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UNIVERSITY OF CALIFORNIA
SANTA CRUZ

**NANOPORE OPTOFLUIDIC DEVICES FOR SINGLE
MOLECULE ANALYSIS AND MANIPULATION**

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

DOCTOR OF PHILOSOPHY

in

ELECTRICAL ENGINEERING

by

**Md. Mahmudur
Rahman**

December 2019

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Abstract

Md. Mahmudur Rahman

Nanopore optofluidic devices for single molecule analysis and manipulation

Integrated optofluidics are one of the prominent choices for particle detection and manipulation which fuses optics and microfluidics in a single platform that enables sufficient sensitivity to probe individual particle, and especially single biomolecules. Among different optofluidic devices, anti-resonant reflective optical waveguides (ARROWs) form the basis of one of the most sensitive biosensors, also allowing integration of electrical single molecule sensing via solid state nanopores. Nanopores, which are small openings in a thin insulating membrane, are a fast-growing tool for label free electrical detection of single particles. Though young but nanopores have already proven their capabilities in sensing a variety of biomolecules with the greatest attention in nucleic acid sequencing which is being commercialized and used around the world. An innovative integration of two extremely powerful technologies like optofluidics and nanopores in a single ARROW platform enables particles to be probed with dual modalities, i.e. both optically and electrically. As the ARROW devices are primarily used for biosensing with facilities of further manipulation and trapping, there is huge room to add multiple functionalities with integrated nanopores including the use of nanopore as a smart gate to controllably deliver particles towards the optofluidic region with great precision. This work deals with the incorporation of nanopores with

optofluidic devices to achieve new functionalities in the area of (chip-based) single molecule analysis. A first major breakthrough is the development and implementation of a feedback control system with the nanopore optofluidic device which is capable of detecting particle deliveries in real time and making further decision based on user's instruction. With the feedback control nanopore gating, it is possible to turn off the electrical voltage across the nanopore after a single particle insertion which ensures isolation of a single particle and delivering that single particle into the fluidic micro-channel. This functionality is demonstrated by delivering single 70S ribosomes and DNA molecules into the optofluidic channel through feedback control nanopore gating. The feedback system is versatile for a wide range of biomolecules which have been justified by gating a variety of biomolecules including ribosomes, proteins, nucleic acids and NaCMC molecules. The feedback control gating offers reconfigurable settings thus, it is possible to adjust the gating functionalities based on user's/experimental necessity. With the reconfigurable settings, deliberate delivery of two and three 70S ribosomes are demonstrated which can be set to any number if desired. Furthermore, automated delivery of 70S ribosomes and λ -DNAs is demonstrated with rates of several hundreds/min, which can be further boosted to near kHz range, illustrating the power and efficacy of the system for high throughput particle delivery and analysis. The feedback system is capable of analyzing translocation details (depth and duration) in real time and based on that it is possible to gate selective particles. This functionality has been demonstrated by selectively gating λ -DNAs from a mixture of 70S ribosomes, opening the door to selecting specific molecules for further

study and producing purified subpopulations of particles when coupled with a microfluidic sorting system. The gated particles can be subjected to further analysis such as fluorescence detection and trapping for prolonged analysis. Fluorescence detection of voltage gated λ -DNAs are demonstrated which illustrates the feasibility of integration of feedback system with existing technologies. Furthermore, a sophisticated integration of the feedback system is shown with on chip anti-Brownian electrokinetic (ABEL) trapping. This functionality has been demonstrated by feedback gating and subsequent ABEL trapping a microbead.

ABEL trapping relies on fluorescence particle tracking and provides electrokinetic feedback force to adjust particle movement which is one of the supreme methods of particle trapping due to inherent advantages over optical trapping and other methods. In another part of the work, a novel ABEL trapping platform is developed which is capable of trapping particles in two dimensions (2D, full in-plane confinement) with better trap stiffness than previous 1D implementations. The trapping methodology, particle tracking algorithm is developed which is demonstrated by 2D trapping a microbead with 14x enhanced trapping stiffness compared to the old 1D ABEL trapping.

In the final part of the work, a novel and elegant method for dramatically increasing nanopore capture rates (event frequency) is demonstrated. Although the nanopore is a great tool for electrical detection of particles, most nanopore applications are limited due to the delivery of an insufficient number of analytes close enough to the pore to enable electrophoretic capture and detection. This severely limits the throughput (and

extends the analysis time) and the limit of detection of the assay. An elegant solution to overcome the limitation is demonstrated which relies on preconcentration of targets on a micro-scale carrier bead followed by optical trapping the carrier beads at the vicinity of pore and thermally releasing them close to nanopore thus, increasing local analyte concentration. The practicality and efficacy of the methodology is experimentally demonstrated with ~80x enhanced capture rates by detecting DNAs corresponding to a melanoma cancer gene. As the method relies on accumulating targets and releasing them close to nanopore it should, in principle, be possible to detect targets at low concentrations. This functionality is demonstrated by Zika ns1 detection down to 2ng/mL which is a clinically relevant concentration. This demonstration illustrates the practicality and promising potential of the methodology which can be further developed towards diagnostics and possibly early stage disease detection.

**To my parents Md. Hafizar Rahman and Mst. Mahfuja Begum as well as my
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Chapter 1 Introduction

This chapter starts with a brief introduction to optofluidics and gradually proceeds towards a brief description of our anti-resonant reflecting optical waveguides (ARROWs) platform with their principle of operation and fabrication details. Next, a brief discussion about particle detection and manipulation on ARROW device is presented, including nanopore based particle detection.

1.1 Optofluidics

Optics is one of the major fields of research and popularly used for particle detection and manipulation due to its proven reliability, sensitivity and other numerous advantages [1]–[3]. With numerous existing application such as imaging, microscopy, spectroscopy etc. several optical methods are being developed around the world. Several breakthroughs have been pioneered based on optical methods, and the Nobel prize has been awarded twice (2014 in Chemistry and 2018 in Physics) for the scientific advancement focused on optical methods [4] which illustrates the importance of this field. One of the most interesting and exciting applications of optics is probing and analysis of biomolecules [5]–[7]. As biomolecules usually reside in a fluidic environment, it is necessary to manipulate them in their native fluidic system without compromising their function. Microfluidics is one of the promising systems that deals with fluid handling on the micro-scale and an ideal tool for biological research [8]–[10]. Numerous applications of microfluidics have been reported, including micropumps and microfilters [11], [12], transport and mixing [13], [14], flow cytometry [15], [16] etc. With the emergence of nanotechnology, small compact

devices are being fabricated to perform multiple integrated small-scale laboratory processes in a single device/platform called lab-on-a-chip (LOC) [17]–[19]. Different powerful techniques are now being integrated to create multi-functional platforms. One prime example of such LOC is optofluidics which is a combination of optics and microfluidics that enables light to interact with liquid on the micron-scale with high sensitivity [20]–[23]. As two individually powerful and versatile techniques are merged together, optofluidics has ushered in a new era of research with significant impactful applications such as tunable dye lasers [24], [25], tunable fluidic lens [26], [27], optical switches [28], [29], interferometer [30], ring resonator [31], [32], filters [33], [34] etc. One remarkable feature of an optofluidic system is that it facilitates even more technologies to be integrated with it to form a hybrid integrated platform which is capable of performing complex tasks within a chip such as mixing, filtering [35]–[37], particle trapping [38]–[40], automated fluid handling [41] etc. Apart from other applications, the most exciting application of optofluidics is detection, analysis and manipulation of biomolecules in the field of life sciences. Microfluidic systems provide the native fluidic environment for biomolecules and optics provides non-invasive and highly precise analysis methods. The combination of both provides an ideal platform for bioanalysis.

1.2 Principle of ARROWs

As the concept of optofluidics relies on the interaction of light with liquid, it is essential to develop a structure (preferably a fluidic channel) that confines both light and fluid

in the same structure. However, the fluids (water, buffers) usually have lower refractive index than that of the usual materials (standard Si based materials) used to fabricate the

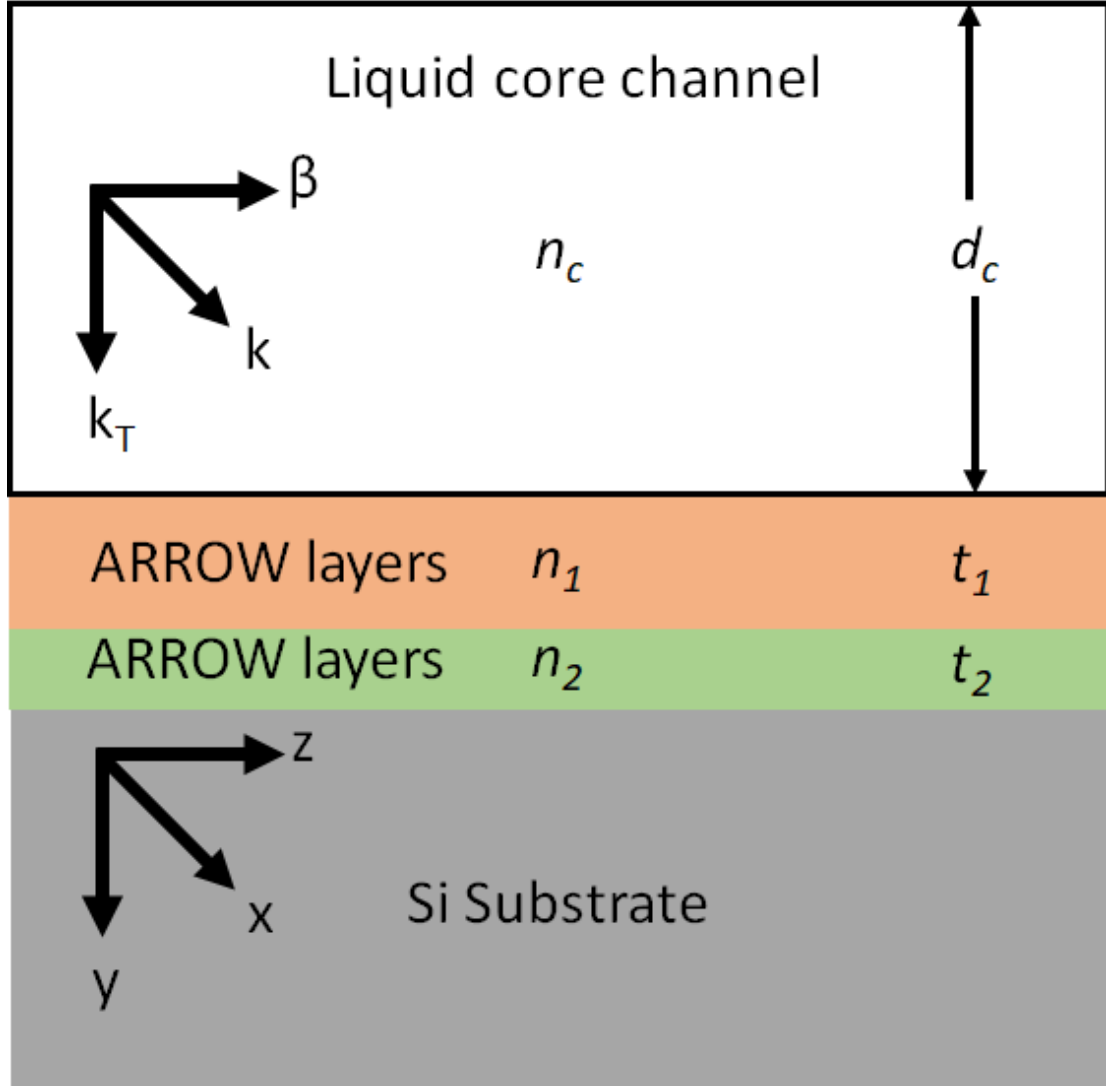


Fig. 1.1 Schematic representation of usual ARROW structure with wave vectors.

channels. This creates a problem as light propagation is not possible within the liquid channel based on conventional total internal reflection methodology. Researchers have developed several methods to overcome the issue to confine both lights and fluidics in the same structure such as slot waveguides, Teflon AF waveguides etc. [42]. However,

in our devices, a more effective method based on periodic structure is implemented to create a Fabry-Perot etalon that allows light to propagate along the liquid channel which is discussed in detail in the following section. Fig. 1.1 shows a generic ARROW structure where the hollow channel/waveguide is surrounded with periodic layers. In our device, the periodic layers are formed with SiO₂ and TaO₂. The light propagates along z direction with a propagation constant β inside the liquid core (LC) channel which has a thickness of d_c and refractive index of n_c . The cladding layers form a Fabry-Perot etalon in y direction with thickness t_1/t_2 and refractive index n_1/n_2 with a wave vector k_T . Based on the refractive indices, the thickness of the cladding layer is carefully designed to meet the anti-resonant condition. At the anti-resonant condition, light is reflected in transverse (y) direction thus, light is confined within the liquid core channel. The thickness (t_i) of the cladding layers to satisfy the anti-resonant condition can be expressed as follows-

$$t_i = \frac{(2N-1)\lambda}{4n_i \sqrt{1 - \frac{n_c^2}{n_i^2} + \frac{\lambda^2}{4n_i^2 d_c^2}}} \quad N = 1, 2, 3, \dots \quad (1.1)$$

where, n_i is the refractive index of i^{th} cladding layer and λ is the design wavelength. Our device is designed for the visible wavelengths with core dimensions of $\sim 5\mu\text{m}$ and core refractive index ~ 1.33 - 1.46 which requires the ARROW layer thicknesses in the order of hundreds of nanometers.

1.3 ARROW device fabrication

The ARROW devices are fabricated by the Hawkins research group at Brigham Young University following standard silicon fabrication techniques. Fig. 1.2 shows the different fabrication steps where the left and right column show the fabrication steps for solid core waveguide and liquid core channel respectively. The devices are usually fabricated on top of a <100> oriented Si substrate. At first, six alternating dielectric layers of SiO₂ (265nm thickness) and Ta₂O₅ (102nm thickness) are sputtered on the substrate which serves as the ARROW layers. The ARROW layers are industrially deposited by Evaporated Coatings Inc. which is shown in Fig. 1.2 (a). The device fabrication requires further etching steps and a 100nm chrome payer is deposited as downstream etch stop layer for this purpose. For the liquid core channel, a sacrificial core is formed using SU-8 lithography with typical dimension of 5μm x 12μm as shown in Fig. 1.2 (b). This sacrificial core is removed in future steps to create a hollow channel. The waveguides are created on a self-aligned pedestal which requires deep reactive ion etching (DRIE). Before etching, a positive photoresist (AZ4620) is employed with subsequent Ni lift off procedure to protect the self-aligned pedestal region which is shown in Fig. 1.2 (c). After employing the protection coating, the self-aligned pedestal is created by anisotropic DRIE of the ARROW layer and Si substrate as shown in Fig. 1.2 (d). Once the pedestal is formed, the next subsequent step is to create the waveguides. For this purpose, a 6μm thick SiO₂ layer is deposited on the whole wafer as depicted in Fig. 1.2 (e). To form the ridge SC waveguide, a ~15μm

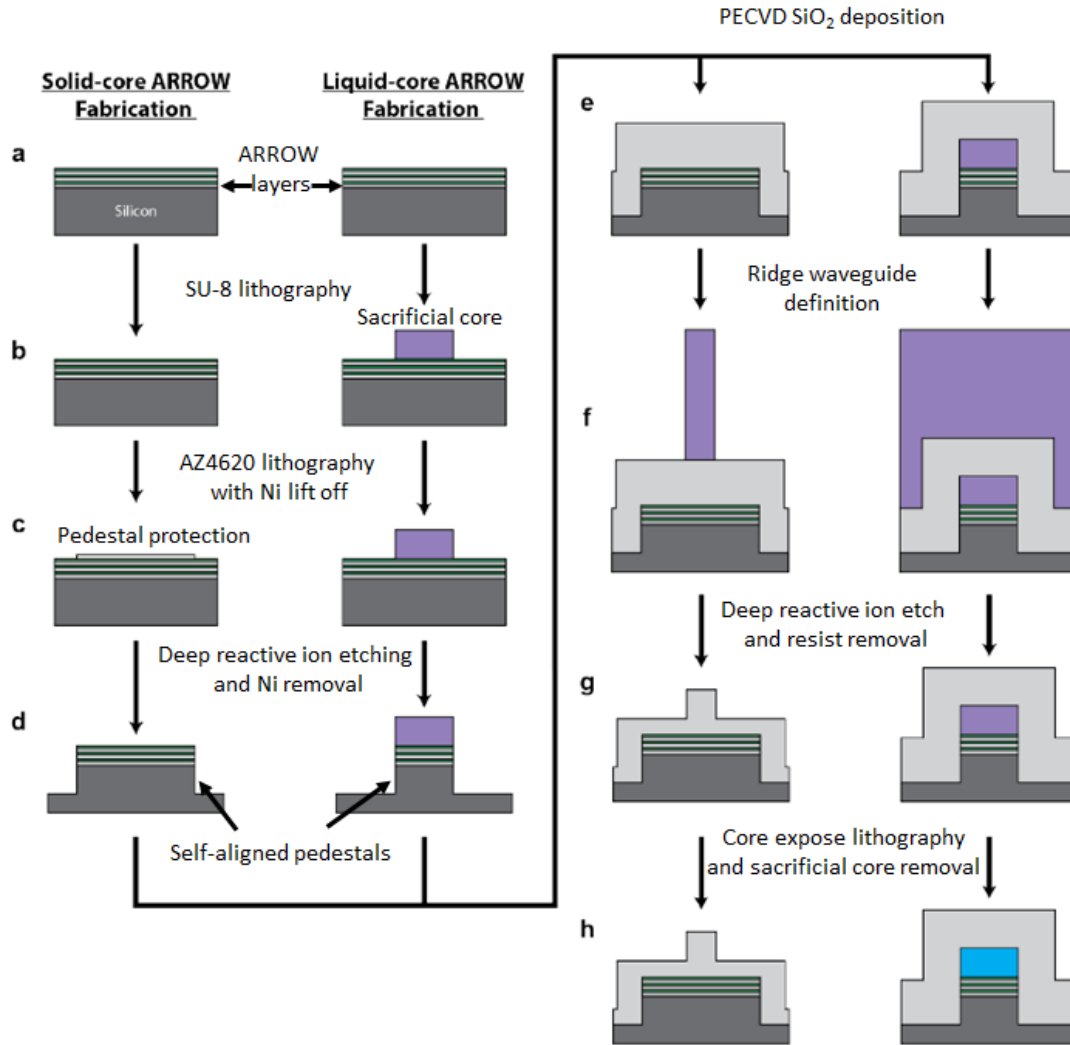


Fig. 1.2 (a-h) Schematic representation of different steps during ARROW waveguide fabrication. [from reference 41]

thick SU-8 etch stop layer is formed on a selective portion of the pedestal whereas the whole LC pedestal is covered with SU-8 as shown in Fig. 1.2 (f). Next, the whole wafer is etched down using DRIE except for the regions that are covered with SU-8 as described in the previous step. In a subsequent step, the SU-8 is lithographically removed which creates a ridge SC waveguide as shown in Fig. 1.2 (g). Next, oxides (SiO₂) are removed from the both sides of LC channel end down to the chrome etch

stop layer using buffered hydrofluoric acid. Finally, the SU-8 sacrificial core is removed using an optimized piranha etching which leaves a hollow channel that can be filled with experimental solution for device functioning. Fig. 1.2 (h) shows a typical schematic structure of final SC and LC waveguides.

The SC and LC dimensions are usually designed to mitigate multimode behavior and allow a single optical mode to propagate through the waveguides [43]. Furthermore, the waveguides can also be tapered to necessary dimensions to mitigate multimode behavior [44], [45].

1.3 Particle detection and manipulation on ARROW platform

Though the ARROW devices are used for a variety of different applications, the primary application of ARROW devices is fluorescence based biomolecular detection. A typical schematic of an ARROW device is shown in Fig 1.3 with intersecting LC (blue) and SC (gray) for optical excitation and fluorescence collection. The LC channel ends are usually terminated using glued reservoirs which are used to introduce fluidic sample. Once the reservoirs are filled with target particles (usually fluorescently tagged biomolecules or fluorescent particles) they can flow through the channel. Usually, fiber coupled laser lights (with proper wavelength) are introduced to the device through the excitation SC waveguides as shown in Fig. 1.3. Once fluorescently labeled particles interact with the laser light, they emit fluorescence light which can be collected via the orthogonal LC SC waveguides as illustrated in Fig. 1.3. These fluorescence signals are

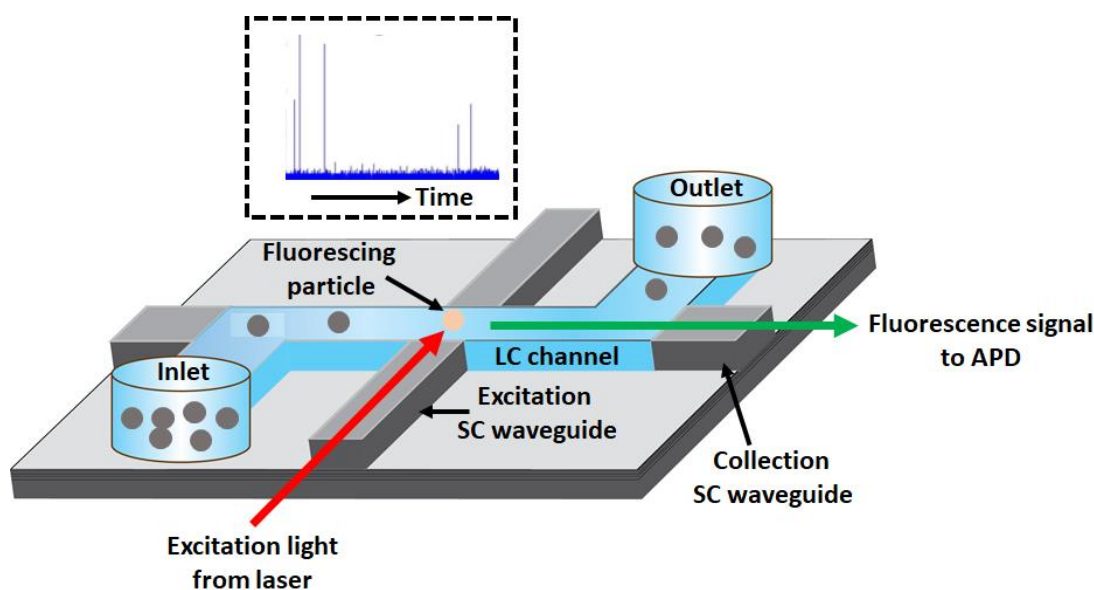


Fig. 1.3 Principle of fluorescence based particle detection in ARROW optofluidic device, inset: an actual APD trace of particle detection.

then sent to a sufficiently sensitive photodetector which generates detection spikes as shown in the inset of Fig. 1.3. For usual dimensions, the device can probe particles at femtoliter excitation volume which is ideal for single molecule analysis (SMA). The device provides sufficient sensitivity to detect single viruses [46], [47] and nucleic acids [37]. The device has been employed for a variety of different applications including but not limited to particle trapping and manipulation [40], [48], [49], optical filtering [33], [34], particle sorting [50], atomic spectroscopy [51], [52], SERS detection [53], multiplexed particle detection [47], [54] etc. One of the prominent features of the ARROW devices is the integration of nanopores with it. Nanopores are another powerful tool for electrical detection of particles including nucleic acids, viruses, proteins etc. [55]–[58] which itself is a vast field of research with tremendous potential. This integration of optofluidic ARROW device with nanopore allows two

very powerful SMA tools to merge and combined in a single platform with a variety of potential exciting applications. With the nanopore ARROW device, successful detection of single ribosomes has been reported [59]. A combined simultaneous electrical and optical detection of single particles including single DNAs and viruses has also been reported before [46], [60].

In the above mentioned nanopore experiments, there was no control over the particles delivered through nanopore which is an utmost desire to isolate and analyze individual single particles. A part of this thesis work demonstrates how precise control can be achieved over particle delivery through nanopore using a feedback control mechanism which is elaborately discussed in chapter 2. Using feedback control, a user defined number of particle(s) can be delivered to the fluidic analysis region where they can further be subjected to fluorescence detection which has been demonstrated by selectively delivering single 70S ribosomes and gated combined electro-optical detections of λ -DNAs. The next conceptual step is to subject the single delivered particle for prolonged analysis using particle trapping. This has been demonstrated by gating and Anti-Brownian electrokinetic (ABEL) trapping a single fluorescent microbead. An ABEL trap has several advantages over conventional optical trap which has already been reported on our ARROW device. However, the existing one-dimensional (1D) ABEL trapping system has some crucial drawbacks including lack of confinements thus, it is necessary to develop a new form of ABEL trap to overcome the limitations. In chapter 3, the design, operating principle and demonstration of a new two-dimensional (2D) ABEL trapping platform is discussed. Next, a major limitation

of nanopore sensing which is the insufficient availability of analytes close to nanopore to enable electrophoretic capture and detection is addressed in chapter 4. As few analytes are present close to nanopore, the capture rate or event frequency is significantly affected and reduced which limits the nanopore detection rate. An elegant solution to improve nanopore capture rate (event frequency) is demonstrated by increasing local analyte concentration with the assistance of optical trapping. Significant improvement ($\sim 80\times$) on nanopore capture rate is experimentally demonstrated. This methodology is further implemented to detect targets at low and clinically relevant concentrations which has high potential to find its application in diagnostics and early stage disease detection. Finally, a summary and outlook of the whole work is presented in chapter 5.

Chapter 2 On demand delivery and manipulation of single molecules on a programmable nanopore optofluidic device

Single molecule analysis usually deals with the analysis of single particles. Most single molecule analysis relies on optical and electrical methods[61]–[64]. In a most common case scenario, single particles are delivered in the analysis chamber on a successive basis without having any control over the delivered particles. In many cases, the introduction of a second particle hampers the analysis of an existing particle[40], [65], [66]. Additionally, for prolonged analysis of a single particle, it is desired to stop further particle delivery until the analysis of the existing particle is over. So, it is an utmost desire in single molecule analysis to have a precise control over the delivery of particle. To achieve this functionality, it is necessary to introduce a smart gate that allow single particles to be delivered one at a time and can also be closed (shut) if desired. Nanopore is the most prominent candidate for this purpose as the size of the pore can be tuned to fit only single particle and can also be used as a smart gate that can deliver the particles on demand with additional feedback control over the nanopore. Some preliminary attempts have been made to get control over nanopore “on off” and particle delivery. One rudimentary step towards nanopore feedback control is to recapture a translocated molecule where researchers first allow a particle to translocate through the nanopore then recapture the translocated molecule by voltage reversal [67]–[69]. Some researchers have attempted to turn “on” and “off” the nanopore by wetting and de-wetting the pore using some chemical modification of the nanopore[70]–[72]. A more

complete approach towards on demand single molecule delivery has been reported by Ivanov et al. where authors demonstrated delivery of single λ -DNAs on demand using a nanopipette[73]. However, their system relies on a pulsating DC source without really implementing any feedback control. Moreover, the whole system is designed upon the assumption that the first λ -DNA will translocate within a certain time from the voltage is applied. However, if the first DNA arrival time is not constant then this methodology can not work. A more realistic and practical approach will be to implement a feedback control over the nanopore to deliver single particles on demand.

