependence of the capture rate on rising voltage with a rate of 0.1 $s^{-1}V^{-1}$ (Fig. 4-5d), indicating the
The capture process is governed by the thermal diffusion,[170] which is consistent with our finding from Fig. 4-5c. In a diffusion limited capture process, molecules within the capture radius migrate to the nanopore under the electrical bias. The capture radius can be calculated with \( r = R/(2\pi CD) \), where \( R \) is the capture rate, \( C \) is the concentration and \( D \) is the diffusion coefficient of molecule, respectively. Here, \( C \) is \( 9.4 \times 10^9 \) molecules/mL and \( D \) is assumed to be \( 6 \times 10^{-9} \) cm\(^2\)/s.[171] At 4.5 V, the nanopore had a capture radius of 4.9 µm, which is comparable to values found in previous work.[172]

Figure 4-6. (a) Scatter plot of blockade depth versus duration. Data were from a 20nm nanopore fabricated on another device. Voltages across the nanopore were 3.6V, 6.3V, and 9V. (b) Comparison between two devices. Presented are the plots of blockade depth versus voltage, and the linear fit lines. (c) Comparison between two devices. Presented are the plots of capture rate versus voltage, and the linear fit lines.

In order to assess the reproducibility of the integrated nanopore approach, a 20nm diameter nanopore was fabricated on a different chip and a different batch of \( \lambda \)-DNA.
molecules was analyzed. The results for single molecule detection with this nanopore are shown in the figure above. Fig. 4-6a clearly shows that the electrical signal has different blockade depths under different voltages. Again, the blockade durations are more widely spread at lower voltages, consistent with our findings and interpretations discussed above. Fig. 4-6b shows that the average blockade depth increases linearly with applied voltage, again in agreement with Fig. 4-5c. Here, we find an increase in blockade depth with a rate of 500 pA/V, which is only 8% different from the value in Fig. 4-5c. Fig. 4-6c shows that, once again, a linear dependence of the capture rate on rising voltage across the nanopore is observed, confirming the diffusion-limited nature of the translocation process. We extract a dependence of the capture rate on applied voltage of 0.093 s^{-1}V^{-1} (Fig. 4-6c), which is 7% different from the value in Fig. 4-5d. Finally, we note that the above measurement was run under different applied voltages (here: 3.6~9V, manuscript: 4.5~6.3V), suggesting the reproducibility over a wider voltage range.
Figure 4-7. (a) Typical electrical blockades (black) and optical signals (red). (b) The cross correlation function between electrical and optical signals. (c) The autocorrelation function of the optical signal (black dots), and the fitting curve (red line).

The major advantage of our nanopore-optofluidic platform is the combined electrical and optical single particle detection, which is ensured by nanopore’s gating function of limiting the single particle entry into the channel. Fig. 4-6a shows the clear synced electrical and optical signals of λ-DNA, where not only the single particle detection is
confirmed, but an obvious correlation between two traces can be seen. Again, since electrical and optical signals have different shapes and durations, the peak locations of all the electrical and optical signals were extracted for the construction of normalized artificial pulses. Computing the cross correlation between the artificial pulses gives us a strong cross correlation peak at 0.18 s, indicating λ-DNA is travelling with a velocity of 8.3 mm/s, in consideration of the 1.5 mm distance between the nanopore and waveguides intersection.

Fluorescence correlation spectroscopy analysis was also applied to the optical signal. The autocorrelation function calculated using experimental data is plotted in Fig. 4-6c. Since the background of fluorescence signal was low, background correction was unnecessary. The autocorrelation function was fitted with a model developed for ARROW.[53] The flow velocity in the channel was fitted to 9 mm/s, which is a good match with what the cross correlation calculation between two types of signals suggests, confirming the feasibility of both analysis methods.
**4.2.2 Determination of optical mode location**

Figure 4-8. (a) Blue lines present the trajectories of 100 particles in the liquid core channel. The dark red cylinder is a diagram of laser beam. (b) Top: A cross section view of the particle distribution. Bottom: The optical mode in the solid-core waveguide. The dotted lines show the location of the center of the optical mode. The length of the scale bar is 2 µm.