In this chapter, we demonstrate the implementation of a microcontroller based real time feedback control over the nanopore. The feedback control system continuously monitors for translocation spikes and can turn on/off the pore according to user defined instructions. Moreover, the system facilitates reconfigurable user defined setting which allows the user to tune the system according to the experimental requirements. We, first, validate the system by delivering single ribosomes on demand. One remarkable advantage of our system is, it can be implemented on a broader range of biomolecules. To validate our claim, we show the delivery of variety of individual biomolecules including ribosomes, nucleic acids, proteins and NaCMC molecules. It is also possible to deliver any number of bioparticle upon necessity which has been showed by delivering two and three ribosomes on demand. The system can be configured to automatically (without manual interface) apply the voltage across nanopore after any user defined interval. This functionality is tested by re-applying the nanopore voltage 10, 20, 50 and 100 msec after a translocation. Using the system, molecules can be

automatically delivered at rapid succession. Successive delivery of ribosomes and λ -DNAs have been shown at rates up to several hundreds/min which can be boosted up to near kHz. It is expected that different biomolecules will produce different translocation pattern (width and height) depending upon their size and electrical charge[74]–[79]. As our system is capable to analyze the translocation height and width in real time, this functionality can be utilized to differentiate individual particles from a mixture when individual particles produce distinguishable translocation spikes from each other. A demonstration of such selective target gating is shown by voltage gating λ -DNAs from a mixture of ribosomes and λ -DNAs based on the translocation patterns specific to λ -DNAs. Furthermore, the feedback system can be integrated with existing analysis methodologies such as optical detection, trapping etc. One example is shown by optically detecting the voltage gated λ -DNAs which show the practicality of the feedback control system[80]. All these reconfigurable settings and functionality at near kHz delivery rate paves the way towards high throughput single molecule analysis on demand on a nanopore optofluidic chip.

2.1 Nanopores

The Greek word “Pore” means microscopic opening or aperture. In the field of nanoscience and sensors, nanopores are usually referred as nanoscale pores (opening) found in nature or artificially fabricated on a thin electrically insulating membrane. Nanopores are generally used to study the physical properties of a biomolecules or as a single molecule sensor by measuring the change in current as the individual molecule

passes through the pore[57], [81], [82]. Generally, both sides of the nanopore are filled with ionic solution and the nanopore makes a contact bridge through the membrane. An applied voltage across the pore results in a steady ionic current through it. As a charged particle traverses from one side of the membrane to the other side (referred as translocation), it transiently modulates the ionic current which is then recorded by a sensitive amplifier. The modulated current spikes are considered as the signature of particle translocation[83], [84]. Depending on the salt concentration and the chemical properties of the pore, the current modulation manifests as a decrease or increase in the ionic current[85]–[87]. As nanopores are highly sensitive, it is possible to precisely resolve the modulated current levels when individual bases of nucleic acids pass through the pore[88]–[90]. As different bases of nucleic acids generate distinguishable current levels, it is possible to read out the nucleic acid bases based on the nanopore current level thus, sequence the nucleic acid. This fundamental yet powerful technique forms the basis of leading next generation nucleic acid sequencing which are already being commercialized[91], [92]. Even though nanopores are being used in sequencing applications, several challenges remain to be addressed such as fast translocation speed, long DNA sequence length etc.[56], [93]–[95]. Though the most prominent application of nanopore is sequencing, it has significant importance in other studies as well. Nanopore has the potential to be used to study, model and understand the underlying physics and process of different biomolecules[79], [81], [96]. Nanopore has already established itself as a successful biomolecular sensor and detector. Researchers have already reported successful demonstration of different biomolecule detection using

nanopore including nucleic acids, proteins, ribosomes, viruses, metabolites etc. [46], [59], [97]–[99]. Recently, nanopores opened up a new avenue in the field of single molecule analysis especially, as a label free single molecule analysis tool[100]–[102]. Additionally, nanopores are widely being integrated with other technologies to form an integrated single molecule analysis platform. In fact, integrated nanopore technology has opened up a new avenue for research and successful implementation of integrated nanopore devices are being reported including our ARROW device[46], [60], [103]–[106]. In our case, we have used the integrated nanopore devices in a whole new perspective. In addition to its conventional detection application, we have used nanopores as a “smart gate” which can precisely deliver particles to the analysis chamber with user defined reconfigurable settings which will be discussed in detail in the later part of this chapter.

There are two types of nanopores that are used for nanopore applications:

2.1.1 Biological nanopores

The most commonly used biological nanopores are α -haemolysin (α -HL) and Mycobacterium smegmatis porin A (MspA). The biological nanopores have a very small inner diameter (~ 1 - 1.4 nm) which only allow single strand DNA or RNA to translocate through the pores[107], [108]. Fig. 2.1(a) and Fig. 2.1(b) show the pictures of α -HL and MspA nanopores respectively. In general, α -HL is more popular than MspA and widely used for biological nanopore research. α -HL is a protein secreted by

Staphylococcus aureus bacteria as a toxin, which forms nanopores with a smallest

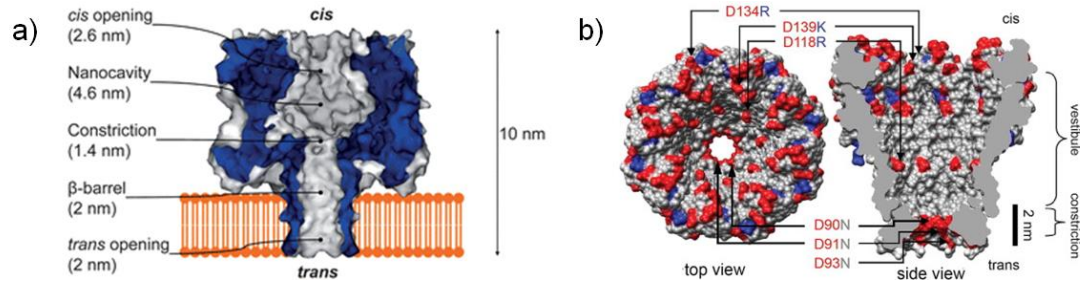


Fig. 2.1 (a) Molecular photograph of an α -HL pore [from reference 107]; (b) Picture of a MspA nanopore [from reference 108].

diameter of around 1.4nm. The first application of biological nanopore has been reported in 1996[109] and since then a vast amount of studies have been reported which has drawn significant attention of researchers and even put them on the way to replace existing sequencing methodologies[95], [110]. Despite some advantages such as low background noise, precise inner diameter, better compatibility with biomolecules etc. biological nanopores have several shortcomings such as non-configurable diameter, limited lifetime, non-reusability etc.[57], [97]. Moreover, it is comparatively difficult to integrate biological nanopores with existing on-chip SMA technologies. These drawbacks lead researcher to fabricate nanopores artificially with the help of advanced nanotechnology which is the second category nanopore usually referred as solid state nanopores or synthetic nanopores.

2.1.2 Solid state nanopores

Solid state nanopores (ss nanopore) are artificially fabricated pores (holes/opening) on a thin insulating membrane. In 2001, the journey of ss nanopore begun[111] and since then it has been a hot spot in the research field. Several methods for ss nanopore

fabrication have been reported including ion beam drilling[112], chemical etching[113], dielectric breakdown[114] etc. Using these fabrication methods, researchers were able to fabricate ss nanopores down to the size of single nanometers[112] and even in the range of sub-nanometers[115]. ss nanopores can also be fabricated as arrays to facilitate parallel and multiplexed sensing[116]. Fig. 2.2(a) and 2.2(b-c) show typical SEM images of a single ss nanopore and nanopore arrays

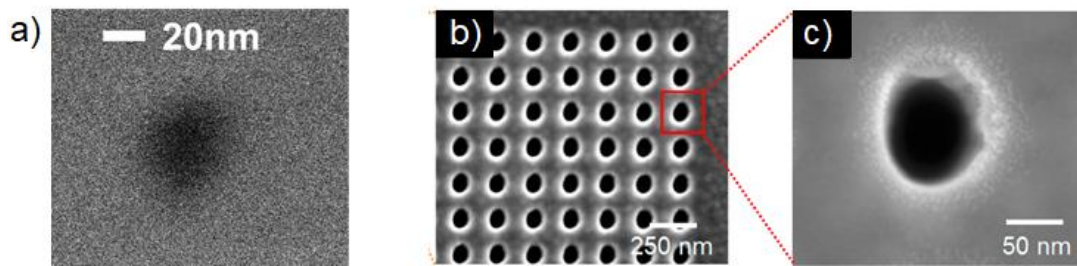


Fig. 2.2 (a) Typical SEM image of a single ss nanopore; (b) SEM image of a ss nanopore array; (c) Zoomed in view of a single nanopore within the array of Fig. 2.2(b) [Fig. 2 (b-c) are from reference 116].

respectively. One remarkable feature of ss nanopore is the size adjustability which allows the user to precisely tune the nanopore size according to their requirement[117]. The membranes commonly used for ss nanopore fabrication are low stress Si_3N_4 [112], SiO_2 [86], Al_2O_3 [118], SiC [119] and graphene membranes[120]. ss nanopores offer several favorable advantages over biological nanopores such as versatility, robustness, diameter tunability, mass producibility, reusability, adjustable surface properties etc. Due to these advantages, ss nanopores have become a popular choice for sensing and detection of biomolecules and are widely being used in those areas including single molecule analysis. Some preliminary works have also been done towards nucleic acid

sequencing using ss nanopore[121] however, some challenges are still unmet towards full scale sequencing.

2.2 Nanopore integration with ARROW devices

As mentioned earlier, one remarkable advantage of ss nanopores is the facility to integrate with other technologies. In our case, we have integrated the ss nanopores with ARROW optofluidic devices. ARROW devices can be employed for optical detection, manipulation, trapping and variety of other applications which have already been demonstrated before[40], [47], [48], [50], [122]. The most popular methods of SMA are optofluidic approach and electrical SMA methods. The integration of nanopores with ARROW optofluidic devices combines these two powerful SMA techniques in a single platform which has tremendous potential to make substantial improvements in the field of SMA.

2.2.1 Nanopore fabrication

The nanopores were fabricated on ARROW devices using ion beam drilling method similar approach which have been reported before[46], [60], [123]. The drilling was done using a dual beam SEM/FIB microscope (FEI Quanta 3D FEG Dual Beam SEM/FIB) where Gallium (Ga) ions were used as the ion beam source. The drilling principle is simple, where high energy Ga ions hit the target material and sputter away the target materials creating a hole in it. Fig. 2.3(a) illustrates a schematic of the cross-sectional view of the nanopore fabrication process. To fabricate nanopores with

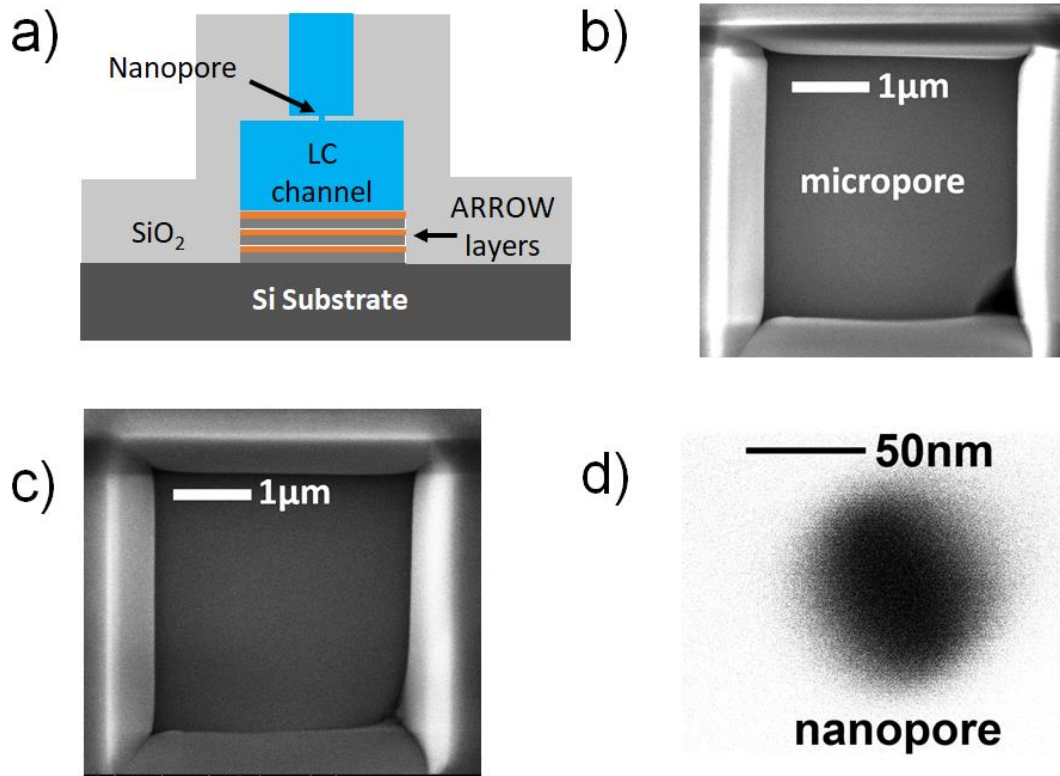


Fig. 2.3 (a) Schematic of the nanopore fabrication process; (b) Square well fabricated on the LC channel with drilling stopped when crack appears at the bottom right corner; (c) Insulation deposition process to cover the crack; (d) SEM image of a nanopore.

ARROW devices, first, it is necessary to remove the top thick oxide layer ($\sim 6\mu\text{m}$) of the LC channel which is usually referred as “micropore”. It should be noted that the drilling process is stopped as soon as any crack is seen in the square micropore as shown in the Fig. 2.3(b) (bottom right corner). The membrane is considered to be sufficiently thin to drill a nanopore once the crack(s) appears. Next, the crack(s) is closed using insulation deposition creating a thin intact membrane suitable for nanopore fabrication as shown in Fig. 2.3(c). Finally, a nanopore is fabricated on the leftover thin membrane using a 1.6pA-10pA, 30kV Ga beam for suitable time period. The exposure of ion beam

current was controlled by the Nanometer Pattern Generation System (NPGS, JC Nabity) to fabricate a nanopore with expected diameter. Furthermore, it is possible to finetune the nanopore size to get better precision in order to fulfil the experimental requirements. Fig. 2.3(d) shows an SEM image of a typical nanopore fabricated in ARROW device.

2.3 Single molecule delivery on demand

To this end, the nanopore optofluidic device is being used as a SMA platform which can deliver particles on a demand basis. The key objective is to achieve a precise control over the particle delivery such as the nanopore can be kept “on” for delivery particles for any desired amount of time as well as it can be kept “off” for any desired amount of time to stop further insertion of particles. Moreover, it is desired to make the platform more versatile and configurable so that the user can modify/function the platform according to their specific requirements. These functionalities have been achieved by implementing a microcontroller based feedback system over the nanopore. The microcontroller looks for a molecular translocation in real time. Once a translocation is detected, it can turn the pore “off” to prevent further particle delivery. Moreover, different user defined functionalities can be achieved by adjusting the microcontroller program.

2.3.1 Feedback control gating principle and methodology

The on demand particle delivery platform is designed on an ARROW optofluidic device as mentioned in Chapter 1. Fig. 2.4 shows a typical nanopore experimental setup

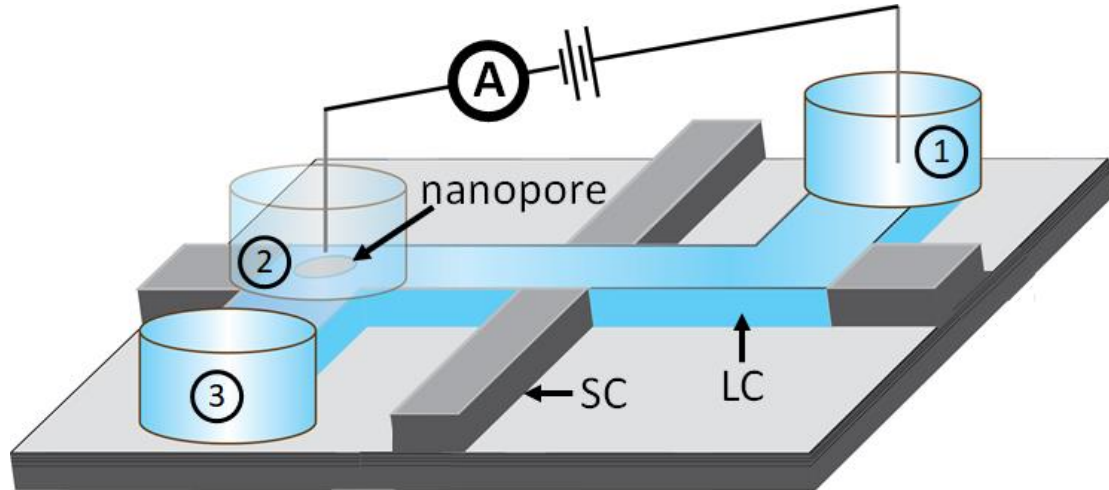


Fig. 2.4 Typical experimental setup of nanopore experiment without feedback control.

(without feedback) on an ARROW device. The LC channel (blue) ends are terminated with glued (wax, Crystalbond 509-3) reservoirs (reservoir 1 and reservoir 3). Another reservoir (reservoir 2) is placed over the nanopore which is fabricated using the methods described in the previous section. The reservoirs are used to introduce fluidic samples into the LC channel as well as to apply electrical and mechanical forces on target particles. In a typical nanopore experiment, target samples are introduced into the nanopore reservoir (reservoir 2) and an electrical bias voltage is applied between reservoir 1 and 2 as shown in Fig. 2.4. The voltage is usually applied to the device using a Digidata (Molecular Devices) via Ag/AgCl electrodes. The corresponding current through the nanopore is recorded using

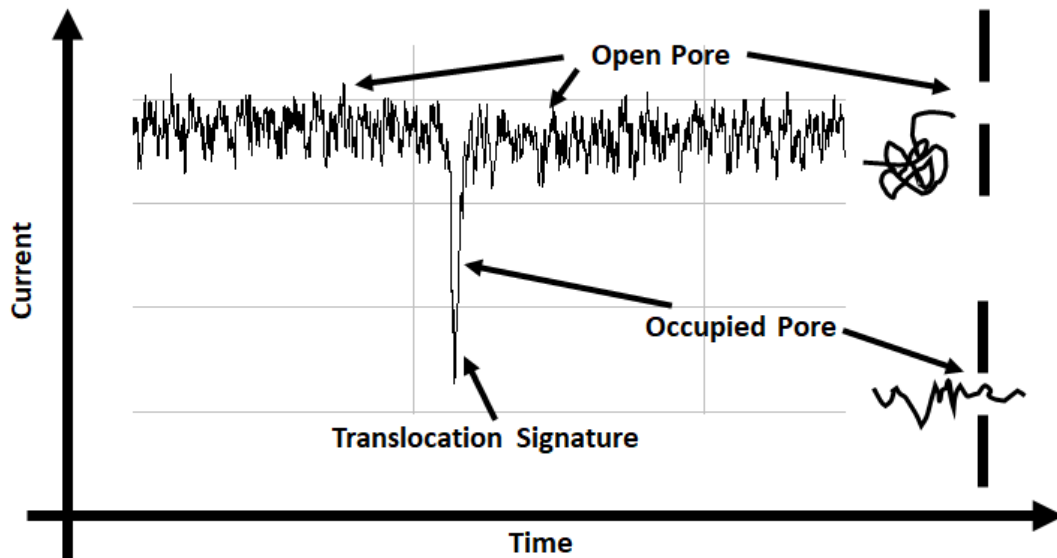


Fig. 2.5 Typical current trace through nanopore with translocation spike.

a patch clamp amplifier (Axopatch 200B, Molecular Devices). As mentioned earlier, an applied voltage across the nanopore generates a steady ionic current through the pore which usually referred as baseline current as shown in Fig. 2.5. For nanopore experiments, the targets are usually electrically charged particles. The electrokinetic force from the bias voltage drives the target particle through the nanopore. As soon as the charged particle translocate through the pore, a transient current spike is generated which is considered as the particle detection signal as depicted in Fig. 2.5. As the ARROW device has interconnected SC (gray, in Fig. 2.4) and LC channels, it is possible to integrate further optical methods to analyze and manipulate the translocated particle. Such integrated electro-optical simultaneous detection of single particles has already been demonstrated[46], [60]. The schematic of the electro-optical multimodal

particle detection principle is shown in Fig. 2.6(a) where an electrical bias voltage

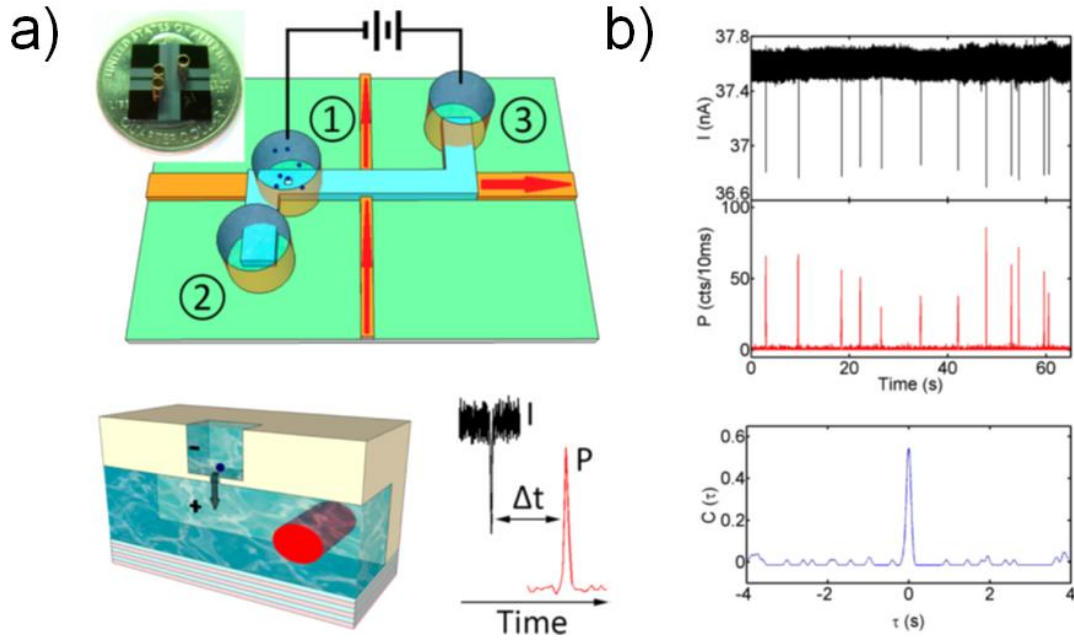


Fig. 2.6 (a) Schematic and principle of electro-optical detection of single particles [from reference 46]; (b) Previous experimental results of dual-mode detection of single viruses [from reference 46].

across reservoir 1 and 3 pulls the particle to the

LC channel. Then the fluorescent particle is excited using proper laser light which is coupled to the SC waveguide and the generated fluorescence signal is collected via the orthogonal LC-SC waveguides as depicted in the top panel of Fig. 2.6(a). The bottom left panel of Fig. 2.6(a) shows the cross-section of the LC channel revealing how the particle is optically excited when passing through the excitation spot. The bottom right panel of Fig. 2.6(a) shows the electrical (black) and optical (red) detection spikes originated from a single particle. Fig. 2.6(b) shows the previous results of electro-optical detection of single viruses[46]. The black trace of Fig. 2.6(b) shows the electrical detection spikes whereas the red trace shows the corresponding optical

detection traces. As both spikes are originating from the same particle, they are highly correlated, and the corresponding correlation signal is shown in the bottom blue trace. Previously, electro-optical detection of single particles has been demonstrated with up to 100% accuracy[46], [60]. The previous nanopore platform lacked feedback control over the particle delivery. As the nanopore bias voltage is kept on (which is the usual case), particles translocate through the nanopore in a rapid succession without any controlled fashion. A true single molecule analysis platform which requires only one particle to be present in the interrogation region at a given time, will require controlled deliver of particles thus, a control over the applied voltage. One possible solution to this problem is to turn off the applied voltage as soon as a translocation spike appears in the current signal to restrict further particle translocation which forms the basis of feedback control over the nanopore.

In this case, the whole feedback gating principle relies on continuous monitoring of the ionic current in real time and identifying the translocation spikes as a translocation occurs. This functionality has been achieved by integrating a microcontroller (PSoC, Cypress Semiconductor) along with necessary circuitry to the nanopore[80]. A schematic of the whole feedback control gating system is depicted in Fig. 2.7. Here, an additional feedback control system is integrated over a typical nanopore setup. The non-scaled voltage signal corresponds to nanopore current (V_{INP}) is sent to the built-in analog to digital converter (ADC) of the microcontroller. A (100 k Ω) potentiometer is used for this purpose which can be adjusted to fine-tune the voltage level for the ADC. Rather than applying the bias voltage directly to the device,

it is sent to the input terminal of a solid-state relay (Vishay Semiconductors). The

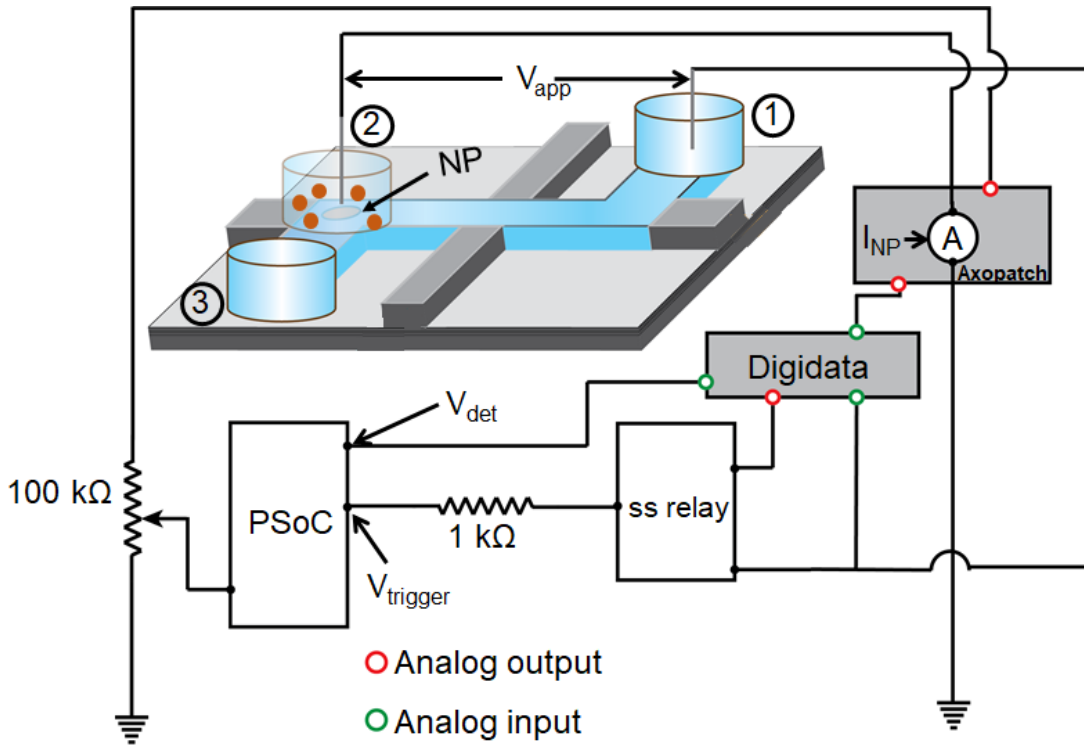


Fig. 2.7 Schematic of the feedback control system on a nanopore optofluidic device with interconnected LC (blue) and SC (gray) waveguides.

microcontroller decides whether to apply the bias voltage to the device or not based on the user instruction. To apply the bias voltage to the device, the microcontroller outputs a logical 1 voltage signal ($V_{trigger}$) to the relay control terminal, and the voltage is being applied across reservoirs 1 and 2 (V_{app}). Due to the voltage application, the corresponding nanopore current is continuously being monitored by the microcontroller. As instructed, the microcontroller waits for translocation(s) to occur. As soon as the microcontroller detects the desired number of translocation(s), it sends a logical 0 (zero voltage) signal to the relay terminal to disconnect the circuit, resulting in zero applied voltage across the reservoirs. Once the circuit is disconnected, there is

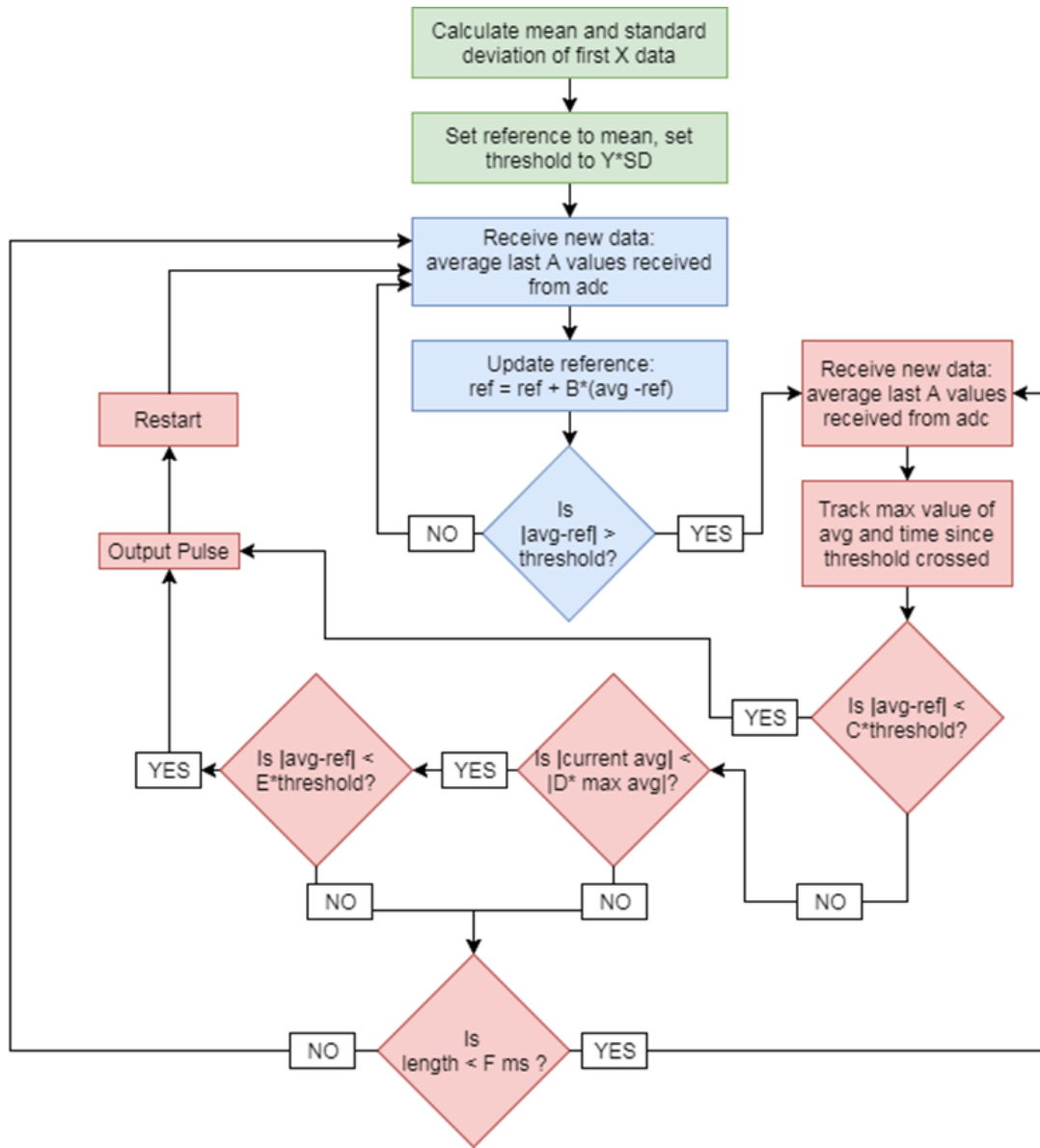


Fig. 2.8 Flow chart of the feedback gating algorithm.

no bias voltage across the reservoirs and accordingly no further translocation occurs. As a confirmation of translocation detection, the microcontroller generates a pulse voltage (V_{det}) which is sent to the Digidata for recording the number of translocation events.

A flowchart of the translocation detection algorithm is shown in Fig. 2.8 which illustrates the details of the microcontroller operating principle. The whole algorithm can be subdivided into three sections, the initialization stage (green boxes), baseline tracking (blue boxes) and decision making stage (pink boxes). During the initialization stage, the microcontroller sets up the necessary variables. First, the standard deviation (SD) is calculated from a certain number of initial samples (X , usually 2000) and a threshold (Y , varies upon target, one example is $6SD$) is defined, which is usually adjusted based on the translocation details of the target molecule. The microcontroller takes certain number of samples per iteration (A) which is defined by the user (in our case it was 5 samples/iteration). Next, a reference is initialized by calculating the mean of the samples. At the baseline tracking stage, an average is calculated from the number of samples in each iteration and the average point is compared with the reference to check whether it exceeds the threshold. If the average point doesn't exceed the reference, the reference is updated, and it proceeds to next iteration. If any average point exceeds the user defined threshold value, it is considered as the start of a translocation. Next, the peak height is determined, and it is monitored whether the oncoming average points fall back to the lower threshold value (C , varies upon target, one example is $2SD$) within a characteristic time period (F , varies upon target, one example is 60msec). The spikes which do not fall back to the predetermined lower threshold within the time period are discarded as baseline noise, the variables are reset, and the microcontroller seeks for next translocation. There is an option available for exceptional situations, where some taller translocations don't fall back to the lower

threshold value. In those specific cases, the tall pulse criterion (D, used only in special cases) can be selected by the user and the lower threshold can be readjusted (E, used only in special cases) upon user's instruction. However, if the tall pulse scenario doesn't appear in case of any target, this condition can be ignored based on user's command.

2.3.2 Feedback control gating simulation

At first, the algorithm is simulated in MATLAB to verify whether the gating

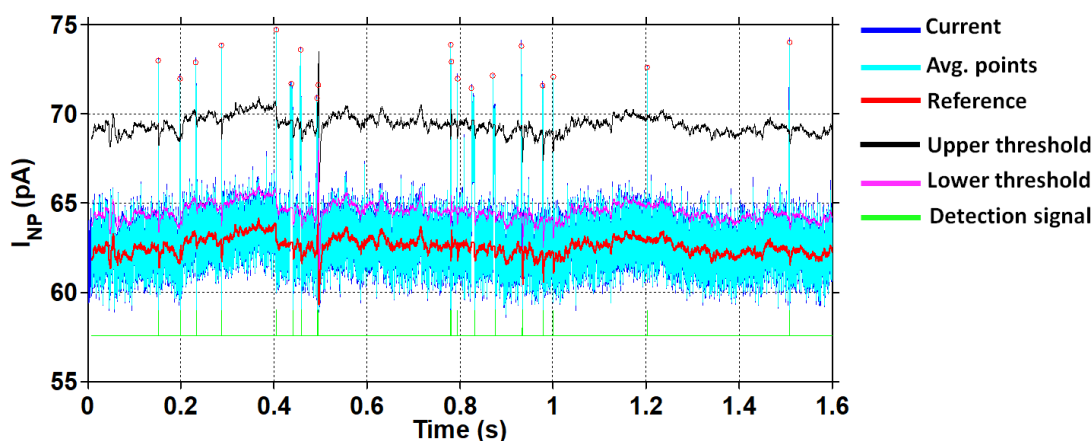


Fig. 2.9 MATLAB simulation of the feedback control gating algorithm.

functionality performs as expected. To do so, an actual current trace (previously recorded) of molecular translocations was input to the MATLAB program and satisfactory translocation detection was seen. The simulation result is shown in Fig. 2.9 where the blue trace corresponds to the actual current signal, the cyan trace shows the average points, the red trace shows the reference points, the black trace shown the upper threshold, the magenta trace shows the lower threshold and the green pulses show the translocation detection signal.

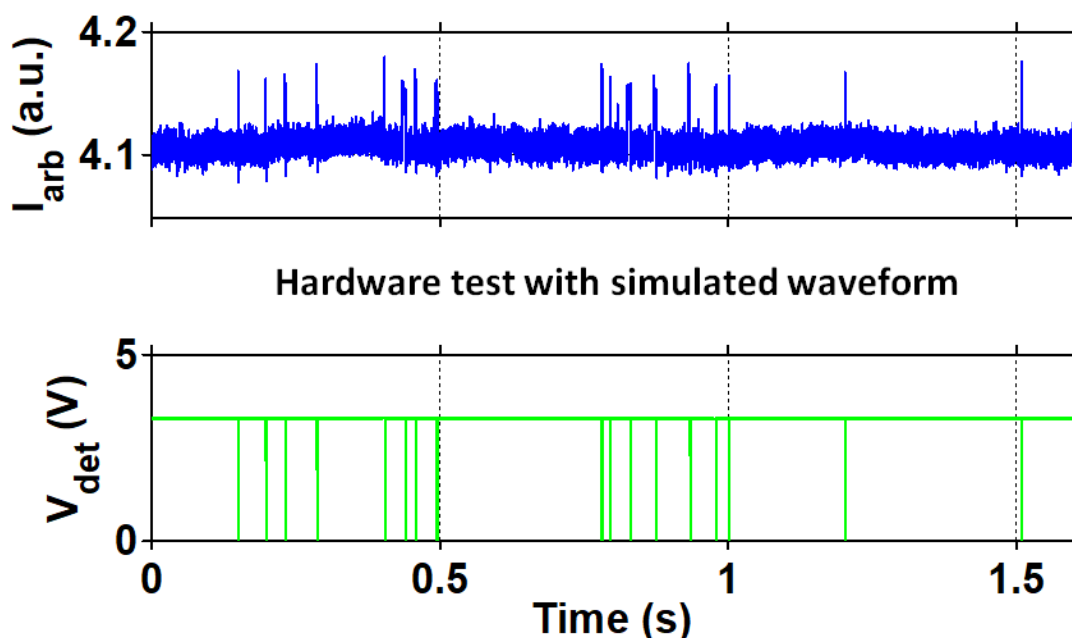


Fig. 2.10 Feedback gating hardware test by mimicking the current trace using an arbitrary function generator.

Once the translocations are successfully detected in MATLAB, the microcontroller is programmed mimicking the MATLAB code. Next, a second stage simulation is performed to verify whether the microcontroller and gating circuitry is functioning properly. An arbitrary function generator was used to generate a signal which mimics the molecular translocation trace. This generated trace is then fed to the microcontroller as an input signal and it is observed whether the microcontroller responds properly and able to detect translocations. This second simulation allows the user to troubleshoot the circuitry if necessary, debug the program and finetune the necessary parameters. A typical hardware test result is shown in Fig. 2.10 where the top blue trace represents the current signal fed to the gating circuitry from an arbitrary function generator and the bottom green pulses represent corresponding translocation detection signals from the microcontroller. Once the parameters are tuned and the

microcontroller performs as expected, the whole feedback system is implemented over the nanopore optofluidic device.

2.3.3 Feedback control gating demonstration

The validity of the feedback control system is justified by voltage gating individual biomolecules passing through nanopore. First, 70s ribosomes were selected as a target molecule and samples were prepared by L. Lancaster in the Noller lab. Ribosomes were purified from *E. coli* MRE600 cells grown at 37 °C to mid-log. Cells were lysed through a French press at 18,000 psi. The lysate was clarified by centrifugation at $30,000 \times g$ for 30 min in a JA20 rotor (Beckman) before layering onto cushions containing 1.1 M sucrose, 10 mM Tris-HCl (pH 7.5), 500 mM NH₄Cl, 10 mM MgCl₂, and 6 mM β ME, and ultracentrifuged in a Beckman Ti70 rotor for 20.5 h at $106,000 \times g$. The resulting ribosome pellet was resuspended in 20 mM Tris-HCl (pH 7.5), 500 mM NH₄Cl, 10 mM MgCl₂, and 6 mM BME and re-pelleted twice at $223,000 \times g$ for 2 h in a Ti70 rotor (Beckman). The resuspended pellet was then loaded onto 10-35% sucrose gradients containing 20 mM Tris-Cl (pH 7.5), 100 mM NH₄Cl, 6 mM MgCl₂, and 6 mM β ME, and ultracentrifuged in a Beckman SW28 rotor at $48,000 \times g$ for 17 h. The 70 S ribosome peak was collected from the gradients using a BioComp Piston Gradient Fractionator, then ultracentrifuged in a Ti45 rotor (Beckman) at $101,000 \times g$ for 22 h. The ribosome pellet was resuspended in 50 mM KHepes (pH 7.9), 100 mM KCl, 10 mM MgCl₂, and 6 mM β ME (ribosome buffer). Aliquots were flash-frozen in liquid N₂ and stored at -80 °C. Before each solution preparation steps, target-specific buffers

were filtered with 20nm Whatman Anotop syringe filters (GE Healthcare) to remove any unwanted contamination. To translocate the ribosomes, a nanopore of comparable diameter ($\sim 38\text{nm}$) was drilled on the ARROW device using the dual beam SEM which is shown in the bottom inset of Fig.2.11. As the most desirable aspect of the feedback system is to achieve the capability to deliver a single particle, the feedback system was

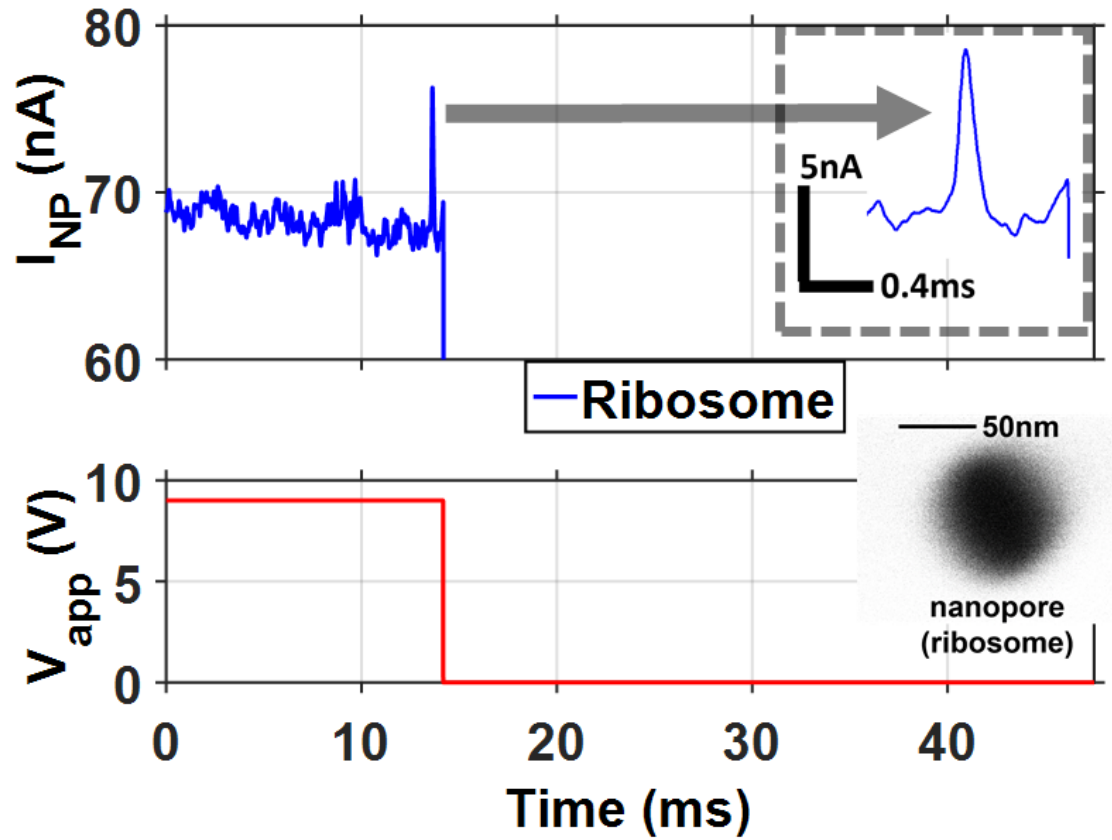


Fig. 2.11 Voltage gated single ribosome delivery on demand; top trace (blue): current through the nanopore, bottom trace (red): applied voltage across the pore; top inset: zoomed in translocation; bottom inset: SEM image of the nanopore used to deliver single ribosomes.

implemented to shut off the voltage across the pore once a single ribosome is delivered.

Fig. 2.11 depicts the experimental demonstration of voltage gated delivery of single ribosome where the blue trace shows the nanopore current and the red trace indicates

the voltage applied across the pore. As soon as the microcontroller detects a translocation (zoomed in view shown in Fig. 2.11 top inset), the voltage is turned off as shown in the red trace. This demonstration validates the practicality and key feature of the feedback system.

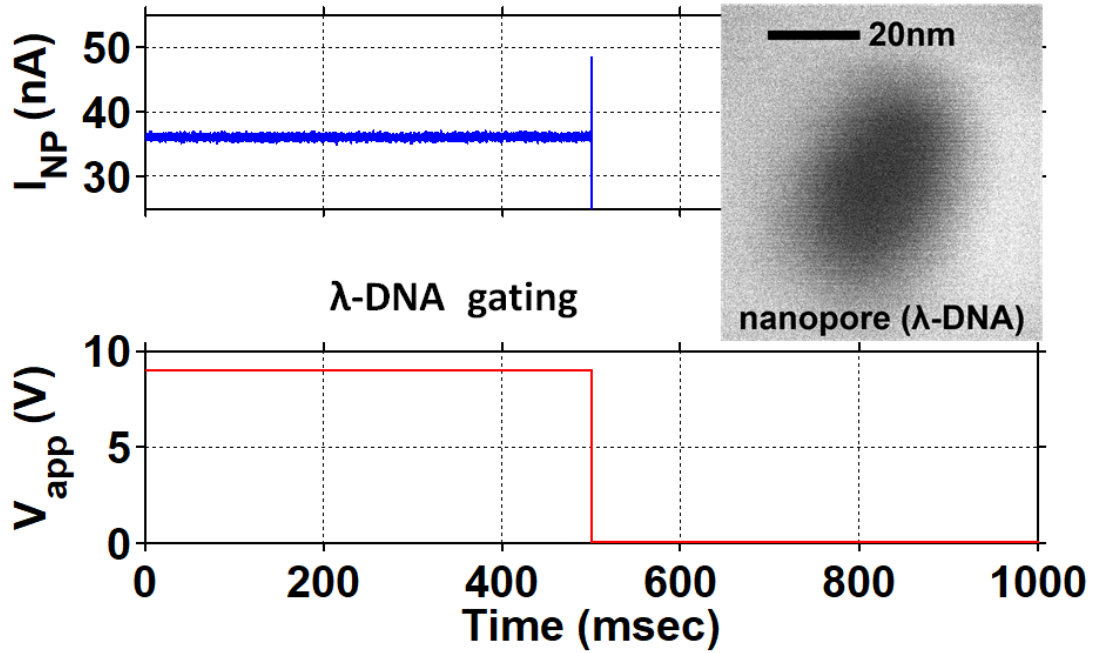


Fig. 2.12 Single λ -DNA delivery on demand; top trace (blue): current through the nanopore, bottom trace (red): applied voltage across the pore; inset: SEM image of the nanopore used to deliver λ -DNA.

One notable feature of the feedback system is it works of a diverse range of biomolecules in different aqueous environment. This statement is justified by individual voltage gating of single λ -DNAs, Zika virus NS-1 proteins, and sodium carboxymethyl cellulose (NaCMC) molecules[80]. First, λ -DNAs were voltage gated through a ~ 20 nm nanopore (inset of Fig. 2.12). λ -DNAs (New England Lab) were diluted into the 1xT50 buffer (10mM Tris-HCl, 50mM NaCl) to a final concentration of 9.55×10^{11} /mL. The λ -DNA voltage gating result is shown in Fig. 2.12 where the blue

trace shows the current through nanopore with λ -DNA translocation and the red trace shows the voltage applied across the pore.

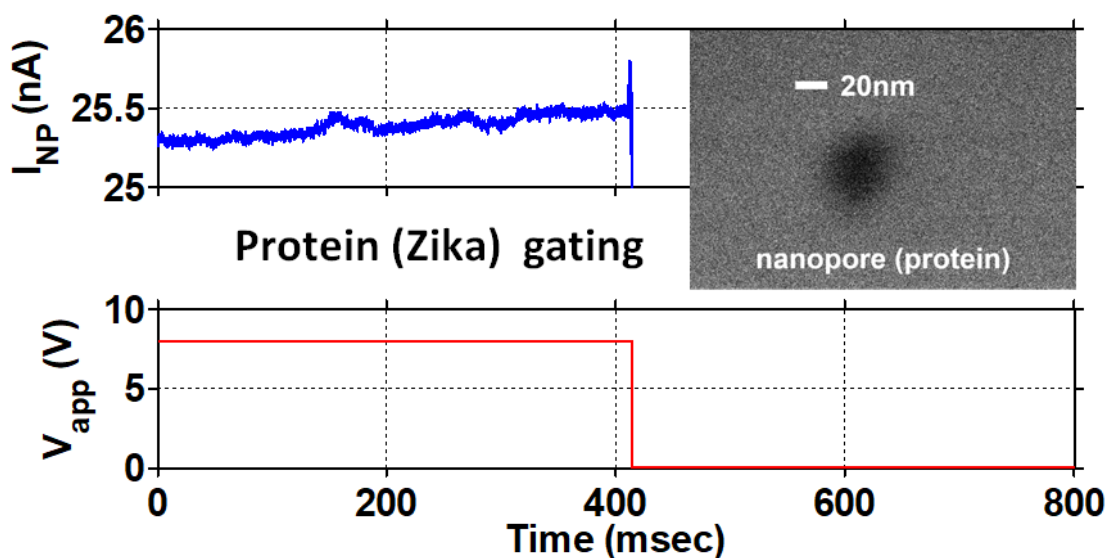


Fig. 2.13 Single Zika protein delivery on demand; top trace (blue): current through the nanopore, bottom trace (red): applied voltage across the pore; inset: SEM image of the nanopore used to deliver Zika proteins.

Next, single Zika virus nonstructural 1 (NS-1) protein (EastCoast Bio) was delivered to the LC channel using the feedback gating methodology. To prepare the solution, Zika proteins were diluted in 1xT50 buffer and diluted to a final concentration of 5.12×10^{12} /mL. Single protein delivery result is shown in Fig. 2.13 where the blue trace shows the current through nanopore, the red trace shows the voltage applied across the pore and the inset represents the SEM image of the nanopore used in this experiment.

This time, sodium carboxymethyl cellulose (NaCMC) molecules were used as the target molecule and single NaCMC molecules were delivered to the LC channel on demand. NaCMC solution was prepared by dissolving 26 mg of NaCMC powder

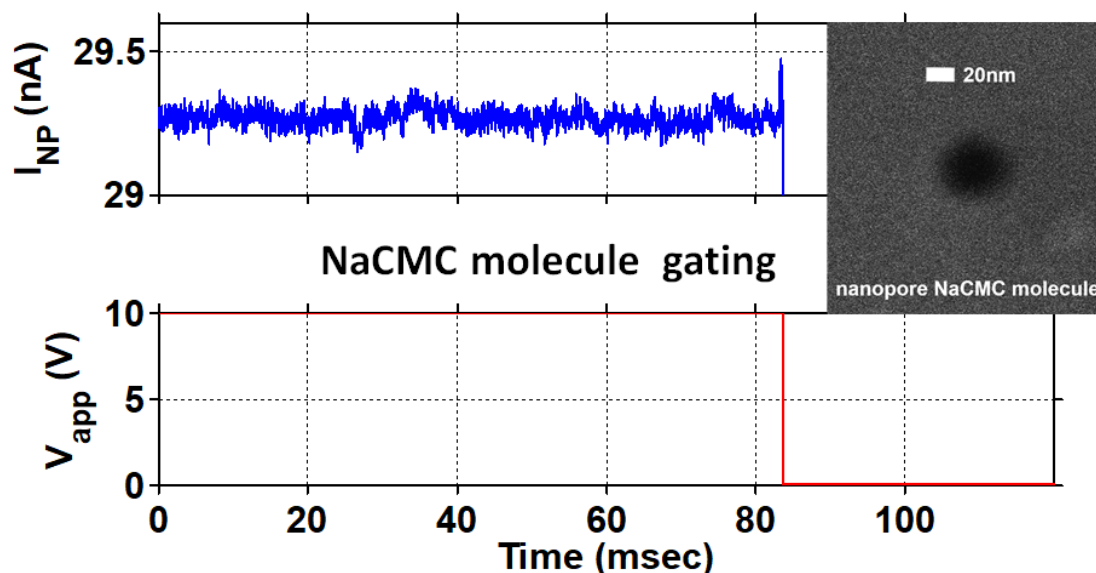


Fig. 2.14 Single NaCMC molecule delivery on demand; top trace (blue): current through the nanopore, bottom trace (red): applied voltage across the pore; inset: SEM image of the nanopore used in the experiment.