Besides the information of corresponding electrical signal and flow velocity, optical signal also carries the information of nanoparticle location in the optical excitation area. After particles are loaded into the channel, where they are interacting with the exciting light is of great interest and importance, since it is potentially helpful for developing more efficient optical detection. The optical mode location can always be acquired by taking a mode image at the end of output solid core waveguide; however,
where the optical mode locates inside the liquid core channel cannot be acquired directly without destroying the chip. Here we propose a method to determine the optical mode location by comparing the simulated and experimental optical signal intensity distributions from λ-DNA.

In order to simulate the nanoparticle distribution in the ARROW, a 3D model consisting of a liquid core channel and a nanopore inlet was built with COMSOL Multiphysics (Find details in appendix.). Laminar flow in the channel was simulated based on the Navier-Stokes flow. 100 solid particles were introduced through the nanopore inlet. Particle trajectories were simulated by using time-dependent solver in COMSOL Particle Tracing Module (Fig. 4-7a). The positions of the nanoparticles at the excitation area are obtained from the simulations and imported in Matlab. This distribution across the 5 × 12 µm liquid core is shown in Fig. 4-7b (top) and shows a clear concentration of nanoparticles near the top of the channel due to the entrance pathway via the nanopore.

The mode location of the excitation light shown in Fig. 4-7b (bottom) was acquired by taking a mode image at the end of output solid-core waveguide. The red dotted line in both top and bottom figures present the center of the optical mode measured from the mode image. However, as we stated, where the optical mode locates inside the liquid-core channel cannot be acquired directly without destroying the chip.

Now the question is how we can confirm the mode location with simulated nanoparticle distribution. As we can see from Fig. 4-7b, when the nanoparticles’
distribution is fixed, if the optical mode (red dotted line) moves up or down, we will get different optical signal intensity distributions. Fig. 4-8a shows the intensity distribution of optical signals obtained from the experiment. A Poisson distribution fitting reveals that the experimental average intensity is 2.7. Theoretically, we can get a pretty accurate mode location by calculating the signal intensity distributions when the mode locates at different heights until a good match is found between the simulated and experimental intensity distributions.

Optical excitation and collection mode profiles were combined to obtain the signal intensity of each nanoparticle, depending on its position. At last, histogram plot was created based on the intensity statistics. Shown in Fig. 4-8, as the distance of optical mode above the channel bottom is increased from 3.1 µm to 3.9 µm, simulated intensity distribution gets a rising expected value. A good match between experimental and simulated intensity distributions won’t be found until the optical mode is 3.6 µm high (Fig. 4-8g).
Figure 4-9. Comparison between experimental and simulated intensity distributions. (a) Experimental intensity distribution and the Poisson fit curve. (b-j) Simulated intensity distributions with optical modes at different vertical locations (3.1 – 3.9 µm above the bottom of the liquid core channel), and the Poisson fit curves.

The analysis above shows that single particle analysis is able to provide meaningful statistical analysis tools beyond bulk averages to analyze the individual electrical and optical signals, or, alternatively, the optical (mode position) or fluidic (flow profile) device properties.

4.2.3 Effects of nanopore shape on detection of λ-DNA configuration

Figure 4-10. (a) The cross section image of a nanopore shrunk by SiO2 deposition. (b) A simulated blockade. Inset: the structures of nanopore and particle. (c) An experimentally observed blockade.