(Sigma-Aldrich, Product Number: 419273) into 1 mL of 1xT50 buffer. The experimental result of the single NaCMC molecule delivery is shown in Fig. 2.14 where the blue trace shows the current through nanopore, the red trace shows the voltage applied across the pore and the inset represents the SEM image of the nanopore used in this experiment. It should be noted that except for ribosome translocation, though small but there is a possibility that more than one particle may pass through the nanopore due to the relatively larger size of the pore compared to particle size. Nonetheless, these demonstrations show the effectiveness and versatility of the feedback system and proves its ability to be implemented in a wide range of applications with a variety of biomolecules.

2.3.4 User defined number of particle delivery on demand

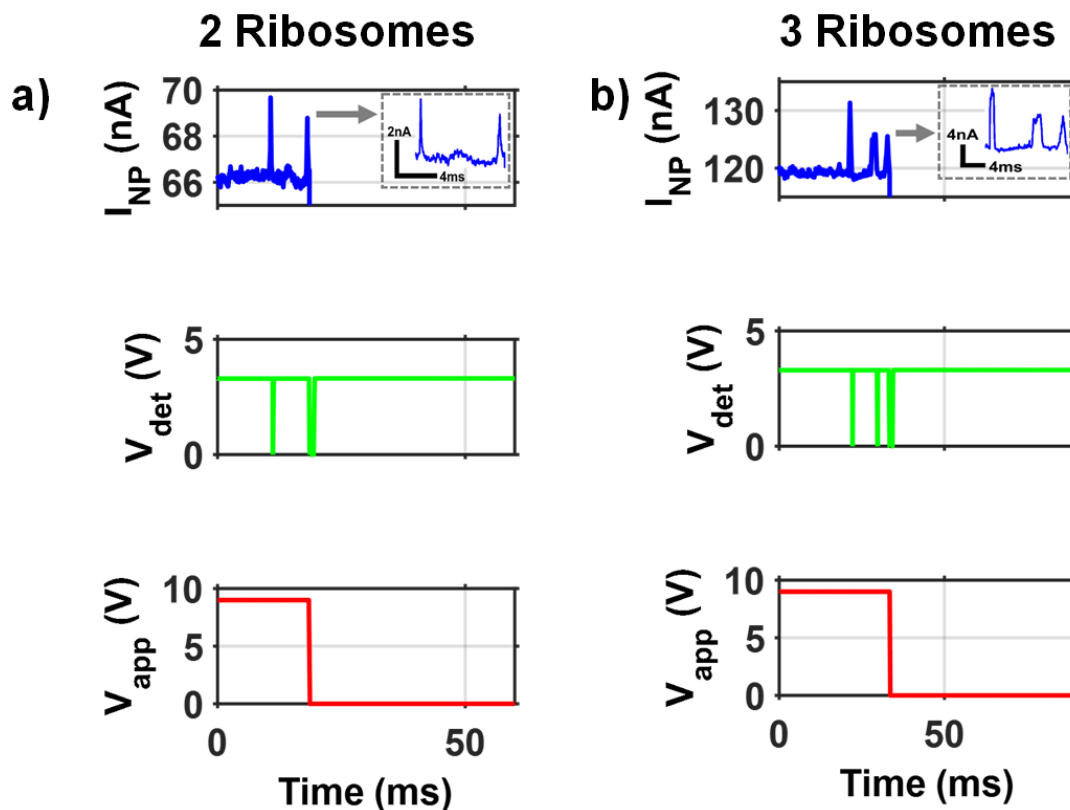


Fig. 2.15 User-defined number of particle delivery on demand into the fluidic channel; (a) two ribosomes delivery; (b) three ribosomes delivery; For both figures, (top: current through pore (inset: zoomed in translocations); center: signals in detection circuit of digitized identification of particle translocation; bottom: voltage across pore turned off after the desired number of particles has been detected.

As the microcontroller is programmable, it provides many reconfigurable features. One such feature is the ability to deliver any desired number of particles instead of just single particle delivery. Fig 2.15 shows an example of two and three ribosome delivery into fluidic channel where the top panel shows current through the pore (inset: zoomed in translocations), the center panel shows the particle detection pulses (V_{det}) and the bottom panel shows the voltage across pore which was turned off after the desired

number of particles has been detected[80]. Similarly, any number of particles can be delivered by adjusting the microcontroller program accordingly.

2.4 Automatic delivery of particles with programmable off-time

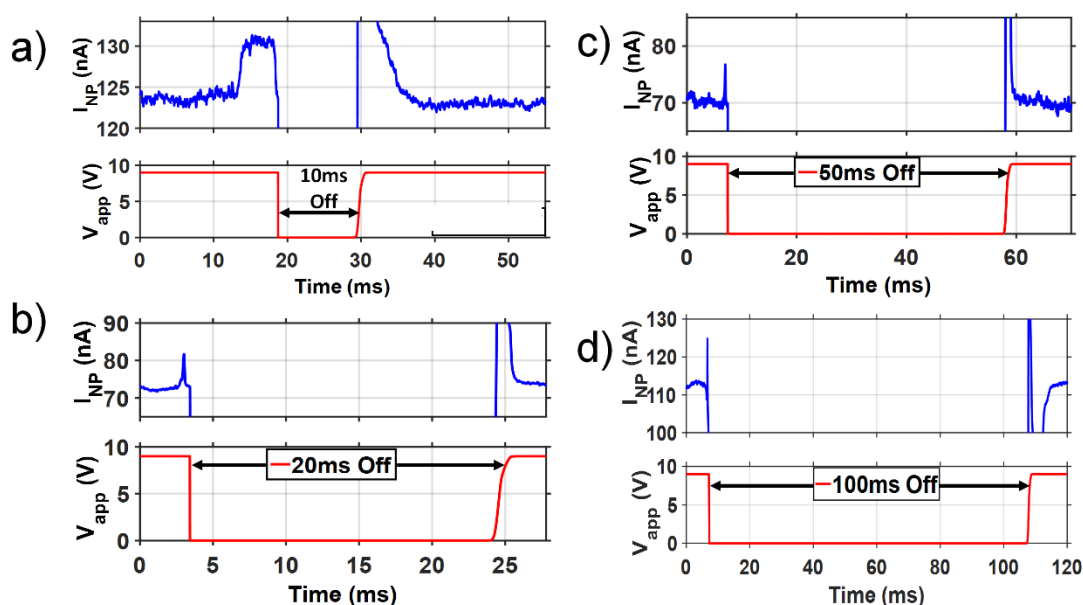


Fig. 2.16 Automatic re-application of voltage across the pore after user-defined time delays; here, (a) 10 msec, (b) 20 msec, (c) 50 msec and (d) 100 msec during which the pore was closed. For all figures: top trace represents the current through the pore and the bottom trace shows the applied voltage across the pore.

A key feature of the feedback system is automatic re-opening of the nanopore gate after a desired time interval following a translocation event meaning, automatic particle delivery after a specific off time. In other words, the pore can be kept in its off state for a user-defined amount of time before it is automatically re-opened. Fig 2.16 shows an example of automatic voltage re-application after four different duration 10, 20, 50 and 100 msec (Fig. 2.16 a-d respectively) of nanopore off time followed by a molecular

translocation which can be set to any desired value based on requirement[80]. In an application, where the analysis time of a single molecule is known or predicted, the off time can be set to that desired duration for successive single molecule delivery in an automated fashion.

2.5 Successive delivery of single biomolecules

As the system facilitates automatic particle delivery, it can be used for rapid, highly controlled delivery of single molecules into the fluidic channel at high rates. An example of such successive 70S ribosome delivery is shown in Fig. 2.17 (a) where the top panel shows the current trace with translocation events, the center panel shows corresponding detection pulses, and the bottom panel shows the voltage applied across the pore. A total of 48 gated translocation events are seen in less than 6 seconds which corresponds to a delivery rate of >500/min. A zoomed in small time segment of Fig. 2.17 (a) is shown in Fig. 2.17 (b) which more clearly illustrates the high controllability of the delivery process. Every time the microcontroller identified a translocation, the voltage across the pore was turned off, preventing further translocation and the pore was automatically re-opened 100 msec after each translocation. In the present configuration, the feedback system can rapidly deliver single particles up to rates of

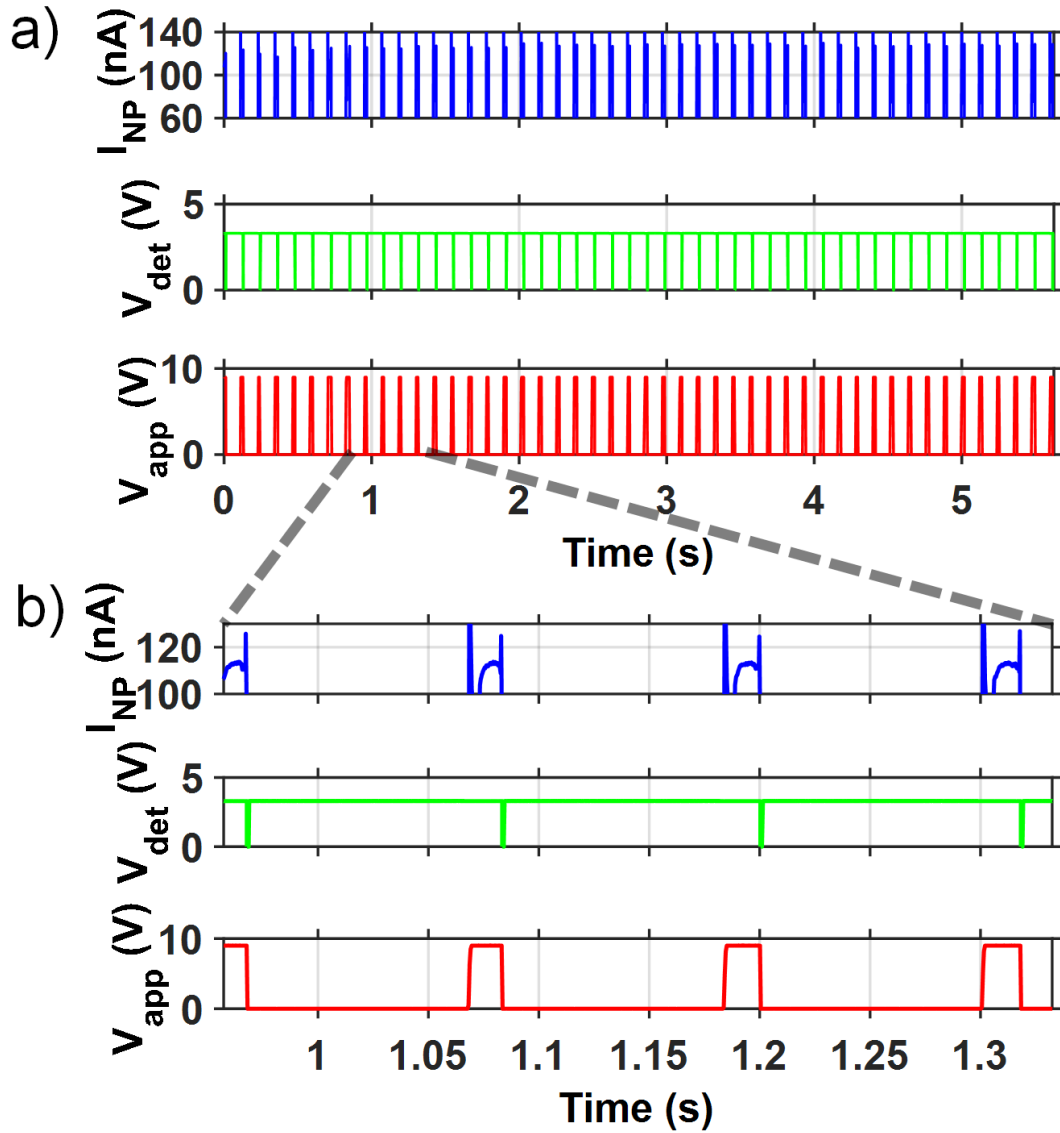


Fig. 2.17 (a) Automatic delivery of 48 ribosomes ($\sim 513/\text{min}$) into the LC channel where the voltage was re-applied 100 msec after each translocation; top: nanopore current, center: translocation detection pulses, bottom: voltage applied across across pore; (b) zoomed-in view of Fig. (a) revealing translocation events and how pore was automatically switched on-off based on users instruction.

$\sim 625 \text{ Hz}$ [80]. At the current configuration, the circuit response rate is mostly limited by the voltage re-application time which is approximately 1.5ms. A similar demonstration of automated successive λ -DNA delivery is shown in Fig. 2.18 (a) and

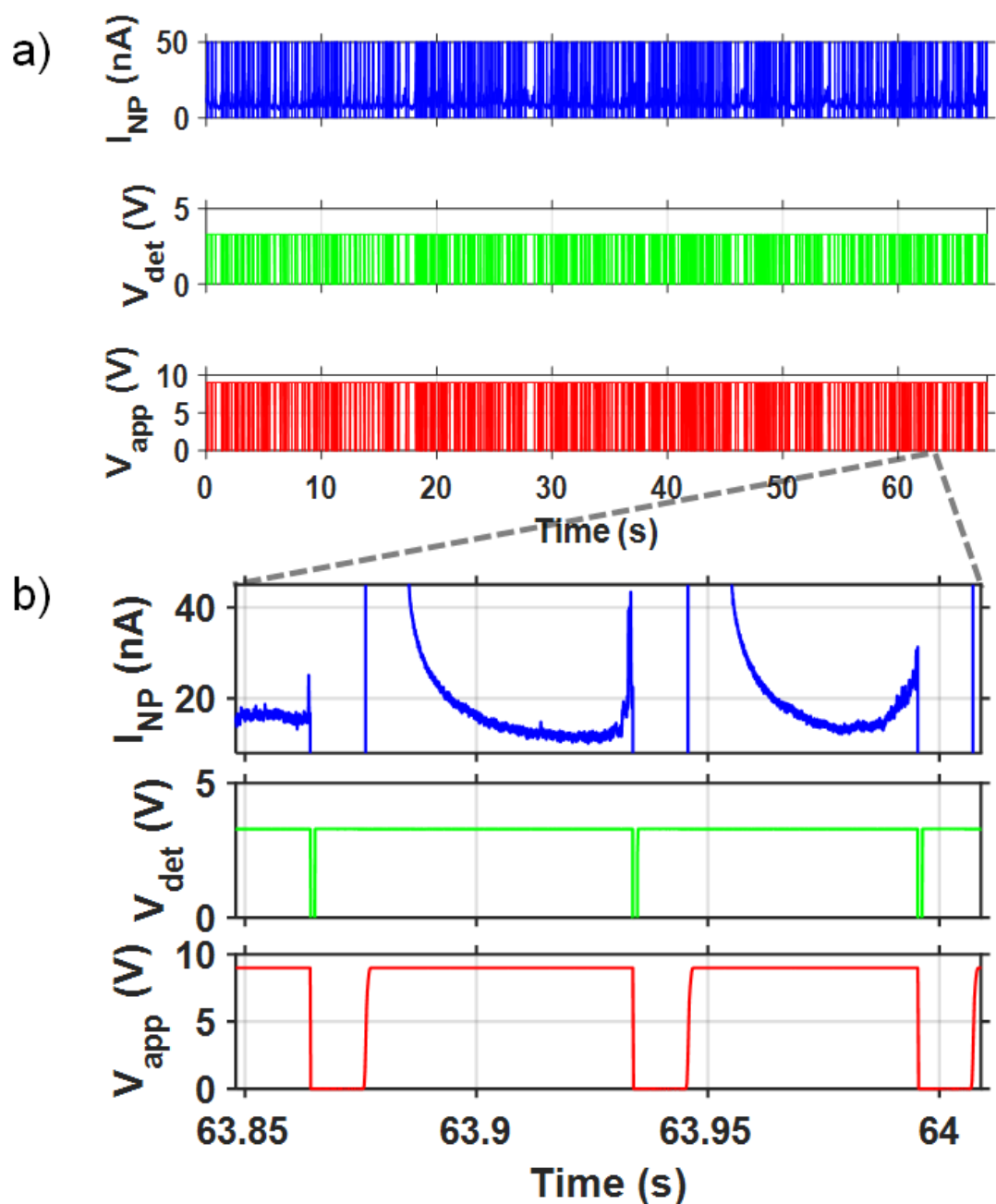


Fig. 2.18 (a) Demonstration of automated delivery of 464 λ -DNA molecules (411/min) into the microfluidic channel where the voltage is re-applied 10 msec after each translocation; top: nanopore current, center: translocation detection pulses, bottom: voltage applied across pore; (b) zoomed-in view of Fig. (a) revealing translocation events and how pore is “switched off” for 10 msec after a translocation has been detected.

a zoomed in time segment of the trace is shown in Fig. 2.18 (b). In this case, 464 gated

translocation events are observed with a delivery rate of >400/min. This time, the nanopore was kept off for 10 msec and every time the pore was turned on 10 msec after a translocation. This demonstration reinforces the statement that the feedback system is capable of handling a wide range of biomolecules with different fluidic environment in a highly controlled way.

2.6 Selective target gating from a mixture of biomolecules

One very promising aspect of the feedback system is the capability of identifying particles based on the real time analysis of their translocation pattern. As different

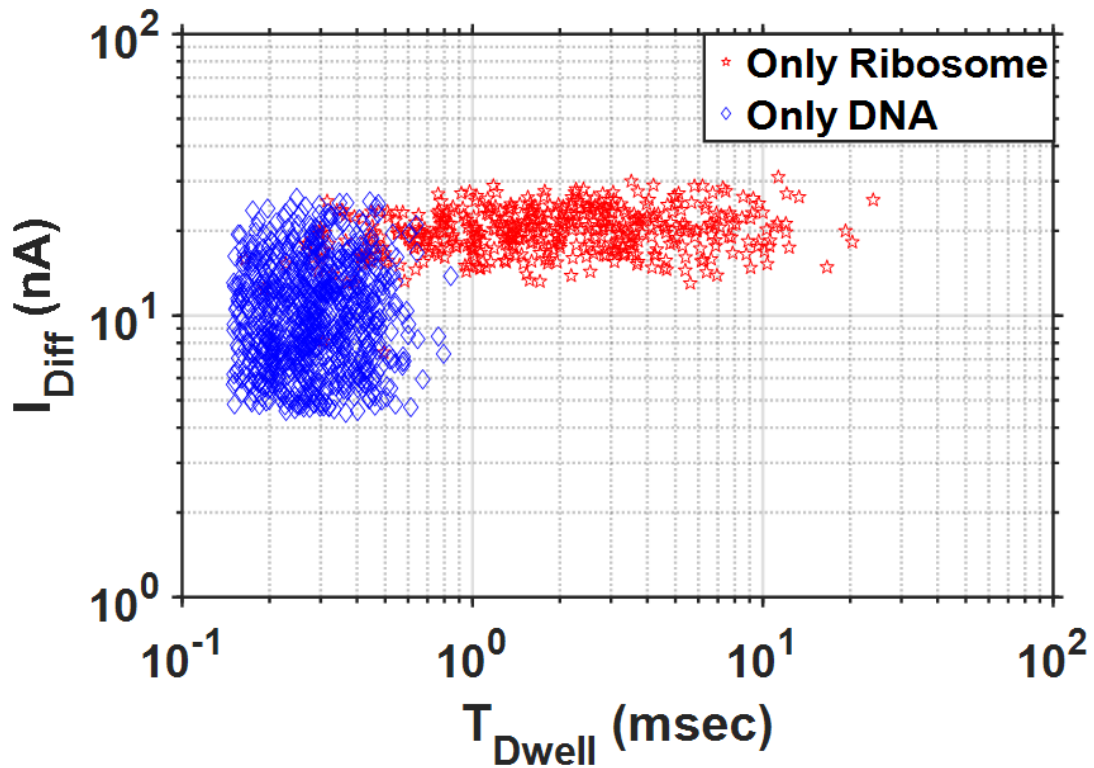


Fig. 2.19 Scatter plot of dwell time vs differential current of each translocation events when only λ -DNAs (blue) and only ribosomes (red) are drawn through the same nanopore.

particles have different size, charge and electrophoretic mobility, it is expected that they will produce different translocation signatures as they pass through the nanopore and this has already been demonstrated by different research groups[74]–[79], [124], [125]. If two particles produce distinguishable enough translocation signatures when they pass through the same nanopore, it is possible to identify the particles based on their individual translocation pattern which forms the basis of our selective target gating approach. In our case, we demonstrate selective voltage gating of λ -DNAs from a mixture of ribosomes and λ -DNAs when they pass through the nanopore. At first, only ribosomes (suspended in ribosome buffer) were loaded to the device and the translocation pattern of individual ribosomes were observed. In general, translocations are characterized and represented by their respective dwell time and differential amplitude[55], [56], [126]. We have also characterized the translocations using their individual dwell time and differential amplitudes. Next, λ -DNA samples (suspended in ribosome buffer) were introduced to the device and their translocations were observed and characterized in the similar way as ribosomes. Fig. 2.19 shows a scatter plot of dwell time vs differential current of each translocation events when only ribosomes (red) and only λ -DNAs (blue) translocate through the pore. As seen in the Fig. 2.19, translocations with differential amplitude less than 10nA and dwell time less than 0.8 msec are generated by λ -DNAs, which are used as criteria for selective gating of DNAs. The differential amplitudes can be monitored by tracking the peak height of individual translocations. The dwell times of the translocations are monitored using the microcontroller loop iteration time. For 5 samples/iteration, each loop run time is

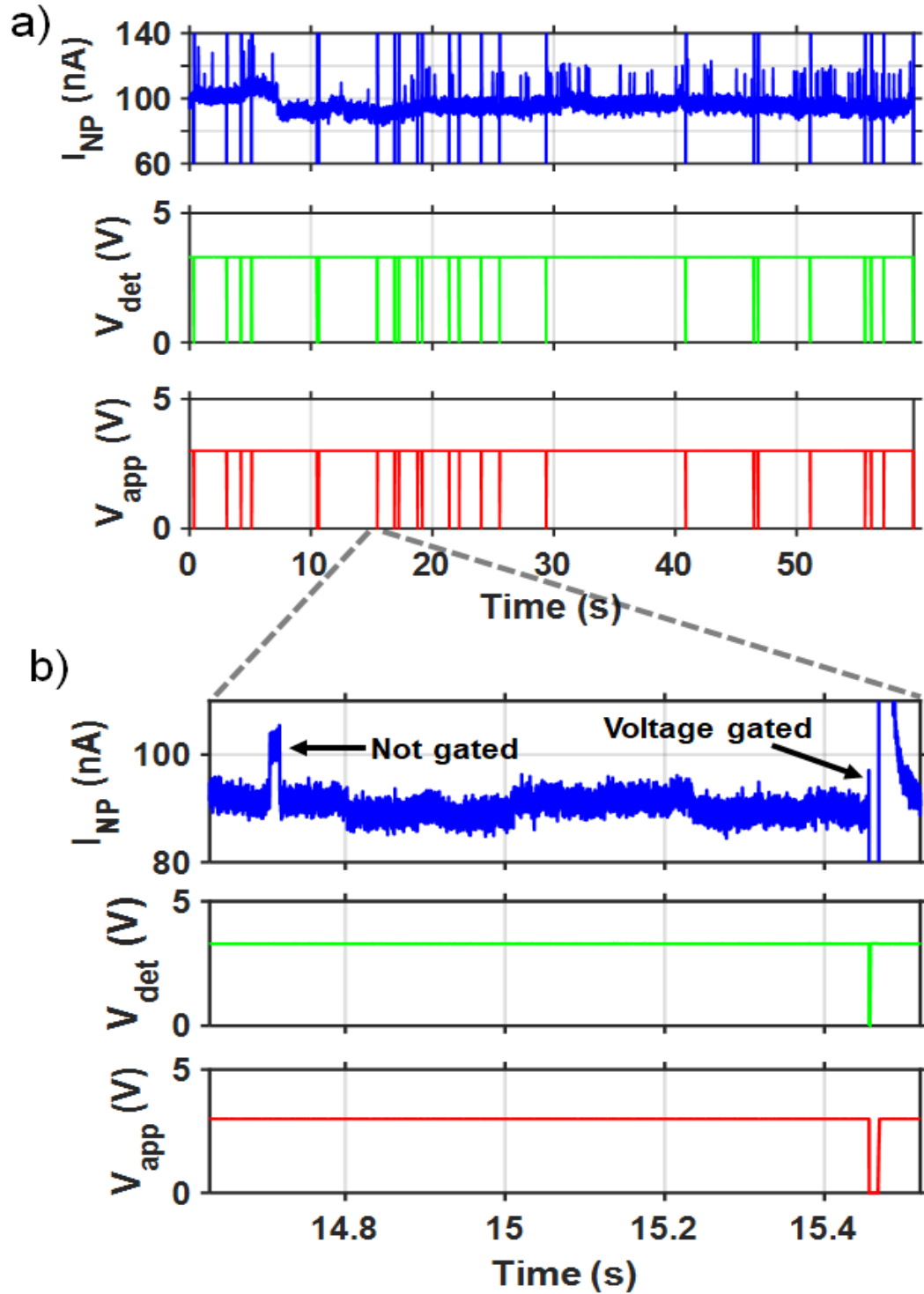


Fig. 2.20 (a) Experimental trace of selective voltage gating of λ -DNAs from a mixture of ribosome and λ -DNA; top: current through nanopore, center: specific translocation detection pulses, bottom: voltage applied across pore; (b) zoomed-in view of panel (a), revealing how specific targets are voltage-gated while others are not gated.

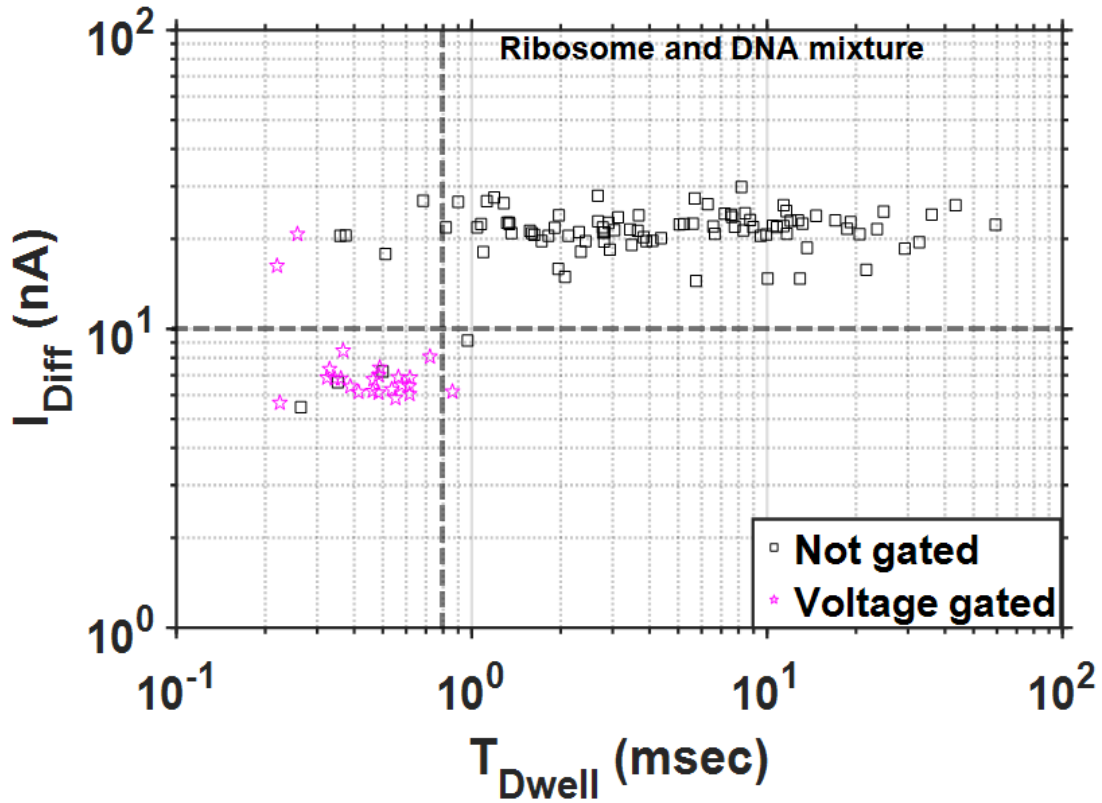


Fig. 2.21 Scatter plot of dwell time vs differential current of each translocation event of Fig. 2.20 (a), where magenta points show voltage-gated translocations, and black points show the translocations which are not gated, with thresholds shown by dotted lines.

mixture of ribosomes and λ -DNAs (suspended in ribosome buffer) were loaded to the device and translocations corresponding to λ -DNAs (differential amplitude less than 10nA as well as dwell time less than 0.8 msec) were voltage gated while the voltage gating was not applied to translocations which didn't fulfill the stated criterion. An experimental trace of the selective λ -DNA gating is shown in Fig. 2.20 (a) where the top trace shows current through the nanopore with translocations generated by the mixture of ribosomes and λ -DNAs. The detection signal for translocations which fulfill the amplitude and duration criteria are shown in the center trace. The bottom trace

shows the applied voltage across the pore which was turned off only when the microcontroller identified the translocations fulfilling the threshold criteria. Fig. 2.20 (b) illustrates a close up view of a small time segment of Fig. 2.20 (a), which reveals how specific translocations are voltage-gated based on predetermined criteria while others are ignored. Next, a scatter plot of translocations (dwell time vs differential current) found in Fig. 2.20 (a) is shown in Fig. 2.21 where the magenta markers represent the voltage gated events and the black markers represent the events which were not voltage gated. The horizontal dotted line shows the amplitude ($\sim 10\text{nA}$) threshold and the vertical dotted line show the duration threshold ($\sim 0.8\text{ msec}$) applied for the selective voltage gating. The accuracy of the gating circuitry is calculated from the 119 translocations considered in the trace shown in Fig. 2.20 (a). Among them, 24 are voltage gated, 3 are missing and 3 are incorrectly gated. This yields a total of 21 true positives (TP) and 92 true negatives (TN). The accuracy is calculated as follows-

$$Accuracy = \frac{TP+TN}{Total} = \frac{21+92}{119} = 94.96\% \quad (1)$$

This demonstration exemplifies the power and efficacy of the feedback system[80]. As the system is reconfigurable, it is possible to voltage gate ribosomes by adjusting corresponding gating thresholds.

This capability can be used to pick out single particles of a desired type for subsequent analysis in the channel. For example, if only DNAs are fluorescently labeled, they can be selected with this mechanism and studied with the optical elements of the chip (Discussed in the next section). It should be noted that unlabeled particles (say ribosomes) may be translocated as well but will not pose a problem. They will not

create fluorescence signals and simply can be discarded while the circuit continues to look for the actual target.

Another potential application of translocation both particle types could be the downstream separation of different particles to different outlets based on positive identification. For example, DNAs are directed to outlet 1, ribosomes to outlet 2, while ambiguous signals result in discarding the particle into a waste outlet. Here, the gating mechanism could be activated after each translocation until the fluidic selection has been completed.

2.7 Integrated electro-optical detection of gated biomolecules

Integration of different technologies in a single platform is a powerful concept and is being employed in different fields including nanopore sensing. Nanopores have already been integrated with optics, microfluidics etc. and different advancements on integrated nanopore technologies have already been reported[127]–[130]. As described before, we also have integrated our optofluidic device with nanopores and multimodal detection of different biomolecules have already been reported[46], [60]. Particles have been electrically driven through the nanopore to the optofluidic channel and are optically detected as they pass through the optical excitation spot. However, the old method lacks a true control over particle delivery as it just successively delivers particles without having control over how many particles are delivered and any off time for further analysis between consecutive particle delivery. The desired control over particle delivery can be achieved using the new feedback system as it can be integrated

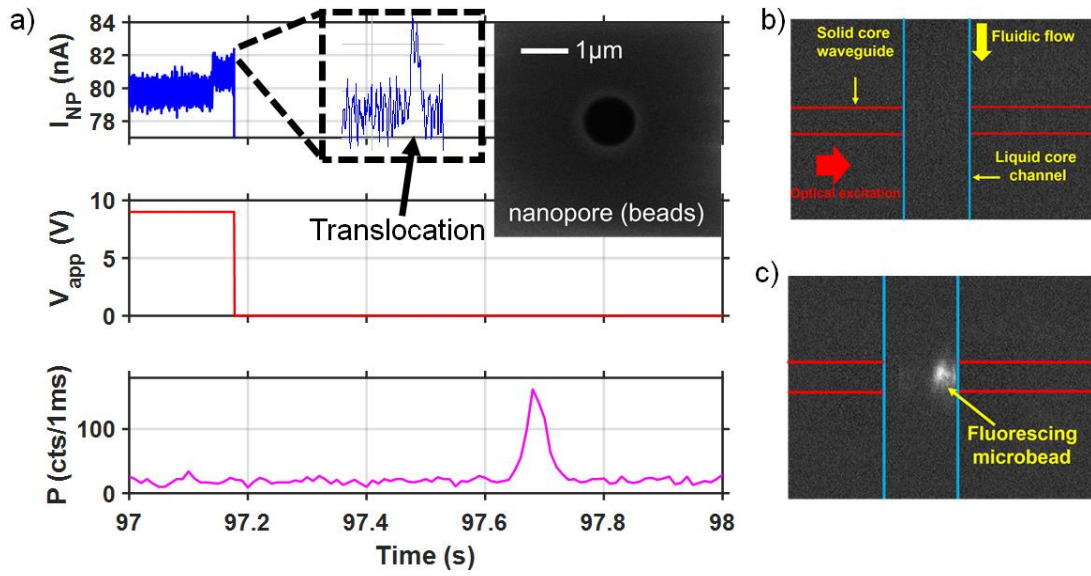


Fig. 2.23 (a) Single particle fluorescence detection on demand; top panel: current through nanopore (Inset: SEM image of the pore) and electrical detection of a single fluorescent microbead entering fluidic channel; center panel: voltage across the pore turned off after the translocation; bottom panel: concurrently recorded optical fluorescence signal showing fluorescent bead detection after characteristic transport time; (b) top down view of waveguide intersection region; (c) still picture of the fluorescing microbead passing waveguide intersection.

the excitation spot via SC waveguide as shown in Fig. 2.22. Once the particle arrives the optical excitation spot, it is optically excited and corresponding fluorescent signal is orthogonally collected using LC and SC waveguide (shown in Fig.2.22) and sent to a photodetector. The same particle is once electrically detected using nanopore as well as optically detected when it passes through the optical excitation spot hence justifies the name electro-optical multi-modal detection. It should be noted that this time, the voltage across the pore is turned off after a single translocation which restricts further particle insertion and delivery to the optical analysis region.

The first demonstration of this scheme is done using synthetic microbeads (Invitrogen). A pore of $\sim 1.4 \mu\text{m}$ diameter (Fig.2.23(a) inset) was drilled on the LC

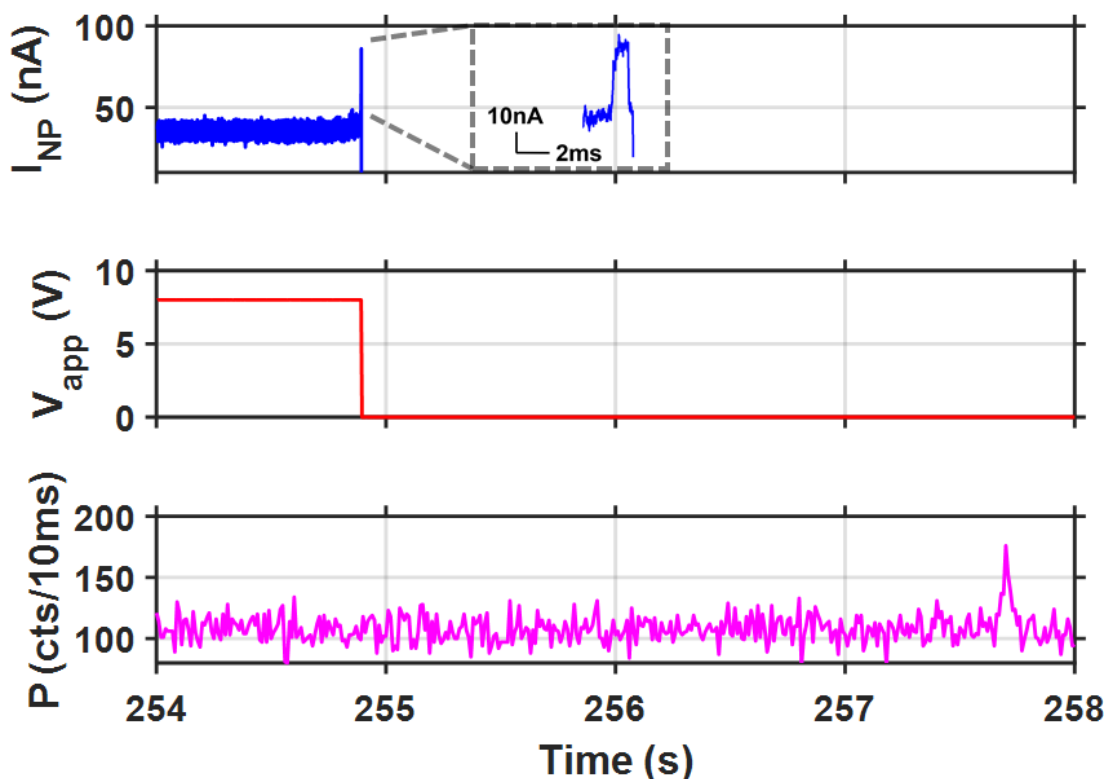


Fig. 2.24 On demand fluorescent detection of voltage gated single λ -DNA; top panel: current through nanopore (inset: zoomed in translocation); bottom panel: voltage applied across the pore; bottom panel: optical detection signal of single λ -DNA.

channel using the ion beam milling method as described before. The fluorescent microbeads were electrically driven through the pore to the optofluidic channel. The voltage across the pore was turned off as soon as the particle traversed through the pore which is shown in Fig. 2.23(a) (top and center panel). The microbead was optically detected as it passed through the excitation region. The optical detection spike is shown in the bottom panel of Fig. 2.23(a). Furthermore, the optical detection of the microbead was recorded using a custom build CCD camera in top-down view. A top down picture of an empty channel is shown Fig. 2.23(b). Fig. 2.23(c) shows a still picture of the

fluorescent microbead during optical detection which independently verifies the practicality of the system.

The optofluidic device is sensitive enough to optically detect single λ -DNAs[54], [60]. This time, the analysis system is used to optically detect the voltage gated λ -DNAs through a $\sim 20\text{nm}$ pore. The DNAs were labeled with SYBR Gold (Invitrogen) intercalating dye ($45\mu\text{L}$ of $9.55 \times 10^{11}/\text{mL}$ λ -DNA aliquot was mixed with $5\mu\text{L}$ of 2x SYBR Gold).

Fig. 2.24 shows the experimental traces of voltage gated electro-optical detection of single λ -DNA. The top panel shows the current trace with DNA translocation and the center trace shows how the voltage was turned off as soon as the DNA translocation was over, thus isolating of single λ -DNA from its group. The bottom trace shows the subsequent optical detection trace of the gated λ -DNA demonstrating on demand optical detection of single λ -DNA on a chip. The flow velocity of the particles can be measured from the electrical and optical detection signal and the physical distance between the nanopore and excitation waveguide. In this case, the λ -DNA velocity is measured to be $33.9 \mu\text{m}/\text{sec}$.

The subsequent optical signal not only serves as a confirmation of electrical detection but also reveals detail features of biomolecule(s) which can be used for screening single particle delivery. The electrical detection of λ -DNAs may generate ambiguous translocation signals with multiple humps which may arise from folded DNAs or even multiple DNAs translocate simultaneously if the pore size is relatively big enough to allow more than one DNA to pass through[79], [124], [125]. In our case,

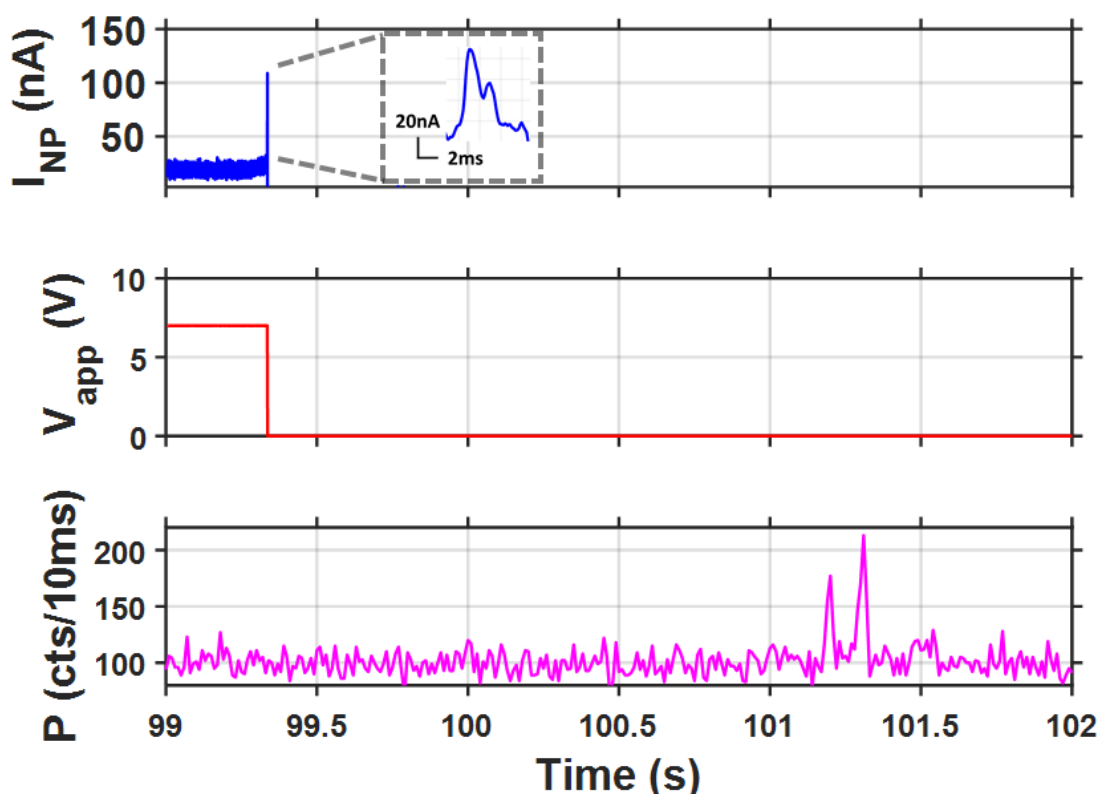


Fig. 2.25 Voltage gating of an ambiguous translocation (top trace and inset); bottom panel: voltage applied across the pore; bottom panel: the ambiguity of the double-peak electrical signal is removed by the optical detection after both DNA molecules have separated in the channel.

though most λ -DNA translocations are observed from single DNAs, some multi-peak signals were also seen. As we have the optical detection modality, it can now be used as a second source of verification on single molecule delivery. Fig. 2.25 shows such an example where a double-peak translocation signal was generated from λ -DNAs (top panel). The center panel shows the applied voltage trace which was turned off after the translocation. The bottom panel shows the subsequent optical detection trace with two clear peaks which reveals that the double-peak signal in fact generated from two λ -DNAs which were separated enough while passing through the LC channel. It should be noted that it will be difficult to conclude the nature of translocation from the

electrical signal alone without any subsequent optical detection[80]. In a true single molecule analysis system, multi-particle events could be discarded based on the optical trace and the gated optical detection can be repeated and even automated (as described before) for high throughput optical analysis of single biomolecules on demand at rapid succession.

2.8 Remaining challenges for future improvements

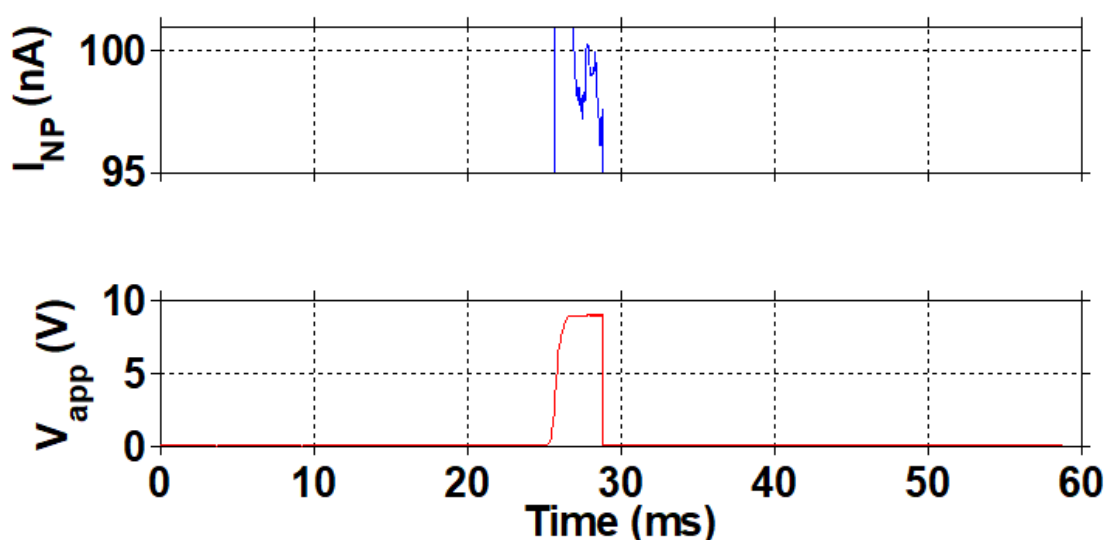


Fig. 2.26 Target gating at capacitive falling edge; top panel: nanopore current with translocation at falling edge; bottom panel: voltage applied across the pore.

In the current configuration, the built-in ADC (integrated with PSoC board) is used to convert the analog current signal to the digital domain. The ADC has a sampling frequency of 48kHz which is comparatively slower than the processor speed which can be boosted up to 67MHz. With 5 samples per iteration, the ADC takes ~ 0.104 msec to acquire the number of samples. Thus, it becomes challenging to detect narrow pulses (< 0.2 msec). Moreover, due to the slow sampling, it may become challenging to trace the sharp rising and falling edges of translocation pulses. Therefore, the ADC is one of

the major performance limiting factors of the current system. However, this problem can be addressed by incorporating a faster ADC along with the system. A faster ADC should provide more details about a translocation in real time which could be useful for proper identification and further decision making such as specific target gating. Another issue of the current feedback system is to detect translocations at the falling edges (due to the system capacitance as shown in Fig.2.16 after voltage application or re-application). Some preliminary attempts show that it should be possible to voltage gate target particles at the falling edges. One example of voltage gating at the falling edges is shown in Fig. 2.26 where one translocation appears during the capacitive falling edge and the feedback system was able to detect the translocation and voltage gate subsequently. However, further improvements are necessary to improve the falling edge target gating functionality with confidence. Though challenging, but one possible solution to this problem can be the use of a faster and dedicated system such as Field programmable gate array (FPGA) with faster ADCs. The code can also be suitably modified to address such issues. Furthermore, though the overall code and algorithm performs satisfactorily, there is room to improve the code for better performance. Some additional filters or even digital signal processing can be employed to specifically identify translocations from noisy baselines if necessary. There is room to make the feedback system even more automated and user friendly for example optical feedback can be incorporated for rapid multimodal detection and analysis of single molecules. Additional parameters can be incorporated with the code for specific target gating, sorting and filtering applications if necessary. Overall, the feedback system can be

made compatible to employ across a variety of SMA methodologies including trapping, multiplexing, sorting, filtering etc.

Chapter 3 Trapping and manipulation of single particles on ARROW optofluidic device

Unrevealing hidden information of small bio-particles such as molecular structure, dynamics, functioning, behavior, chemical and biological processes etc. can help significantly to understand the bio particles and possibly make breakthroughs in the life sciences [131]. Single particle analysis is a critical tool which can be used to probe and interact with individual molecules on the molecular level for better understanding. There is a growing interest in prolonged analysis and investigation of single particles in a wide range of disciplines such as molecular biology, analytical chemistry, biomedicine, biophysics, physiology, genomics, and proteomics [65], [132]. Usually, biomolecules are found in aqueous solutions as that is their native and functionable environment [133]. In a fluidic environment, small particles usually jitter and jiggle around with erratic random movement as they continuously collide with the solvent molecules which is known as Brownian motion [134], [135]. The Brownian motion is usually quantified by the particle diffusion co-efficient which can be expressed according to the Stokes-Einstein equation as follows-

$$D = \frac{k_b T}{6\pi\eta a} \quad 3.1$$

where D is the diffusion coefficient, k_b is the Boltzmann constant, T is temperature, η is the fluid viscosity, and a is the radius of the molecule. According to the Stokes-Einstein relation, the diffusion coefficient (D) increases with a decrease in particle which in turn means that smaller particles will diffuse more easily and encounter more

random movement. Unfortunately, this Brownian motion prohibits small particles from being held in one place for prolonged analysis and investigation which is necessary for better understanding. Thus, it is imperative to hold (trap) a single particle at its point of interrogation without compromising the innate functioning. Thus, particle trapping, and manipulation is a ubiquitous choice for bio-scientists. The majority of the particle trapping techniques are developed based on optical trapping methods [132], [136]. However, one recent form of particle trapping which relies on fluorescence tracking and an electrokinetic feedback force commonly referred as Anti-Brownian Electrokinetic (ABEL) trap and has become a popular choice due to several advantages over optical trapping methods. Our ARROW device is a suitable platform to implement different particle trapping methods, including ABEL traps. Implementation of an ABEL trap in our ARROW platform has already been reported with the trapping demonstration of a single *E. coli* bacterium [40].

In this chapter, 1D and 2D ABEL trapping of single particles is discussed. The ARROW devices are miniaturized lab-on-a-chip platforms which facilitates integration of different technologies including electrical integration over the waveguide based optofluidic device. A complex integration is demonstrated by integrating a feedback control system along with the existing ABEL trapping platform. Using the integrated platform, single microbead delivery and subsequent ABEL trapping is demonstrated which illustrates the proof of concept and paves the way towards on demand single particle delivery and prolonged analysis on a chip.

Next, a novel two-dimensional particle trapping (ABEL trap) methodology is demonstrated and implemented which eliminates some drawbacks of the previously demonstrated one dimensional ABEL trap [137]. The design, simulation and characterization of the new 2D ABEL trapping device is discussed in detail. A new trapping algorithm is developed which can identify particle position based on generated fluorescent pattern. The trapping algorithm is validated by trapping a single microbead with 14x improved trap stiffness over the previously demonstrated 1D ABEL trap [137]. Additionally, an alternative architecture for 2D ABEL trapping is illustrated which facilitates the separation of fluidic and optics sub-sections. Trapping demonstration of single microbead is also shown in the modified device.

3.1 Trapping single molecules

Due to their extraordinary appeal, particle trapping, manipulation and analysis techniques have been propelled by recent technological advancement. Researchers have demonstrated different forms of particle trapping and manipulation techniques based on ring resonators[138], [139], photonic crystals and photonic fibers[140], [141], slot waveguides [142], evanescent field trapping [143], loss based trap[48], dielectrophoretic trap[144], surface attachment[145], magnetic tweezers[146], etc. Among them, optical methods are a popular choice for particle trapping and manipulation as they avoid physical contact with the particle of interest [132], [136]. Optical trapping was pioneered by Arthur Ashkin since the first demonstration in 1970 [147] which made a significant breakthrough in the field of particle trapping and

manipulation. Though successful for many applications, optical trapping has some drawbacks including unfavorable scaling towards smaller particle size and the potential for damaging the object of interest [65], [148]. On the other hand, approaches relying on electrical trapping forces such as ABEL trap are very suitable for particle trapping as they scale well with shrinking particle size and require ultra-low power to operate [40], [65]. Due to the advantages over other platforms, ABEL trap has recently flourished as a prominent choice for particle trapping.