It can be seen from Fig. 4-9 that a thin layer of deposited SiO2 is protruding from the top of the initial nanopore, and form a pointed end. In order to simulate what kind of
electrical blockade we can get from a nanopore with such a cross section shape, a structure with similar shape was modelled in COMSOL (inset in Fig. 4-9b). The minimum diameter of the structure is 20 nm, and the nanopore is 250 nm deep. The blue particle formed by two connected cylinders (18nm wide 100nm long, and 9nm wide 100nm long. These values were chosen with the considerations: a) try to make the volume of this artificial particle close to that of λ-DNA and b) try to make the diameter of the thick part close to the thinnest aperture) to imitate a highly folded DNA molecule. The radius of the thicker cylinder is twice as large as that of the thinner cylinder (R₂ = 2R₁). After moving the particle through the nanopore and calculating the ionic current at each point, we got a step-like blockade (Fig. 4-9b). We also found that the ratio between the amplitudes of two steps equals to the ratio of the cross section areas of two cylinders (I₂ / I₁ = πR₂² / πR₁² = 4). However, when the same particle was moved through a regular cylinder, step-like blockades are not observed, indicating that the fine structure was not detected. The cross section analysis and the simulation suggest that the nanopore sensitivity can be enhanced by using a nanopore shrunk by SiO₂ deposition due to the introduction of a limiting aperture that is very sharply defined in the vertical direction. The simulation matches well with some experimentally observed blockades, as seen in Fig. 4-9c. Because I₂ / I₁ ≈ 4, based on the above discussion, we can deduce it could be caused by a folded λ-DNA molecule of which the thicker part is twice as thick as the thinner part. Note that the thick end always translocate first, which is possibly because of the higher charge in the thick end.
The above findings are potentially important and useful in different applications. According to the simulation, because the nanopore fabricated on ARROW is always thick (100~200nm thick), it won’t be sensitive enough to detect small changes in particle configuration. However, this problem can be solved by using deposition shrinking method, which can create a small and short nanopore. The simulation also provides us a way to estimate the relative cross section areas of particles by comparing the step heights of blockades.

4.3 Electrical detection of polysome

Besides nanoparticles, viruses, and DNA molecules, we also want to use our chip to electrically and optically measure some biological particles with complex structures. Ribosomes link amino acids together in the order specified by messenger RNA (mRNA) molecules to synthesize proteins. Several ribosomes may translate a single mRNA molecule at the same time, forming a polysome chain. Polysomes may appear as clusters, linear polysomes, or circular rosettes on microscopy, but mainly circular in vivo.[173]–[175] The spatial arrangement of individual ribosomes in the context of polysomes during active protein synthesis is of great interest. Though it is challenging, several methods have been applied for the study on this subject, such as cryoelectron tomography or ribosome profiling.[176]–[178] Since our nanopore-optofluidic platform is sensitive enough to identify and distinguish single nanoparticles, we expect that our platform can be used for counting the number of ribosomes on a polysome chain, or measuring the spacing between ribosomes.
In this section, the ARROW chip is used for the measurements of polysomes. We are going to show that we can distinguish monosomes, disomes, and trisomes by analyzing electrical signals. And we possibly observed signals caused by 1ps to 7ps, as well. Since the structures of polysomes are complicated and still not clear to us, in this work, only electrical measurements are presented. More research is still under way.
Figure 4-12. The result of the polysome gel analysis (courtesy: Dr. Harry F. Noller).

1PS: Fairly pure monosomes; 2PS: Almost equal amounts of mono- and disomes, with smaller amounts of tri- and tetrasomes; 3PS: Almost equal amounts of disomes and trisomes, and nearly as much monosomes, and some tetrasomes; 4PS: Nearly equal amounts of tri- and tetrasomes, with lesser amounts of mono- and disomes and some pentasomes. 5PS: Nearly equal amounts of mono-, di-, tri-, with less tetra and even less pentasomes.

The polysomes used in our measurements were provided by Dr. Harry F. Noller, and they were stored in a ribosome buffer: 100 mM NH₄Cl, 10 mM MgCl₂, 0.01% Nikkol, 6 mM β-mercaptoethanol, 30 mM Tris–Cl, pH 8.3. As is shown in Fig. 4-10b, polysomes used in the measurements were not monodisperse species. This may be due to (1) limited resolution of preparative ultracentrifugation, (2) dissociation of ribosomes from mRNA or (3) cleavage of mRNA by ribonucleases.
4.3.1 Electrical discrimination of monosome, disome and trisome

Figure 4-13. (a) Scatter plot of blockade amplitude versus duration of electrical signals got from monosome, disome and trisome measurements. (b)-(d) Blockade amplitude histograms of monosome, disome, and trisome.