3.2 Anti-Brownian electrokinetic (ABEL) trap

Unlike optical trapping where the trapping relies on momentum exchange due to elastic collision between light photon and target particle, ABEL trapping relies on two basic steps. In the first step, particle position is actively monitored by optical means and finally an electrical feedback force is applied in a way which compensates the particle movement [40], [65]. To track diffusive particles in solution, an active optical tracking with feedback mechanism was proposed by Enderlein [149]. Utilizing this idea, design and implementation of first ABEL trap has been demonstrated by Cohen with a trapping demonstration of fluorescently labeled tobacco mosaic virus [65], [150], [151]. The device was designed on a microfluidic platform which uses non-planar confocal microscopy and real time video processing to track the fluorescently labeled particles. In 2006, Armani developed another form of ABEL trap in a PDMS based microfluidic system which can not only steer single particle but is also capable of steering multiple particles[152]. In 2013, King reported a 3D-ABEL trapping

platform with a trapping demonstration of 40nm fluorescent nanoparticles [153]. Proposed algorithm and ABEL trapping guidelines have also been developed by other groups [154], [155]. We have previously demonstrated an optofluidic ABEL trap platform that provides one dimensional position control along a fluidic channel [40]. In all forms of ABEL trap, it requires significantly lower optical power compared to optical traps and is suitable for trapping smaller bio-particles which are significantly challenging to trap by other means [40], [65]. In fact, manipulation of particles as small as a single dye particle has been demonstrated using ABEL trap [66]. The ABEL trap also facilitates trapping biomolecules in commonly used buffer solutions or in distilled water [65]. ABEL trap has been used to explore previously hidden aspects of molecular dynamics which signifies the effectiveness and ability of ABEL trap [131], [156]. Hence, ABEL trap is becoming a premier choice of molecular trapping, especially at the small, molecular scale.

3.3 Feedback gated 1D ABEL trap

Prolonged analysis of single particles is an utmost desire in SMA. However, mostly, bioparticles are found as a group with their comrades. Thus, many SMA techniques study and analyze particles as a group resulting in an ensemble measurement rather than studying a single particle [157], [158]. Here, we demonstrate an elegant solution to this problem. As described before, the feedback control gating can be used to isolate a single particle from a group. Moreover, the feedback gating facilitates integration over other existing SMA tools. In the previous chapter, integration of feedback gating

with electro-optical detection of single particles has been demonstrated. This time, it is intended to integrate the feedback gating system with single particle trapping platform (1D ABEL trap) for prolonged analysis. The whole experiment can be sub-divided into two consecutive steps. First, delivering a single particle to the LC channel through nanopore following the feedback control gating methodology as described in chapter 2. Next, it is intended to ABEL trap (1D) the translocated particle on the optofluidic device which facilitates prolonged analysis. It should be noted that ABEL trap also requires electrokinetic feedback voltage to enable the trap which is kept off during the nanopore translocation process to avoid electrical interference. As soon as any translocation is seen, the nanopore voltage is automatically turned off and the ABEL trapping electronics are activated. The feedback control methodology is described in detail in chapter 2 and 1D ABEL trapping principle and methodology will be described in the following section.

3.3.1 ABEL trap (1D) in ARROW device

As stated earlier, demonstration of 1D ABEL trap on ARROW optofluidic platform has already been reported. Device schematics and trapping methodology is illustrated in Fig. 3.1. The optofluidic device is shown in Fig. 3.1(a) with interconnecting LC (gray) and SC (white) waveguides. As before, fluidic reservoirs are attached at the LC channel ends. Reservoirs are used to introduce target samples into the LC channel and Ag/AgCl electrodes are immersed into the fluidic reservoirs to apply the electrokinetic

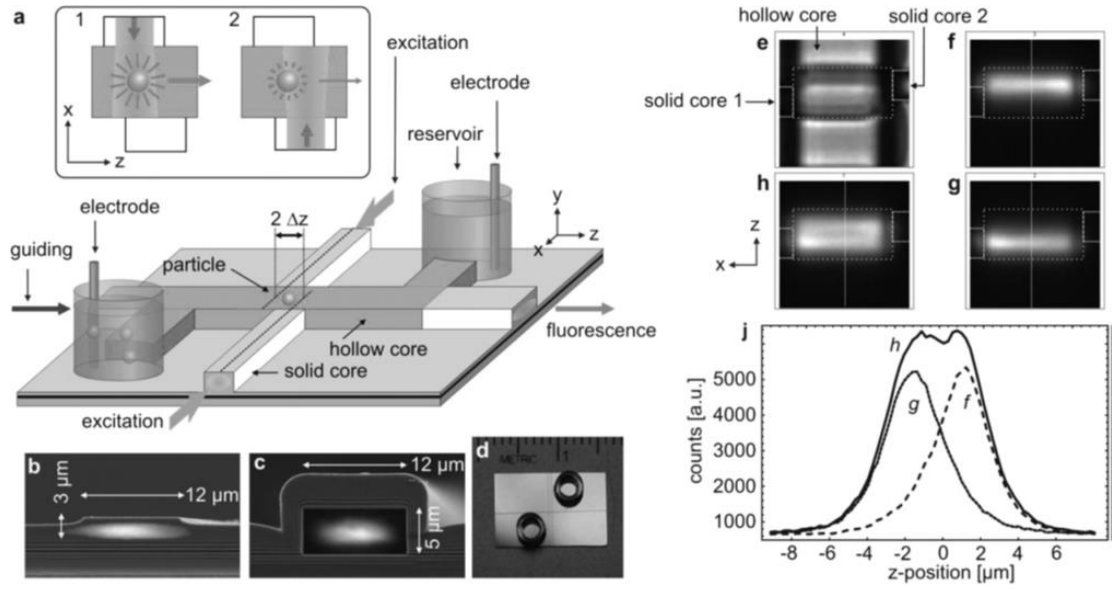


Fig. 3.1 (a) Schematic of 1D ABEL trapping device, top inset: position dependent fluorescence generation principle; (b) SEM image of a SC waveguide (cross-section); (c) SEM image of a LC channel (cross-section); (d) picture of an actual device; (e) top-down image of SC/LC channel intersection; (f) mode profile when SC₁ is excited; (g) mode profile when SC₂ is excited; (h) mode profile when both SC₁ and SC₂ are excited; (j) graphical representation of mode profiles presented in f-h. [Fig. 3.1 is taken from reference 40]

feedback voltage. This time, the two excitation SC waveguides are designed to have an offset ($2\Delta z$) between them [Fig. 3.1 (a)] to generate position dependent fluorescence signal from the target particle (Fig. 3.1 (a) inset). The device was fabricated using standard semiconductor fabrication process as described in chapter 1. SEM images of SC waveguide, LC waveguide and an actual photograph of the fabricated device are shown in Fig. 3.1 (b), (c) and (d) respectively. The SC waveguide dimensions are carefully designed to support a single mode inside the waveguides. Fig. 3.1 (e) shows a top view of the SC/LC intersection region. To visualize the optical excitation mode, the LC channel was filled with fluorescent dye solution and corresponding image was collected using a CCD camera. Fig. 3.1 (f) and (g) show the fluorescent profiles

generated by the individual SC waveguides when excited separately whereas Fig. 3.1 (h) shows the fluorescent image when both SC waveguides are excited at the same time. Fig. 3.1 (j) shows the graphical representation (cross-section view) of the fluorescent images shown in Fig. 3.1 (f)-(h). The two individual SC excitation modes show single mode profiles with maximum intensity in the middle and gradually decaying on the two sides which resemble a gaussian profile. As these optical signals are used to excite fluorescent particles, the generated fluorescent signal depends on the relative waveguide and particle position during the moment of excitation. An example of such a space dependent fluorescent signal is shown in Fig. 3.1 (a) inset, where the brightest fluorescent signal is generated when a particle sits in the middle of an individual excitation path and it becomes relatively dimmer as it sits at the sides of the individual excitation path. The generated fluorescence signal is collected via the orthogonal (relative to the excitation SC) LC-SC waveguides as shown in Fig. 3.1 (a). An ABEL trap requires determination of particle position and in this case, the particle position is determined from the generated fluorescence signal. To determine the particle position, it is necessary to modulate the optical excitation signal in a way that the position information is encoded in the fluorescence signal. This is achieved using an optical chopper (Thorlabs) which was installed in front of the excitation paths in a way that only one excitation path is allowed to go through while the other excitation path is blocked (for details see sections 3.3.2 and 3.4.4). The chopper is usually driven using a chopper driver (Thorlabs) which can be used to control the chopper rotation speed. The modulated optical excitation pattern is tantamount to a square wave as shown in

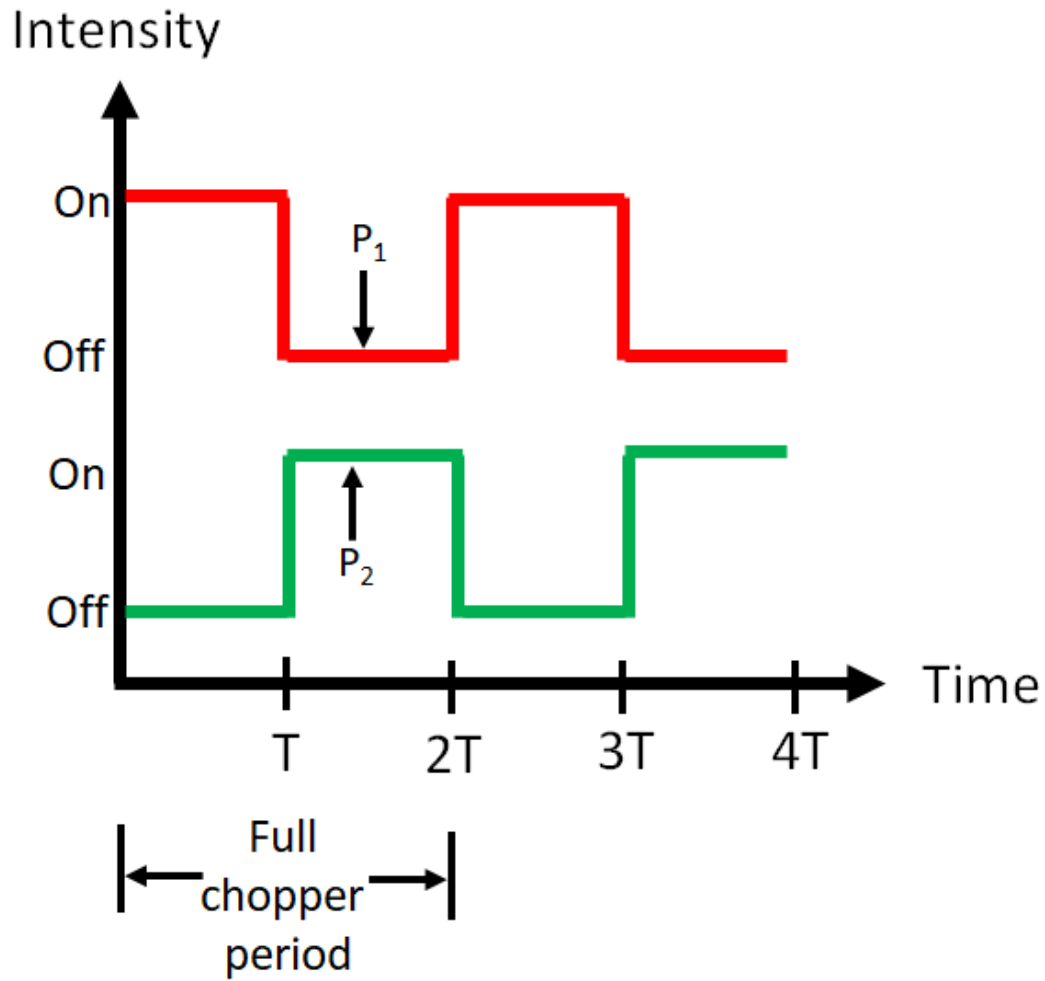


Fig. 3.2 Graphical representation of SC waveguides excitation pattern.

Fig. 3.2 where the red (path 1 or P_1) and green (path 2 or P_2) lines denote the time period (T) for which the individual excitation paths are “on” which means there will be only one optical excitation path activated at any given time. The generated fluorescence signal in each excitation period is collected and send to a single photon counter (Excelitas). The methodology for particle position determination is depicted in Fig. 3.3 where the SCs and LC channel are shown schematically, and the trapping center is shown with an arrow where the two excitation profiles intersect [Fig. 3.3(a)]. If a

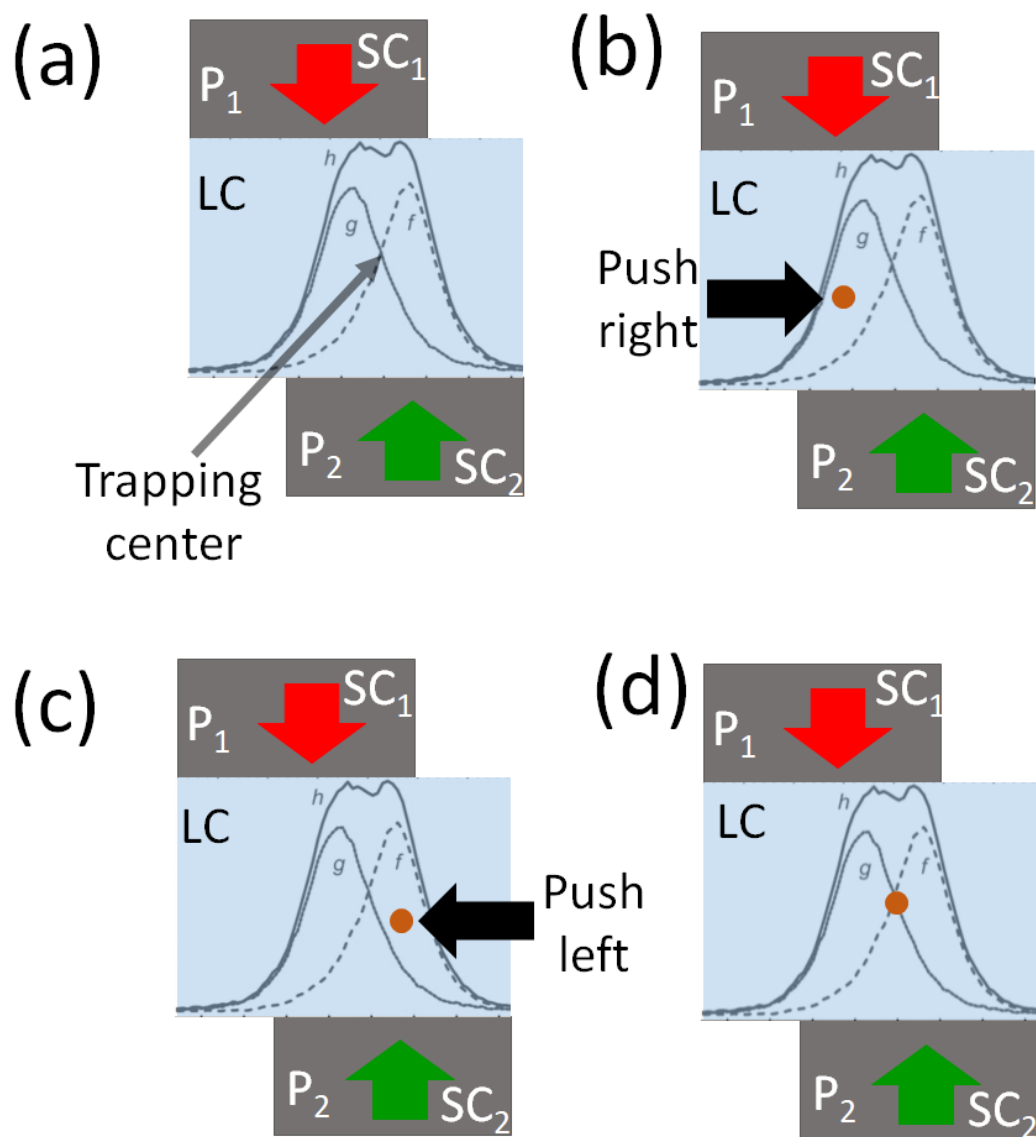


Fig. 3.3 Particle position determination in 1D ABEL trap, (a) SC LC schematics with representation of trapping center; (b) position determination when particle resides left to the trapping center; (c) position determination when particle resides right to the trapping center; (d) position determination when particle resides at the trapping center.

particle moves toward excitation path 1 (SC₁) the photon count (fluorescence signal) during P1 (C₁) will be higher than the photon count during P2 (C₂) due to the relative position dependent intensity difference between P₁ and P₂. This indicates that the

particle is positioned left to the trapping center and need to be pushed right (black arrow) toward SC_2 which is shown in Fig. 3.3 (b). Similarly, if the particle moves toward SC_2 then the C_2 will be higher than C_1 which will indicate that the particle is positioned right to the trapping center and need to be pushed left towards SC_1 as shown in Fig. 3.3 (c). Only when the particle sits at the intersection (trapping center), both C_1 and C_2 will be equal indicating that the particle is sitting at the desired position and no feedback force is required to adjust the particle position which is shown in Fig. 3.3 (d).

In the practical system, the position determination and feedback force application are implemented electronically using necessary components and circuitry embedded in a box which we usually refer as electronic box. The chopper signal and APD pulses (as electronic pulses via connectorized BNC cables) are input to the electronic box and the box outputs the feedback voltage necessary to adjust the particle position. The ABEL electronics can be sub-divided into three primary parts. The first part generates all the timing signals necessary to synchronize the chopper signal and corresponding photon counts in each excitation period (T). The second part consists of an electronic counter which determines the particle position by subtracting the photon counts generated by individual excitation paths. To do this, an 8 bit up-down counter (256 count levels) was used which was initialized at the mid count level (127) which is called “counter base value”. During the first fraction of the excitation period (P_1), the generated fluorescence signal is sent to the electronic counter in the form of APD pulses and the counter increases its count level (C_1 , as stated before) where each count level corresponds to a single APD pulse. In the next excitation period (P_2), the counter

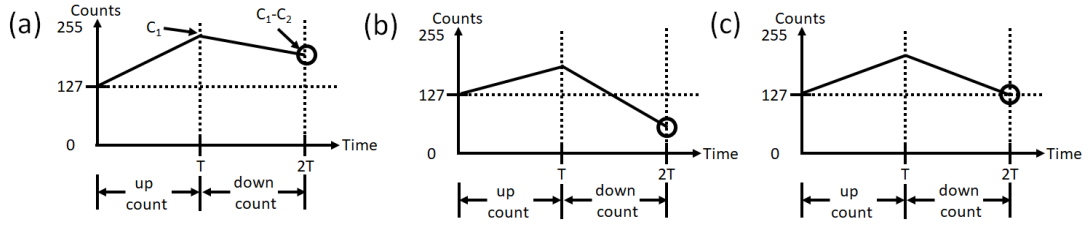


Fig. 3.4 Determination of particle position using an electronic counter, (a) position determination when particle resides left to the trapping center; (b) position determination when particle resides right to the trapping center; (c) position determination when particle resides at the trapping center.

decrements its count level from the previously counted maximum value based on the number of APD pulses received in time period P_2 (C_2). After a full chopper cycle $2T$ (P_1+P_2), the final value of the counter corresponds to the subtraction of photon counts generated in individual excitation periods (P_1 and P_2). The particle position is determined in a similar way as described in Fig. 3.3. In this case, if the final count value after a full chopper period $2T$ is more than 127 [$(C_1-C_2) > 127$] then it indicates that the particle is positioned left of the trapping center and needs to be pushed right toward SC_2 as shown in Fig. 3.4 (a). Similarly, if the final count value after a full chopper period $2T$ is less than 127 [$(C_1-C_2) < 127$] then it indicates that the particle is positioned right of the trapping center and needs to be pushed right toward SC_1 as shown in Fig. 3.4 (b). If the particle produces equal APD pulses in both excitation period and the final value of the counter after a full chopper cycle $2T$ is equal to 127 [$(C_1-C_2) = 127$] then it indicates that the particle is sitting at the trapping center and no position adjustment is necessary which is shown in Fig. 3.4 (c). The final part of the ABEL electronic box is basically a feedback amplifier circuit which generates a feedback voltage (V_F) which counteracts particle movement and pushes the particle towards the trapping center. At

the end of each chopper cycle $2T$ (P_1+P_2), a digital to analog converter converts the digital counter value into analog domain. It should be noted that the polarity of V_F can be manually flipped using a dedicated switch and proper polarity needs to be selected by the user during an experiment. The magnitude of the feedback voltage (V_F) depends on the relative difference of the counter base value (127) and the final counter value (C_1-C_2). The feedback gain can be fine-tuned using a potentiometer (gain knob upto $\pm 12V$) which is available for manual adjustment. Additionally, another potentiometer (offset knob) is also available for adjustment to compensate for unequal optical power in the individual excitation SC waveguides or flow. At the very beginning of the next chopper cycle, the electronic box applies the feedback voltage and resets its counter to the base value and the process repeats.

Using the trapping methodology, prolonged trapping of different particles including trapping of single E.coli bacteria for ~ 20 sec have been previously demonstrated [40]. Compared to optical traps, up to 5 orders of magnitude lower power was required and trap stiffness as high as 140 times higher was demonstrated [40].

3.3.2 Demonstration of gated 1D ABEL trap

To implement a gated ABEL trap, it is necessary to select an ABEL trapping device which has desirable electrical properties (electrical detection using nanopore) as well

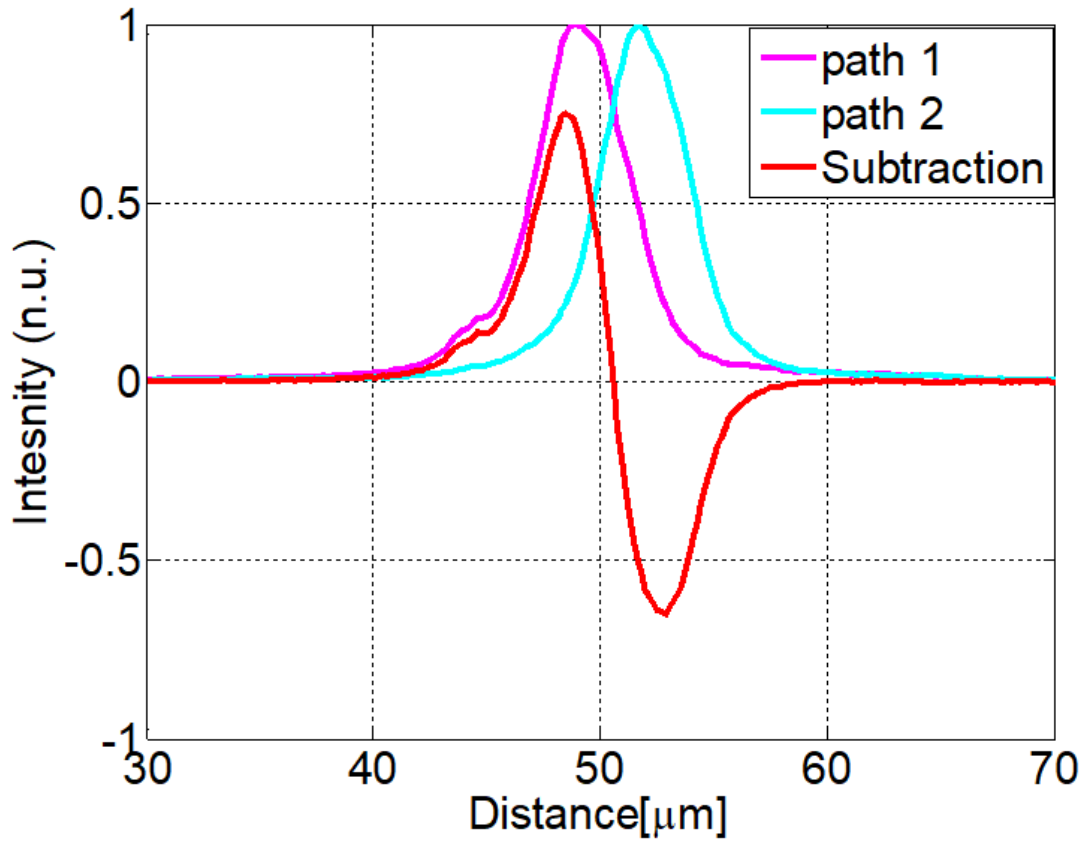


Fig. 3.5 Measured excitation profiles of P_1 (magenta) and P_2 (cyan) and corresponding subtraction (P_1-P_2 , red) curve.

as suitable optical properties (desired excitation profile with desired offset) to enable ABEL trap. At first, the device was optically characterized if that exhibits suitable enough single-mode excitation profile to trap a particle. For this purpose, the LC channel of the device was filled with cy-5 dye and excited with a 633nm HeNe laser (Newport). The optical excitation profiles are shown in Fig. 3.5, where the magenta and cyan traces are showing the profiles of excitation path 1 (P_1) and excitation path 2 (P_2) respectively. The difference (P_1-P_2) between these profiles (red) shows a steep linear region across the symmetry center which enables position recognition. The device generated a single-mode excitation pattern with desired offset which is suitable

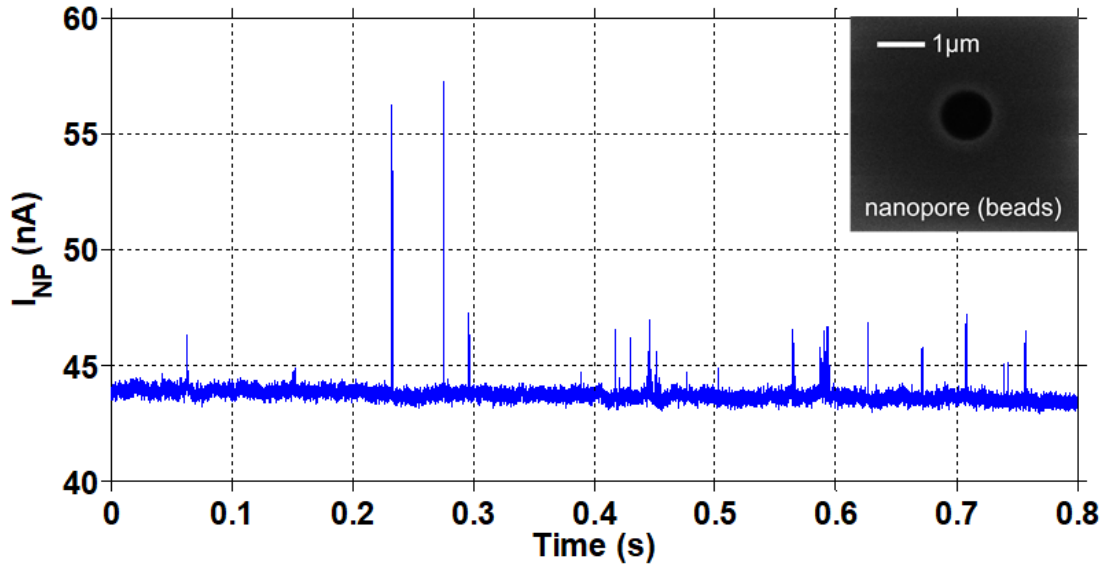


Fig. 3.6 Electrical detection trace of fluorescent microbeads; inset: SEM image of the pore used in the experiment.