Impure polysome samples directly lead to one major issue: different kinds of blockades will show up in the electrical signals. Three different chips were used for monosome, disome and trisome measurements, but the nanopore sizes were very similar. The average nanopore size was about 40nm. Fig. 4-12a shows the scatter plot of blockade amplitude versus duration of electrical signals got from monosome,
disome and trisome measurements. As can be seen from the figure, the blockade amplitudes from all the measurements have a wide distribution, which means the blockades are caused by polysomes with different sizes or configurations. The histograms of blockade amplitude for each measurement gives us a better idea about the diversity of each sample. Since we got a large number of blockades from monosome and disome measurements, the histograms of them look clearer. In the histogram of monosome signals, there is a strong peak around 70pA. There are also some signals between 100 and 200pA, but the number is small. Only a few blockades with over 200pA amplitude can also be found. As for disome, two major distributions can be seen clearly around 76pA and 180pA. Similar to the monosome measurement, some blockades with bigger amplitude can be found, but not many. At this point, we can find the amplitude histograms of monosome and disome match with the gel analysis quite well. In both the gel analysis and the histogram analysis, one and two major peaks are found in 1PS and 2PS samples. The bigger blockades could be from bigger polysomes. And we can also conclude that the blockade amplitudes of monosome and disome are around 70pA and 180pA, respectively.

In the trisome measurement, we didn’t get as many blockades as we did in monosome and disome measurements. It can be explained that trisome molecules have larger sizes when they are folded, which make it hard for them to go through the nanopore. A histogram analysis to the trisome blockades is shown in Fig. 4-12d. Though it is not very clear, we can still see that there is one peak around 77pA, and there is possibly a peak around 168pA. But different from monosome and disome measurements, we got
lots of blockades with higher amplitude. Again, the bigger blockades could be caused by larger polysome molecules. The structure of polysome is still not clear to us, so further research is needed to perfectly explain the histogram.

4.3.2 Analysis of step-like blockades

Blockades obtained from trisome measurement have bigger amplitudes and longer durations, but the most interesting thing is some blockades are step-like, which was not found in either monosome or disome measurement. In 4.2.3, we discussed that the height of the step in a blockade is proportional to the cross section area of the particle. So these step-like blockades are possibly caused by some folded polysomes. Several typical step-like blockades are shown in the figure below.
Figure 4-14. Step-like blockades found in the trisome measurement. They are possibly caused by (a) trisome, pentasome, (b) tetrasome, (c) hexasome, and (d)
heptasome.

Before analyzing the steps, we can see there is a spike at the very beginning of some step-like blockades (Fig. 4-13b and c). The spike can’t be found in normal blockades (no steps). A possible explanation is that all of these step-like blockades are generated by polysomes consisting of at least 3 ribosomes, and the average diameter of the nanopore is only about 40nm, so the big polysome molecule can’t go through smoothly. When the molecule meets the pore, the pore is clogged for a very short period (generating a spike). And then, under the driving electric force, the molecule quickly changes its configuration and thread itself through the nanopore. Similar observations have been reported before on hairpin DNA molecules.[180], [181]

The easiest case for the analysis of step-like blockade is shown in Fig. 4-13a. Here we have two side by side blockades for comparison. According to the simulation result, the ratio between step heights is proportional to the ratio of the cross section areas of the particle. In our case, we assume the cross section area is proportional to the number of ribosomes. In the histograms shown in last section, monosome’s amplitude should be less than 100pA, but the 100pA amplitude is possible to show up for a larger single ribosome. Higher step’s 200pA amplitude is caused by two ribosomes, according to the histogram. Though we don’t have a clear peak for trisome in the histogram, we believe the 310pA is caused by 3 ribosomes. Now we can get an idea about what kind of polysome we are seeing here. Apparently, the left one is from a pentasome, and the right one is from a trisome. Similarly, based on conjecture, the
step-like blockades in Fig. 4-13b, c, and d are possibly from tetrasome, hexasome, and heptasome.

Please notice, step-like blockades have very different durations. When a very large particle with uncertain configuration goes through the nanopore, the duration can be affected by too many factors. So the analysis of dwell time is ignored for now.

Hereby, we demonstrate that our platform has the ability to recognize and distinguish a large number of monosomes and trisomes in a mixture of polysomes. Our platform can also monitor the configurations of 1ps to 7ps, with the help of deposition shrunk nanopore.