for ABEL trapping. Fluorescent microbeads were selected as target particle to verify the viability of gated 1D ABEL trap. Next, a suitable size pore ($\sim 1.4\mu\text{m}$) was drilled on the LC channel using the method described in chapter 2. To electrically test the device, microbeads (suspended in 1xT50) were loaded in the nanopore reservoir and an electrical voltage was applied similarly as described in chapter 2. Due to the bias voltage, particle translocations were seen and a trace of microbead translocation is shown in Fig.3.6. The inset of Fig. 3.6 shows the SEM image of the pore used in this experiment. Once it was verified that the device performs both electrically and optically then these two techniques were integrated together to enable on demand delivery and trapping of single microbead. The schematic with detail experimental setup of gated 1D ABEL trap is shown in Fig. 3.7 where the optofluidic device is shown with offset SC

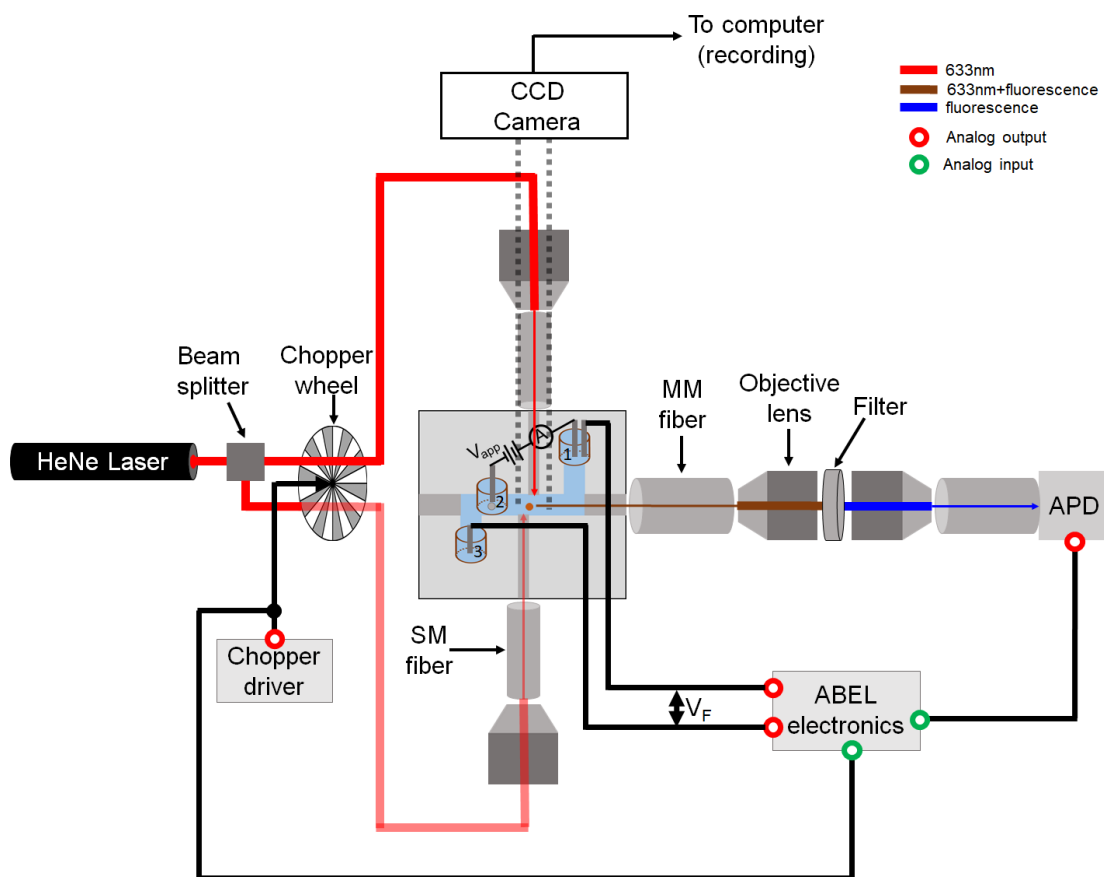


Fig. 3.7 Schematic of feedback gated 1D ABEL trapping experimental setup.

excitation waveguides. The nanopore voltage was implemented using the feedback control methodology (not shown in detail for simplicity). For ABEL trapping, the incoming laser light was split (using a 50:50 beam splitter) into two excitation paths and sent through the chopper wheel which modulated the excitation signal. The optical chopper was driven using a chopper driver which was set to modulate the optical excitation at 1kHz. The modulated excitation signals were launched to the excitation SC waveguides (SC_1 and SC_2) using single-mode fibers. The generated fluorescence signal was collected from the orthogonal (relative to excitation SC waveguides) SC waveguide using a multi-mode fiber. The filtered fluorescence signal (Omega Optical)

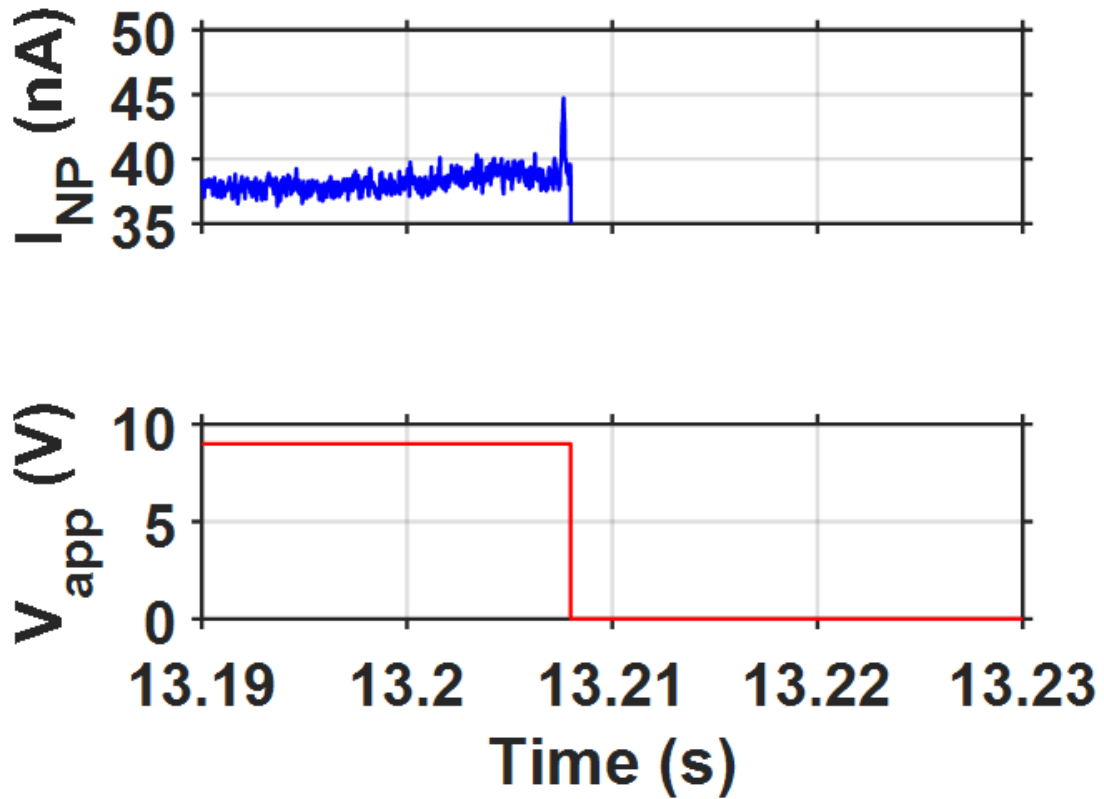


Fig. 3.8 Feedback gated delivery of single microbead; top panel: current through the pore with translocation spike; bottom panel: voltage across the pore.

is then sent to the APD via connectorized multi-mode fiber. The chopper signal was also sent to the ABEL electronic box as a reference for the electronic counter. The output pulses of APD were sent to the electronic box via a BNC cable. The trapping event was observed and recorded using a custom-built CCD camera (Andor Luca). The electronic box outputs the feedback voltage (V_F) which was applied across reservoir 1 and reservoir 3 using Ag/AgCl electrodes. As mentioned earlier, the experiment was performed in two consecutive steps. First, the microbeads were loaded in the nanopore reservoir (reservoir 2) and an electrical bias voltage was applied between reservoir 1 and 2 as shown in Fig. 3.7. As soon as a microbead translocation was detected by the

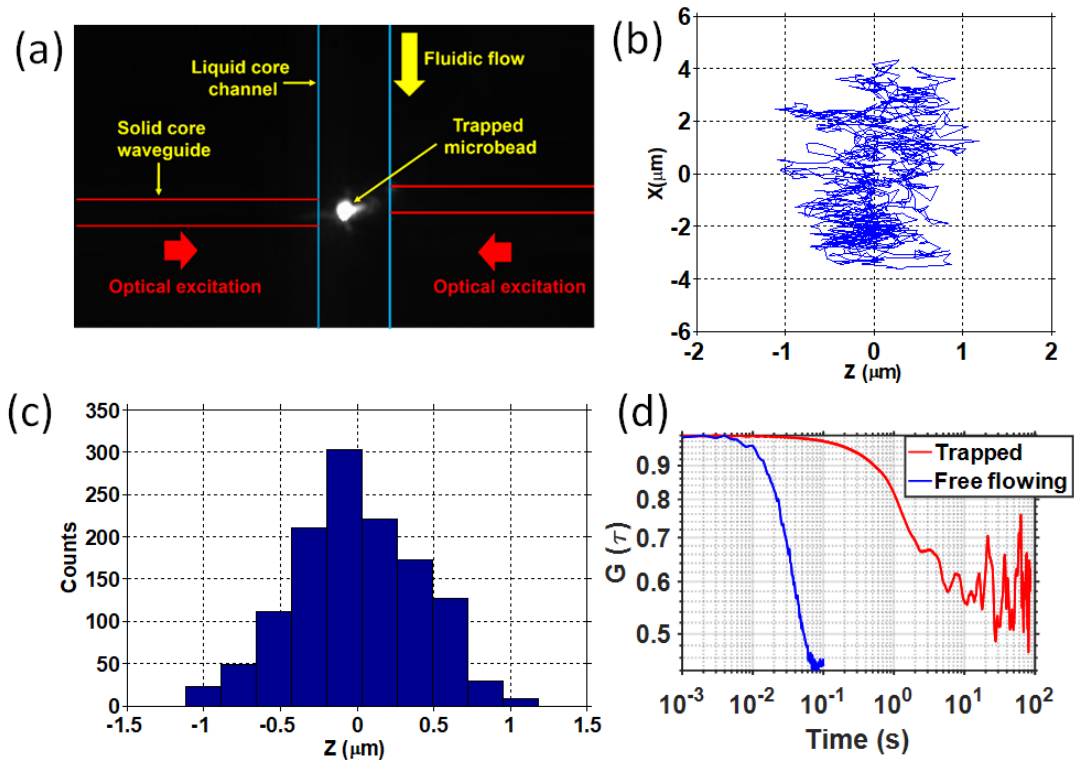


Fig. 3.9 (a) Still picture of the trapped microbead; (b) particle trajectory during trapping; (c) position histogram along the LC channel during trapping; (d) FCS curve of a free-flowing (blue) and trapped microbead (red).

feedback control system, the voltage across the pore was turned and kept off. The voltage and current traces of single microbead gating are illustrated in Fig. 3.8 where the top blue trace shows the current through the pore with the translocation spike and the bottom red trace shows how the voltage was turned off after the microbead translocation. Next, ABEL trapping electronics were activated to enable the translocated microbead trap. The bead was then successfully held in the ABEL trap following the 1D ABEL trapping principle where it was available for further analysis. A still picture of the trapped microbead is shown in Fig. 3.9 (a). The detected fluorescence signal was used to characterize the ABEL trap itself. Fig. 3.9 (b) shows

the actual particle trajectory in the ABEL trapping region which is extracted from the top-down video. From this curve, a particle position histogram (along the LC channel) can be determined which is shown in Fig. 3.9 (c). From the particle histogram, it is seen that the particle was mostly confined within a region of $\sim 2\mu\text{m}$ along the LC channel. ABEL trap can be considered as a harmonic system and an analogy of the trap can be made with the spring mass system which follows the formula-

$$F = -kx \quad 3.2$$

Where, F is the restoring force (trapping force), k is the spring constant (trap stiffness) and x is the relative displacement from the equilibrium position. Trap stiffness (k) is a parameter that quantifies the strength of a trap which can be expressed as follows [40], [151]-

$$k = \frac{k_b T}{\langle x^2 \rangle} \quad 3.3$$

Where, k_b is the Boltzmann constant, T is the temperature, and $\langle x^2 \rangle$ is the variance of particle position while trapped. Using the formula, the trap stiffness is calculated and found to be 25 nN/m which is comparable with concurrent reported values [40], [151]. Fig. 3.9 (d) shows a comparison of the autocorrelation of the fluorescence signal of a trapped versus a free-flowing particle. While the decay of the autocorrelation for the latter is governed by the combination of diffusion and pressure-driven flow, for the trapped particle, it is determined by the trapping time. An increase in residence time by over two orders of magnitude is found and trapping times in excess of one minute were observed.

This proof of concept experiment provides further insight to implement a full-fledged prolonged single particle analysis platform on a chip. The coalition of two powerful techniques like feedback gating and ABEL trap in a single platform opens a new avenue for single particle analysis and paves the way towards on demand prolonged analysis of single molecules on an integrated optofluidic chip.

3.4 2D ABEL trap

ABEL trapping (1D) in ARROW devices has several merits over optical trapping which have already been discussed earlier. However, the previously demonstrated ABEL trap can confine particles in only one dimension along the channel length. Due to the lack of an active trapping force, the particle can still freely move along the width of the LC channel while trapped. For better confinement, it is required to implement active trapping in both dimensions which is one of the major motivations towards developing a platform that enables 2D ABEL trapping. Furthermore, it has been previously seen and reported that the trap performance is limited by the inability to collect fluorescence from a particle diffusing to the channel walls [40]. Fig. 3.10 (a) shows a comparison of fluorescence collection of a particle from the top using CCD camera and side detection with an APD where it is visually clear that the fluorescence collection via APD (which is the actual signal sent to the electronic box for feedback force generation) is suppressed near the LC walls. A quantitative analysis of the relative fluorescence collection efficiency is illustrated in Fig. 3.10 (b) where it is seen that the efficiency significantly falls off near the LC side walls. This is one of the crucial

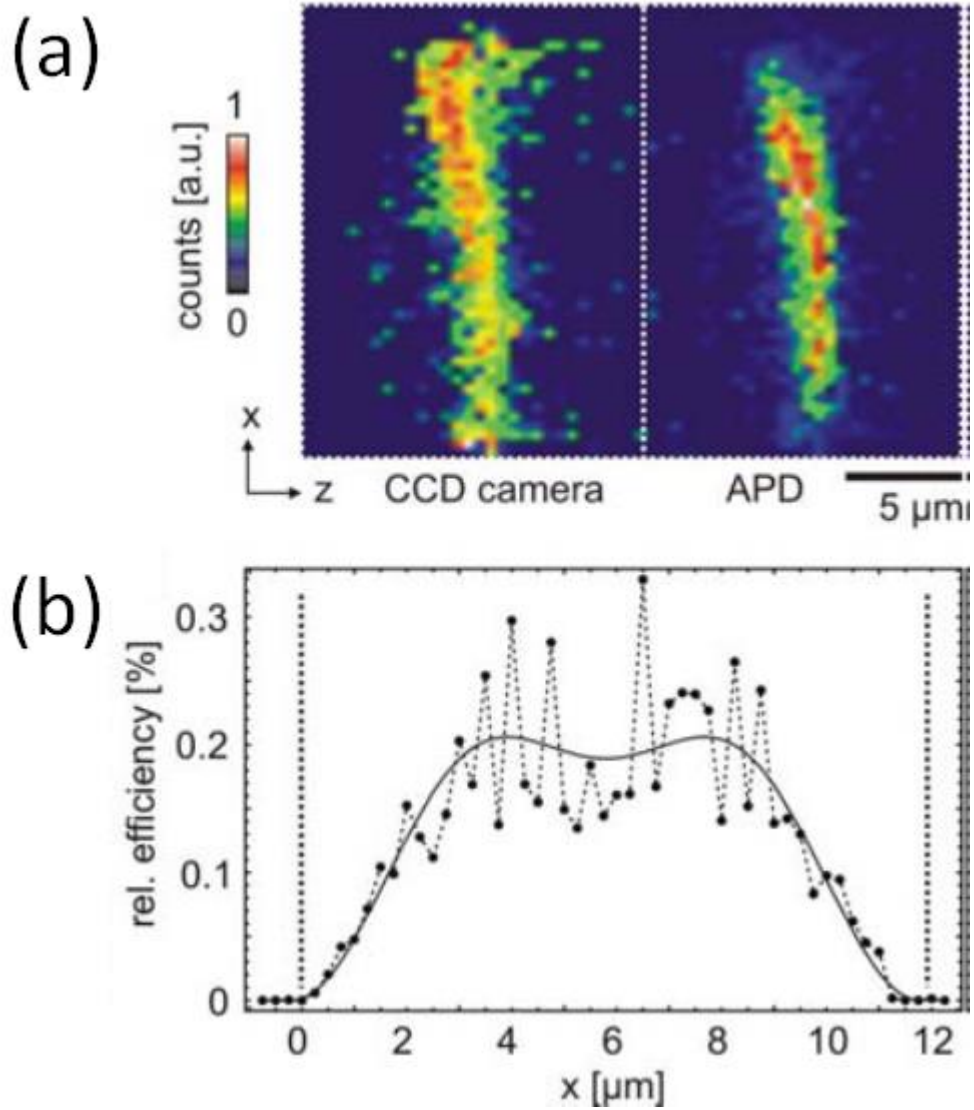


Fig. 3.10 (a) Illustration of poor fluorescence collection when collected using APD compared to a CCD camera; (b) relative fluorescence collection efficiency at different position of the LC channel (along the width). [Fig. 3.10 is taken from reference 40].

motivations towards the 2D ABEL trap which will confine particles in two dimensions prohibiting the particles to move towards the LC side walls.

3.4.1 Device design and simulation

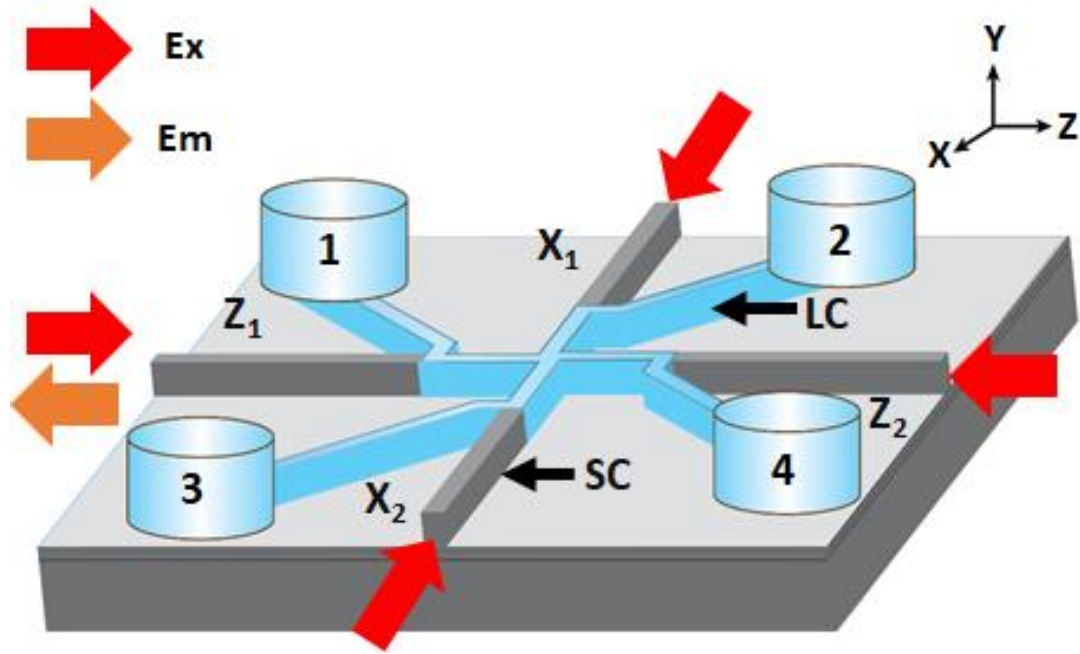


Fig. 3.11 Schematic of the 2D ABEL trapping device.

The new 2D ABEL trap is designed on the same ARROW platform with two sets of offset excitation waveguides (4 excitation waveguides in total). The core idea is to implement two 1D ABEL traps along two individual dimensions with proper correlation to form an integrated 2D ABEL trap. An outline of the new 2D ABEL trapping device is shown in Fig. 3.11 where four interconnected LC channels (blue) converge to a central, square trapping region. The counter-facing LC waveguides along X and Z directions are offset with each other which is carefully designed to enable position recognition. A detail of the particle position determination methodology will be discussed in the methodology section. Two pairs (along Z and X direction) of SC waveguides (dark gray) are connected to the offset LC channels as shown in the Figure. One pair of excitation waveguides (X_1 and X_2) is designed to counteract particle movement in Z direction and the other pair (Z_1 and Z_2) is designed to counteract particle

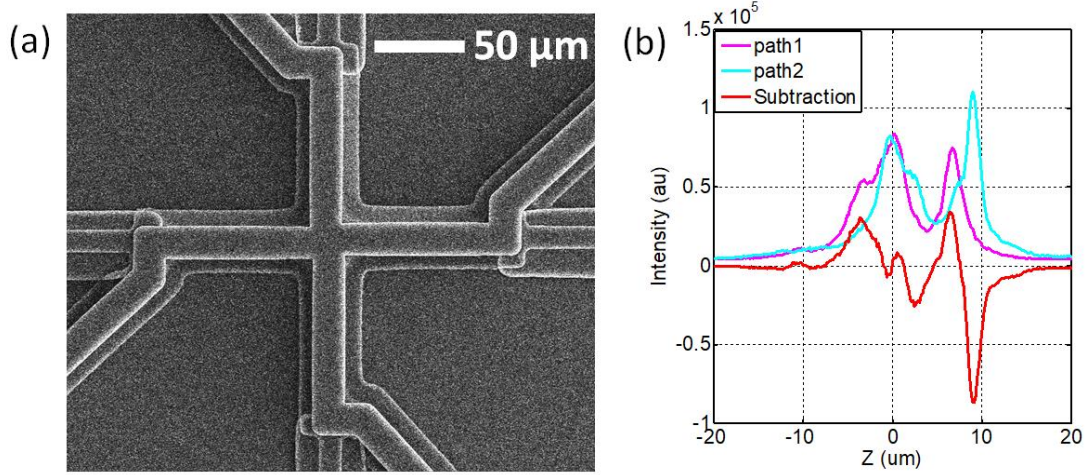


Fig. 3.12 (a) SEM image of the central region in a fabricated device; (b) excitation profiles of the SC waveguides (magenta and cyan) and their subtraction curve (red) showing multi-mode behavior.

movement in X direction. The red and orange arrows show the excitation and fluorescence collection paths respectively. Unlike the previous 1D ABEL trapping device, this 2D ABEL device does not have a dedicated fluorescence collection waveguide. Thus, one of the SC waveguides is intended to be used for both excitation and fluorescence collection purpose using a dichroic mirror.