4.4 Summary

In conclusion, in this chapter, we show that our nanopore-optofluidic chip is capable to electro-optically detect single nanoscale biological particles. Correlated electro-opto detection of single viruses and λ-DNA molecules are demonstrated. Beyond the detection, we also show that single viruses can be distinguished from a mixture of viruses and nanoparticles with similar size, with the help of both characteristic electrical blockades and fluorescence signals detected at specific wavelengths. The optical signals of λ-DNA proves that it can help determine the optical mode location in the liquid channel. By analyzing the blockades got from λ-DNA measurement, we find the deposition shrunk nanopore are potentially useful for the detection of fine structures of nanoparticles. With the help of deposition shrunk nanopore, we successfully observed the blockades of 1PS to 7PS electrically.
Chapter 5 Summary and outlook

5.1 Summary

In last four chapters, we have shown that an extremely sensitive electro-opto-fluidic sensor has been developed. Based on our rich experience in design, fabrication, and characterization of optofluidic devices, and proven on-chip fluorescence single molecule detection technique, we creatively integrated another nanoscale sensor, solid-state nanopore, into our platform. The sophisticated combination of the advanced nanotechnology and reliable optical detection method empowered our nanopore-optofluidic platform to detect nanoparticles electrically and optically on the single molecule level. Before the integration of solid-state nanopore into our ARROW chip, we did thorough studies on the fabrication methods of micropore and nanopore. The micropore can be fabricated by dry etching with a Cr stop layer, or by focused ion beam (FIB) milling. The method of dry etching can be used for mass production, but we found the FIB milling is more flexible. We fabricated nanopores using the methods of direct FIB drilling (DD), electron beam shrinking (EBS), ion beam shrinking (IBS), or SiO₂ deposition shrinking. Systematic comparison between 50nm wide and 50nm deep nanopores manufactured by DD, EBS, and IBS were done by comparing the cross section shapes, elemental compositions, and their different behaviors when being used for single nanoparticle detection. Our study, for the first time, directly revealed the shape of nanopores, and provided evidences for the
prediction of the blockade amplitude and dwell time, as well. After that, we integrated micropore and nanopore into our ARROW chips, and successfully demonstrated the correlated electro-optical detection of single nanoparticles (500nm, 200nm, and 100nm), single viruses, and single λ-DNA molecules. Especially, we proved the extremely high sensitivity of our platform by distinguishing and identifying specific particles in a mixed solution of similarly sized particles. Moreover, electrical and optical signals provided even more useful information. By analyzing the electrical signals, we extracted the flow velocity in the channel. Analysis to the electrical signal also helped us find the deposition shrinking nanopore was potential to detect particle sub-structures because of its shape. Meanwhile, besides providing identities of specifically colorized particles, a fluorescence spectroscopy analysis to the optical signal could also show us the velocity information. In addition, it was found the optical signal can help us determine the location of optical mode inside the channel, which couldn’t be easily accessed without physically destroying the chip.

In summary, we developed and demonstrated a novel electro-opto-fluidic sensing platform for single nanoparticle analysis and detection, which could be immediately applied to the research in labs, and is promising to be improved and further developed for clinical applications.

**5.2 Outlook**

This work is just a start of the broad applications of our electro-opto-fluidic platform. There are a lot of things to improve, and there are a lot of potential applications
waiting to be explored.

In order to measure even smaller particles or study the fine structures of some biological particles, we need nanopores to be more sensitive, which means we need to make smaller and thinner nanopores on the ARROW chips. Milling the micropore with FIB is flexible, but it’s tricky to make sure the membrane left is thin enough. Maybe we should revisit the method of dry etching with a stop layer. The attempt to use the photoresist as a stop layer sounds promising, if the Cr layer can cause the issue of low optical transmission.

As stated in Chapter 3 and 4, we can get correlated detection only when the optical mode has a higher location, and that’s because the optical mode is closer to the ceiling of the liquid core channel. Maybe we can try to make the liquid core channel lower, thus the optical mode is automatically closer to the top of the liquid core channel.

We have been using the ionic current change to detect nanoparticles. Since we have the ability to fabricate nanoscale structures with the DualBeam, it is a possible way to increase the sensitivity by making nano-electrodes around the nanopore, and use the tunneling current to detect particles.

Besides improving the sensitivity, we also need to apply this platform to more applications. We have shown that the nanoparticles can be moved, trapped, or sorted inside the ARROW chip before, [37], [182] but it is tricky to manipulate a single nanoparticle, simply because it is not trivial to control the amount of nanoparticles inside the channel. Nanopore is born to be a gating device, which can limit the entry
of nanoparticles into the channel. By monitoring and controlling the voltage across the nanopore, we can definitely precisely introduce a certain number of nanoparticles into the channel.

Moreover, a lot of functional devices have been developed based on ARROWs, such as optofluidic filters, PDMS based hybrid optofluidic devices, and programmable devices.[23], [24], [36] It can be anticipated that in the near future the combination of nanopore and these functional devices will bring more exciting breakthroughs.
Appendix

A. Setup for electro-optical measurement

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
<th>Ref.</th>
</tr>
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<tr>
<td>HeNe Laser</td>
<td>Melles Griot</td>
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<tr>
<td>Argon Laser</td>
<td>Spectra-Physics</td>
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<td>3</td>
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<tr>
<td>Item</td>
<td>Details</td>
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<td>xyz stage</td>
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<tr>
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</tr>
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<td>16</td>
</tr>
<tr>
<td>Homemade shutter</td>
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</table>
Basically, the single molecule setup is a bigger Faraday cage, so what we need to do is just to move the amplifier headstage into the single molecule setup. The amplifier headstage need to be fixed on the stage circled in the figure. Besides, we also need to remove the microscope and cover the whole setup with a metal lid. Be sure that the metal lid is also grounded.
B. COMSOL simulation of nanopore

The best suggestion for anyone want to use COMSOL is “READ THE MANUAL”. Though the following details will help you get some results, you have to read some textbooks and papers to understand the theory, and carefully read the manual of COMSOL.

The simulations in this work were done using COMSOL Multiphysics 3.5. A lot of changes were made in newer versions of COMSOL, including interface, settings, etc. However, since equations are still the same, and the solver in COMSOL shouldn’t be changed a lot, I think the settings I used in COMSOL 3.5 can be easily adapted for newer versions (COMSOL 4.3 or later).

1. Set the space dimension to “Axial symmetry (2D)”, and add all the equations we need.
We need “Incompressible Navier-Stokes”, “Electrostatics”, and “Nernst-Planck without Electroneutrality”.
2. In the Draw Mode, layout the structure for simulation.

As you can see, we actually draw three rectangles here. Two big ones stand for the cis and trans chambers, and the tiny one in the middle stand for a cylindrical nanopore.

After that, select all the three rectangles, and click to merge them into one structure.

3. Set up the constants which will be used in the equations later. Double click on the “constant”, and enter all the names, values, and units in the pop up window.
4. Now, we are going to set up the details of each equation. First, let look at the Incompressible Navier-Stokes equation. Double click on the “Subdomain Settings”, and a window will pop up. There are three subdomains, which are actually the three rectangles. Since those three rectangles are in same structure, the settings for all the subdomains should be the same. Enter the values/expressions as is shown below.

The settings for Stabilization, Init, and Element are shown below.
5. After subdomain settings, we need to enter the boundary conditions. We have 12 boundaries.
All the boundaries along the red line should be set as “Axial symmetry”.
The inlet and outlet boundaries should be set as shown in the two figures above.
Two little boundaries connecting the rectangles should be “Continuity”.
Except the boundaries mentioned above, all other boundaries should be “Wall - no slip”.

6. Do the similar settings to other two equations. Every detail is going to be shown in the following figures. As for the boundary conditions, I’ll skip “Axial symmetry” and “Continuity” boundaries, because they are so obvious.

“Electrostatics”
Subdomain Settings - Electrostatics (es)

Equation

\(-\nabla \varepsilon_0 \mathbf{E} \cdot \nabla V = p\)

Subdomain selection

1
2
3

Initial value

\(V(0)\) 0 V Electric potential

Select by group

Active in this domain

Material properties and sources

Library material: \(\varepsilon\) Load...

Constitutive relation

- \(\mathbf{D} = \varepsilon_0 \mathbf{E}\)
- \(\mathbf{D} = \varepsilon_0 \mathbf{E} + \mathbf{P}\)
- \(\mathbf{D} = \varepsilon_0 \mathbf{E} + \mathbf{P} + \mathbf{P}_{\text{therm}}\)

Quantity Value/Expression Unit Description

\(\mathbf{P}\) \(\mathbf{P}(x,y,z)\) \(\text{mF/m}^2\) Space charge density

\(\mathbf{P}_{\text{therm}}\) \(\mathbf{P}_{\text{therm}}(x,y,z)\) \(\text{mF/m}^2\) Relative permittivity

Relative permittivity

Select by group

Active in this domain

OK Cancel Apply Help
Subdomain Settings - Electrostatics (es)

Equation

\[- \nabla \varepsilon \cdot \varepsilon_0 \nabla V = p\]

Subdomain selection

1
2
3

Group:

- Select by group
- Active in this domain

Element settings

Predefined elements: **Lagrange - Quadratic**

- shape: shlag(2, V)
- gorder: 4
- corder: 2

Boundary Settings - Electrostatics (es)

Equation

V = V_0

Boundary selection

Boundary sources and constraints

Boundary condition: Electric potential

- Quantity: Value/Expression
- Unit: V
- Description: Electric potential

Conditions

Weak Constr.

Color/Style

Boundary condition: Electric potential

- Quantity: Value/Expression
- Unit: V
- Description: Electric potential
“Nernst-Planck without Electroneutrality”
Subdomain Settings - Nernst-Planck without Electroneutrality (check)

Equation:
\[ \nabla \cdot (D \nabla c_2 - u c_2 \nabla V) = \nabla \cdot (\mu \nabla c_2) \]

Species 1
- Library material: ...
- Value/Expression: ...
- Unit: ...
- Description: Diffusion coefficient

Species 2
- Library material: ...
- Value/Expression: ...
- Unit: ...
- Description: Diffusion coefficient

Group: ...
- Select by group
- Active in this domain

Artificial Diffusion...
7. Before running the simulation, we need to set the mesh parameters. For most boundaries, we don’t need to care about the mesh parameters, and COMSOL will take care of it. But we need to set the mesh parameter of the following boundary, because we need the calculation around this area to be more accurate. In the figure, I use 1nm for the mesh size. After entering 1nm, just click “apply” and “remesh”.
8. Click to set the solver parameters. If you just want to simulate the ionic current under a certain voltage, use the stationary solver, as is shown below.
If you want to scan a range of voltages, use the parametric solver, as below.
9. Click 📊 to activate the Solver Manager. You can directly use the following settings to run the simulation. But other setting may be very important or useful for your own simulations, so again, READ THE MANUAL.
The Solve For tab will tell COMSOL which values will be solved. Sometimes, if you select the pressure $p$, maybe the simulation won’t converge, so just uncheck it.
Now, you can click “Solve”. After the simulation is done, you need to calculate the ionic current. Just click “Postprocessing” and “Boundary Integration”. Enter the equations and values as shown in the figure below. Click “Plot” to generate a I-V curve, and click “Apply” to see the value under certain voltage.
10. If you want to simulate a blockade, you need to draw a particle in the model.

Click 🖼️ to enter the Draw Mode. Then draw a particle.
11. Select all the structures in the model, and click . Then the model should look like the following figure.

12. Make sure that all the boundary conditions are still correct after you insert a particle into your model. And other settings are the same.

13. In order to get a simulated blockade, you need to repeat step 10-12 and change the location of the particle along the z axis in each simulation.
C. COMSOL simulation of particle distribution

In chapter 4, we simulated the particle distribution in the liquid core channel, and used that information to confirm the optical mode location. The model was built in COMSOL, and some details are listed below.

Two modules were used here: Laminar Flow and Particle Tracing for Fluid Flow.

- **Laminar Flow (spf)**
  - Fluid Properties 1
  - Wall 1
  - Initial Values 1
  - Inlet 1
  - Inlet 2
  - Outlet 1
  - Equation View

- **Particle Tracing for Fluid Flow (fpt)**
  - Wall 1
  - Particle Properties 1
  - Drag Force 1
  - Inlet 1
  - Outlet 1
  - Force 1
  - Equation View

A section of liquid core channel was built in the software, and the dimension is listed below.
In order to simulate the movement of particles in the channel, an artificial inlet was built on the channel.
After the model was built, we set the boundary conditions as follows:
In order to run the particle tracing module successfully, the Laminar Flow module needs to run first. After that, run the Particle Tracing module separately, using the flow information obtained from the first run. More details can be found in COMSOL’s manual. COMSOL provides enough examples and details in the manual, and we used the exact procedure in our simulation.
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