Briefly, devices were fabricated with usual LC channel dimensions [(5 μm (height) x 12 μm (width))] following standard silicon fabrication methods (as described in chapter 1). An SEM image of the first stage fabricated device is shown in Fig. 3.12 (a). As the LC channel was wide enough to support more than one mode, the devices show multi-peak excitation profile which is not suitable for trapping. An example of such excitation profile is shown in Fig. 3.12 (b). Therefore, it is necessary to determine suitable waveguide dimensions to produce desired excitation profiles to enable ABEL

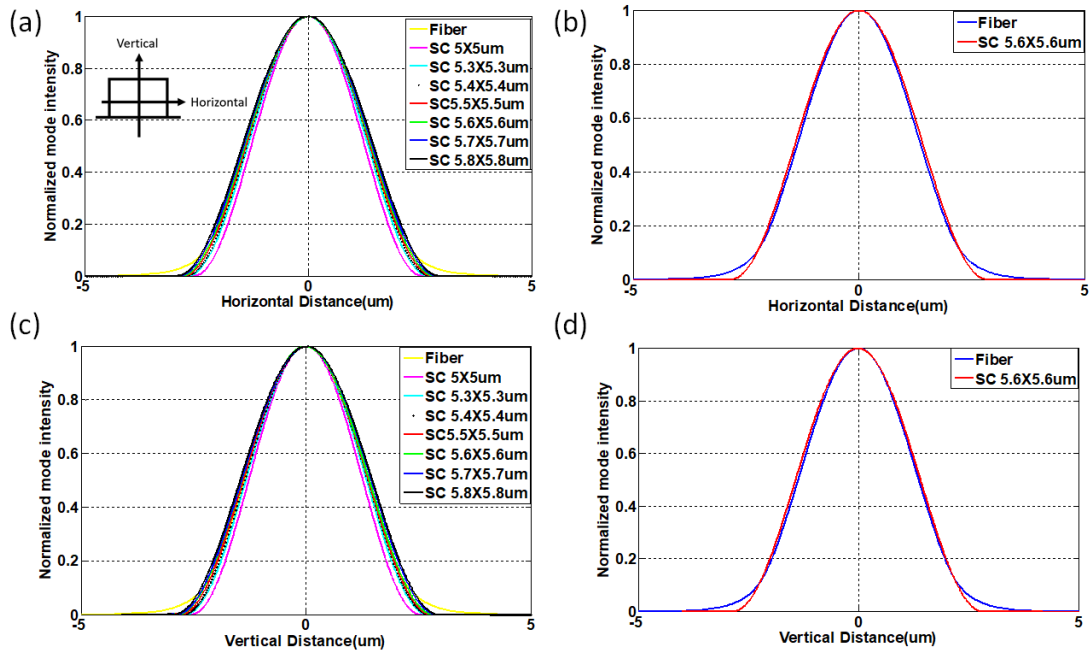


Fig. 3.13 (a) Simulation results for fiber-SC horizontal mode matching; inset: representation of horizontal and vertical direction; (b) fiber mode (blue) and closest matched SC waveguide mode (red); (c) simulation results for fiber-SC vertical mode matching; (d) fiber mode (blue) and closest matched SC waveguide mode (red).

trap. For this purpose, a simulation was performed using FIMMWAVE (a waveguide mode solver from Photon Design) to extract suitable waveguide dimensions. As the excitation light is launched to the SC waveguide via a standard single-mode fiber, it is important that the optical mode of the single-mode fiber matches with the optical mode of SC waveguide which depends on the waveguide dimension. For simulation purpose, the mode field diameter of the single-mode fiber was considered to be $4.3\mu\text{m}$ and the optical wavelength was chosen to be 633nm to match the HeNe laser output. For optical mode matching, the mode intensities are observed in two dimensions (horizontal and vertical as shown in Fig. 3.13 (inset) as well as are graphically represented for quantitative comparison. Fig. 3.13 (a) shows a comparative plot of horizontal mode intensity profiles of SC waveguides for different dimensions. From the graph, it turns

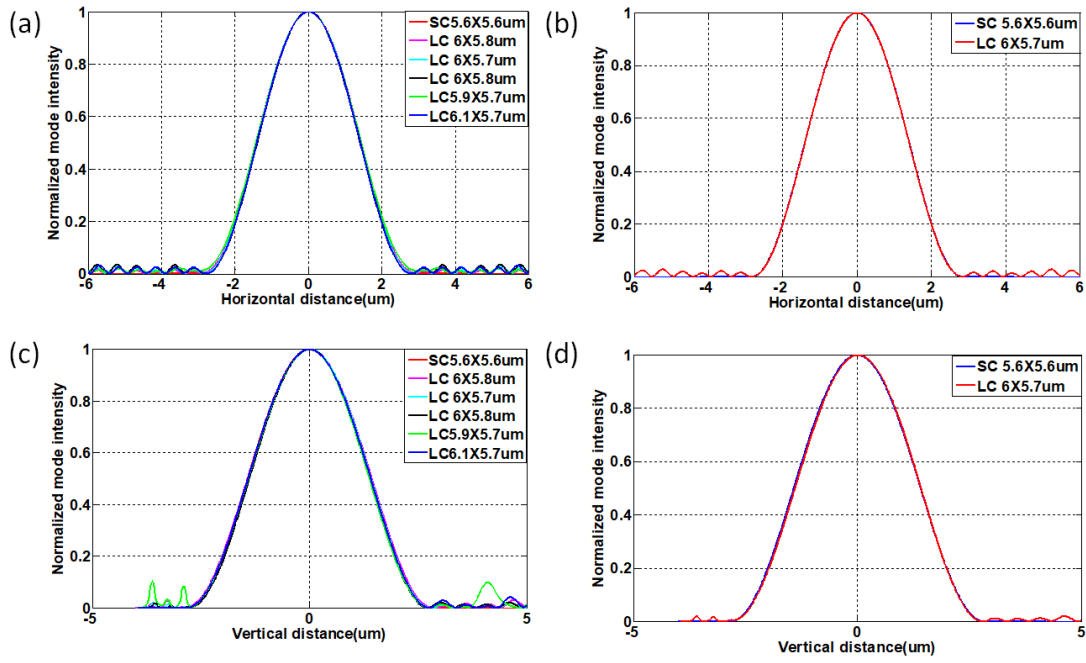


Fig. 3.14 (a) Simulation results for SC-LC horizontal mode matching; (b) SC mode (blue) and closest matched LC waveguide mode (red); (c) simulation results for SC-LC vertical mode matching; (b) SC mode (blue) and closest matched LC waveguide mode (red).

out that a SC waveguide with a width of $5.6\mu\text{m}$ shows best mode matching with a single-mode fiber which is shown in Fig. 3.13 (b). Similarly, the vertical mode intensity is also plotted in Fig. 3.13 (c-d) and it is found that a SC height of $5.6\mu\text{m}$ shows best mode matching with a single-mode fiber. Thus, a SC waveguide with a dimension of $5.6\mu\text{m} \times 5.6\mu\text{m}$ is expected to provide best excitation pattern for ABEL trap. Next, the excitation light is launched to the LC waveguides from the SC waveguide via SC-LC waveguide intersection. Thus, it is also necessary to find suitable LC dimension to match the incoming SC waveguide mode. The horizontal mode intensities for different LC dimensions are plotted in Fig. 3.14 (a) and it is found that a LC width of $6\mu\text{m}$ matches with the SC waveguide mode (Fig. 3.14 (b)). In a similar

way, the height of the LC waveguide is also determined and found to be $5.7\mu\text{m}$. The vertical mode intensity plots are shown in Fig. 3.14 c-d.

Next, it is necessary to determine the required offset between the counter-facing waveguides which provides best trapping performance. The feedback force for particle trapping, thus, the trapping stiffness depends on the offset between the individual excitation profile as shown in red curve in Fig. 3.5. The steeper the difference curve the larger the trap stiffness. Therefore, for best trapping performance, it is necessary to determine the offset in a way that results in steepest difference curve. For simplicity, the excitation profiles are assumed to be gaussian in shape which are offset with each other by an arbitrary distance $2a$. The two gaussian profiles $f_1(x)$ and $f_2(x)$ can be expressed as follows-

$$f_1(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x+a)^2}{2\sigma^2}} \quad 3.4$$

$$f_2(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-a)^2}{2\sigma^2}} \quad 3.5$$

where σ is the standard deviation and a is the center of the gaussian profiles. The difference curve $G(x)$ can now be written as follows-

$$G(x) = f_1(x) - f_2(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x+a)^2}{2\sigma^2}} - \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-a)^2}{2\sigma^2}} \quad 3.6$$

Now, the slope $[S(x)]$ of the difference curve $[G(x)]$ can be found from the derivative of $G(x)$ with respect to x which can be expressed as follows-

$$S(x) = \frac{d(G)}{dx} = \frac{1}{\sigma\sqrt{2\pi}} \left[\frac{-(x+a)}{\sigma^2} e^{-\frac{(x+a)^2}{2\sigma^2}} + \frac{(x-a)}{\sigma^2} e^{-\frac{(x-a)^2}{2\sigma^2}} \right] \quad 3.7$$

The slope of the difference curve at the intersection point ($x=0$) of the two excitation profiles can be expressed as-

$$S(x = 0) = \frac{1}{\sigma\sqrt{2\pi}} \left[-\frac{(2a)}{\sigma^2} e^{-\frac{(a)^2}{2\sigma^2}} \right] \quad 3.8$$

Finally, the optimum separation between the two gaussian beams can be found by simply differentiating equation 3.8 with respect to a , setting it to zero and then solving the equation for a .

$$S_{max}(a) = \frac{d(S(x=0))}{da} = \frac{1}{\sigma\sqrt{2\pi}} \frac{-(2)}{\sigma^2} e^{-\frac{(a)^2}{2\sigma^2}} \left[1 - \frac{a^2}{\sigma^2} \right] = 0 \quad 3.9$$

Since $e^{-\frac{(a)^2}{2\sigma^2}}$ cannot be zero, it can be written that $\left[1 - \frac{a^2}{\sigma^2} \right] = 0$, which results in $a=\sigma$.

Therefore, the two excitation profiles should be separated by twice the standard deviation for optimum trapping performance. From simulation results, the standard deviation (σ) of the excitation profiles is found to be $1.2\mu\text{m}$ (FWHM= $2.8355\mu\text{m}$). Thus, the optimum offset is calculated to be $2.4\mu\text{m}$ (2σ).

3.4.2 Device fabrication and characterization

Once the waveguide dimensions are determined, the modified devices were fabricated by the Hawkins group using standard fabrication procedure. Briefly, the devices were created on top of a 100mm, <100> oriented Si substrate, with the alignment designed for proper facet cleaving on the four edges of the chip. Six alternating dielectric layers of SiO_2 ($n=1.47$) and TA_2O_5 ($n=2.107$) were then sputtered over the whole wafer, by Evaporated Coatings Inc., to thicknesses of 265 nm and 102 nm respectively, forming the ARROW layer stack, which acts as the substrate in subsequent fabrication steps.

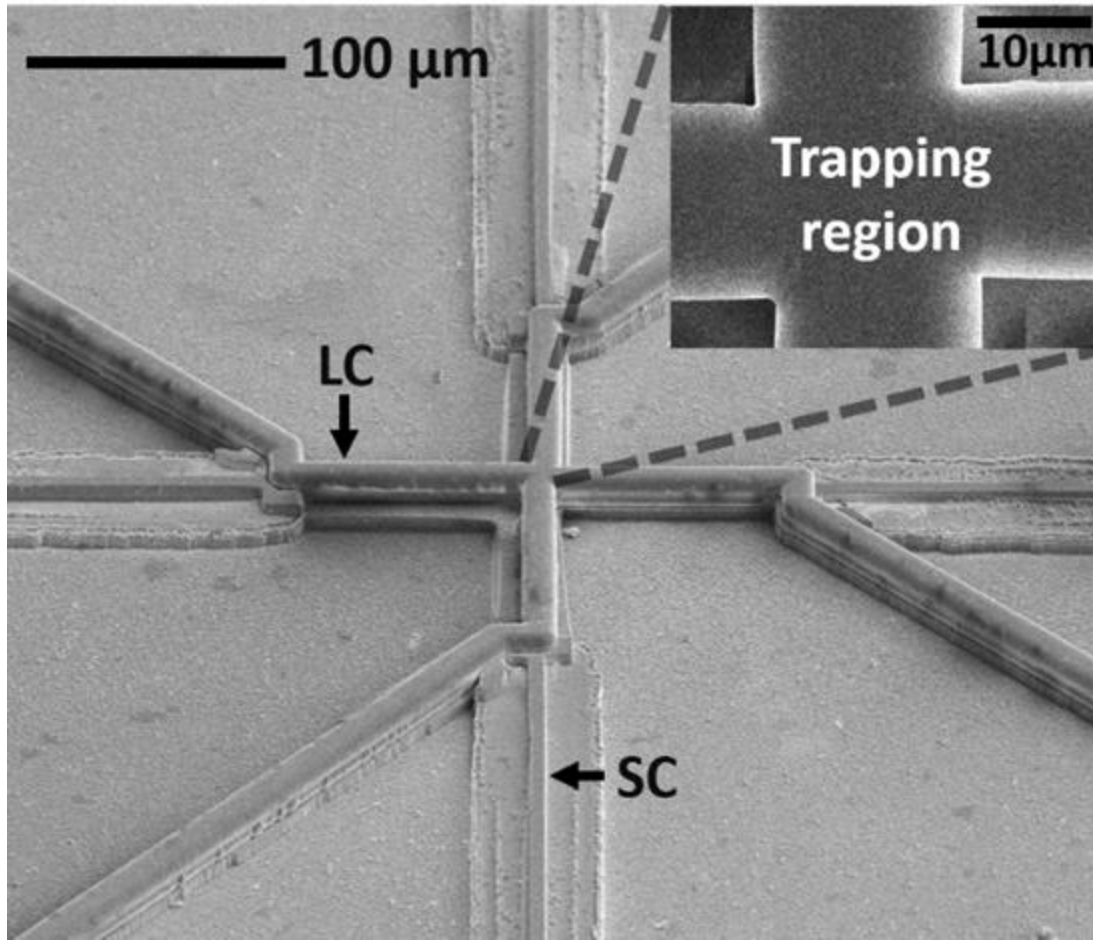


Fig. 3.15 SEM image of the central region in a fabricated device showing how SC and LC waveguides are interconnected; inset: zoomed-in image of the trapping region.

The hollow optofluidic liquid core channels are $6\mu\text{m}$ tall and start at a width of $12\mu\text{m}$ near the fluid reservoirs placed at the corners of the chip. These channels then taper to a width of $6\mu\text{m}$ near the trapping region. The total length of these fluid channels is 4.25mm . The channels were formed by first defining them using standard lithography procedures for SU-8, and then hard baking the layers at a maximum temperature of 250°C to withstand further processing. A self-aligned pedestal was defined by using reactive ion etching to etch through the sputtered ARROW stack and then approximately $3\mu\text{m}$ deep into the underlying silicon substrate. The purpose of the

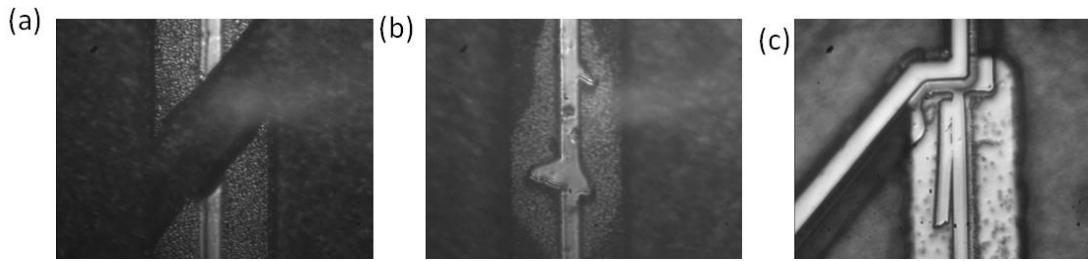


Fig. 3.16 Example of some fabrication glitches; (a) broken SC waveguide; (b) irregular width in SC waveguide; (c) branch in SC waveguide.

pedestal is to improve the structural integrity of the hollow cores in the subsequent sacrificial etch process. Once the pedestal is defined, a $6\mu\text{m}$ thick PECVD oxide layer was deposited over the wafer using a low stress deposition recipe to reduce potential core cracking. Excitation and collection waveguides were then patterned using typical lithography procedures involving a nickel hard mask and etched with an RIE etcher to create $3\mu\text{m}$ tall rib waveguides. The rib waveguides are $5.6\mu\text{m}$ wide and run from the chip edge to interface with the liquid core optofluidic waveguides. The original SU-8 cores used to define the liquid core structures were exposed at the corners of the chip by removing the oxide with buffered hydrofluoric acid. The wafer was then placed in a strong acid to remove the SU-8, hollowing out the liquid core channel. After completion of the microfabrication steps, individual chips were cleaved from the wafer to a size of $10\text{mm} \times 10\text{mm}$. An SEM image of the central region in a fabricated device is shown in Fig. 3.15 which shows how the LC and SC waveguides are interconnected to form the trapping region (inset).

Once the devices were fabricated, they were thoroughly characterized to find an appropriate device suitable for ABEL trapping. At first, the devices were visually inspected, and several fabrication glitches were found during this process such as

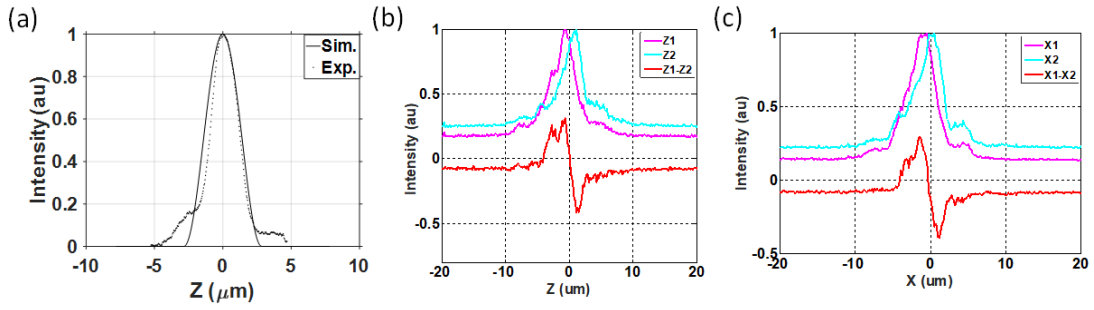


Fig. 3.17 (a) Optical mode profiles of SC waveguide (Solid line simulation, dotted line experiment); (b) optical intensity profiles at central trapping region with Z_1 and Z_2 excited; (c) optical intensity profiles at central trapping region with X_1 and X_2 excited.

broken SC waveguides, anomalies in SC waveguides, branches in SC waveguides etc. which are shown in Fig. 3.16 (a-c). Proper measures were taken to mitigate these glitches in next wafer fabrication. The most important aspect of the characterization process is to observe the optical excitation profiles. At first, the optical mode profile of a SC waveguide was observed as graphically illustrated in Fig. 3.17 (a), where the solid and dotted traces denote the simulated and experimental mode profiles respectively. The profiles show good matching with a FWHM of $2.83\mu\text{m}$ and $2.4\mu\text{m}$ for simulation and experiment, respectively. Next, the LC channels were filled with cy-5 dye and excited with 633nm HeNe laser. In ARROW devices, annealing improves the optical performance which has been seen before [159] and this observation is also consistent with the 2D ABEL trapping devices. Fig. 3.17 (b-c) show the X and Z excitation profiles of a 2D ABEL trapping device without annealing. After annealing the excitation profiles improves significantly which is shown in Fig. 3.18 (a-b). The optical excitation profiles (magenta and cyan) at the central trapping region when only the SC waveguide pair along the Z direction (Z_1 and Z_2) was excited are shown in Fig. 3.18 (a). The red trace represents the difference of the two profiles and shows a steep linear

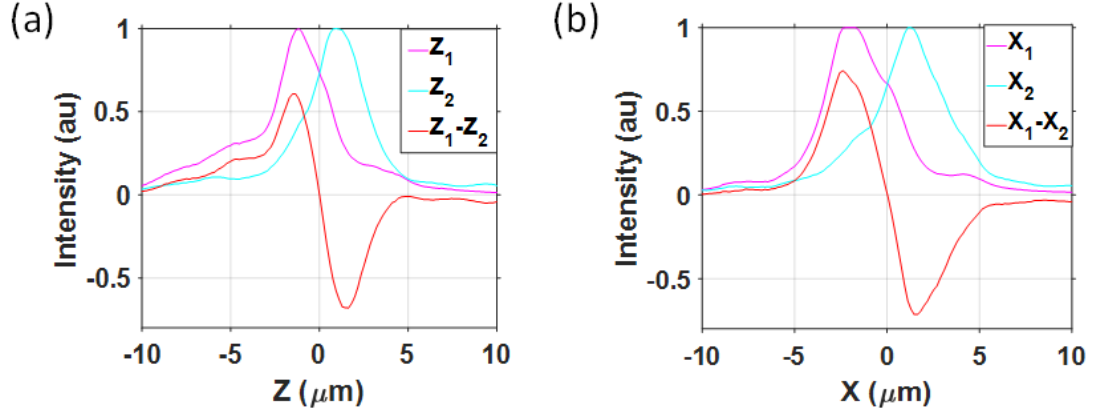


Fig. 3.18 (a) Optical intensity profiles at central trapping region with Z_1 and Z_2 excited; (b) optical intensity profiles at central trapping region with X_1 and X_2 excited.

region across the symmetry center that enables position recognition for applying feedback. Similar optical profiles result when the SC waveguide pair along the X direction (X_1 and X_2) is excited (Fig. 3.18 (b)). The excitation profiles show good enough characteristics to enable ABEL trapping.

3.4.3 Trapping principle and methodology

A major part of ABEL trap is to determine particle position properly. The feedback correction force is generated based on the particle location determination. Thus, it is very important to determine the particle position with precision. An outline of the central trapping region is shown in Fig. 3.19(a) with the bold lined square. Outside the central trapping square, X_1 , X_2 , Z_1 and Z_2 show the outlines of the four individual excitation paths. One excitation light path is shown in Fig. 3.19(a) [light green color] when only the Z_2 waveguide is on. It can be visioned from the figure that the excitation paths will mutually overlap with each other within the central trapping square. Based on the overlapping excitation regions of the four individual excitation paths, the central

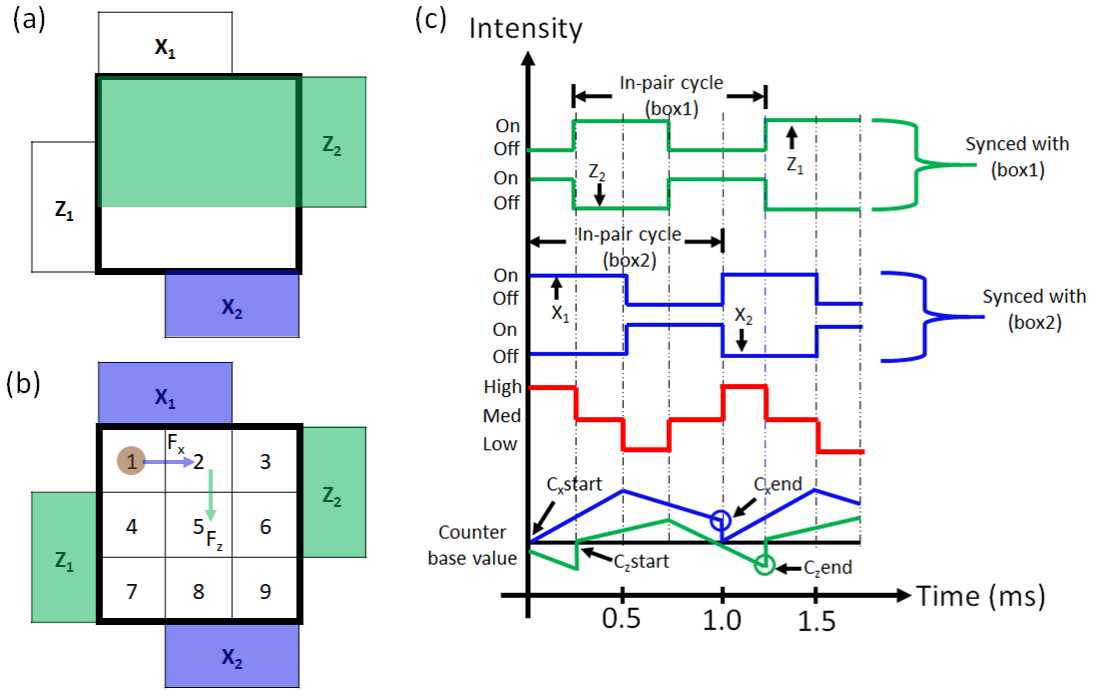


Fig. 3.19 2D ABEL trapping principle; (a) Central trapping region with Z_2 waveguide excitation path (green); (b) subdivision of central trapping region for position identification with a particle sitting at segment 1 (brown circle) and corresponding feedback force directions (blue and green arrows); (c) excitation scheme (top green and blue traces) with an example of encoded fluorescence pattern (red) when particle resides at segment 1, and corresponding counter states (bottom blue and green traces).

trapping region can be sub-divided into nine segments [as numbered from 1 to 9] which is shown in Fig. 3.19(b) where segment 5 denotes the trapping center. As the trapping relies on optical position determination, 2D position information must be encoded into the fluorescence signal generated by the target particle. Thus, it is necessary to devise a SC excitation sequence which can generate distinct a fluorescence pattern for each of the segments to determine particle location. This time, there are four excitation waveguides and the excitation pattern should be correlated with each other to enable particle position determination. The top four traces of Fig. 3.19 (c) illustrates an excitation scheme (top green traces for Z_1 and Z_2 , following blue traces for X_1 and X_2)

where each counter-propagating SC waveguide within a pair is alternately excited (Z_1 is 180° out of phase w.r.t Z_2 , same for X_1 and X_2) whereas the waveguide pairs are 90° out of phase with respect to each other (Z_1/Z_2 is 90° out of phase w.r.t X_1/X_2). Here, two ABEL electronic boxes (similar to the 1D ABEL electronic box) are necessary to generate the feedback force. One in-pair excitation (Z_1 and Z_2) signal is synchronized with one electronic box (same as chopper reference signal of 1D ABEL trap) whereas the other in-pair excitation (X_1 and X_2) signal is synchronized with one electronic box. Each of the excitation signals is designed to be modulated at a frequency of 1kHz. As the excitation signals are designed to have overlapping “on” period, the whole chopper cycle (1msec in total) can be divided into four time-segments (0.25msec each) as shown with the vertical dotted lines in Fig. 3.19(c). From the figure, it clear that any of the two excitation beams are “on” within any time-segment.

Now, the particle can reside in any of the segments (1 to 9) and the core idea of the excitation scheme is to identify the particle location (segment number) based on the generated fluorescence signal. As two excitation paths are “on” within an individual time-segment (0.25msec), three possible excitation scenarios are possible based on how the particle gets excited. To illustrate this, let’s assume that the particle is residing in segment 1 as shown in Fig. 3.19(b). One possible scenario is that the particle gets excited with both excitation paths (when Z_2 and X_1 are “on”) and the corresponding generated fluorescence is referred to as “high” state. The second possibility is the particle gets excited with only one excitation path (when Z_1 and X_1 is “on”) and the corresponding generated fluorescence is referred to as “medium” state. The third

possible scenario is that the particle doesn't fall within the excitation region of any of the excitation paths (when Z_1 and X_2 is "on") and the corresponding generated fluorescence is referred as "low" state. The red trace in Fig. 3.19 (c) shows the whole fluorescence pattern generated by a particle when it resides in segment 1. Similarly, uniquely separable fluorescence patterns are generated when the particle resides in any of the segments.

Next, the encoded location information is sent to the dedicated electronic boxes using a single photon avalanche photodiode. As stated earlier, the overall electrokinetic feedback force is generated using two identical electronic boxes. One of the boxes (box1) generates a feedback voltage to limit particle movement in the X direction which is synced with the excitation cycle of Z_1 and Z_2 [0.25 ms - 1.25 ms is a full cycle as shown in the bottom green trace in Fig. 3.19(c)] whereas the other electronics box (box2) is synced with the excitation cycle of X_1 and X_2 [0 ms – 1 ms is a full cycle as shown in the bottom blue trace in Fig. 3.19(c)] and limits particle movement in the Z direction. Similar to the 1D ABEL electronic box, each of the electronic boxes contains an up-down counter (C_Z/C_X) which counts the APD pulses originating from the trapped particle. The counters count up [increase counter value from 127 (counter base value)] for the first half cycle of the in-pair excitation period (Z_1/X_1) and count down [decrease counter value] for the duration of the second half cycle (Z_2/X_2) [same way as the 1D ABEL counter described in earlier section]. The bottom blue (C_X) and green (C_Z) traces in Fig. 3.19(c) depict the representation of both counter states based on the encoded fluorescent signal when the particle resides in segment 1. Each of the electronic boxes

generates a feedback voltage depending on the respective counter state after corresponding in-pair excitation cycle as encircled on the counter traces in Fig. 3.19(c) [1.0 ms for C_x , 1.25 ms for C_z]. Similar to the 1D ABEL principle, the direction of the feedback force depends on the end cycle count location (greater/smaller than the base value) whereas the magnitude of the feedback force is proportional to the final counter value (relative count difference from the base value). The feedback forces are applied at the very beginning of next in-pair excitation cycles and the counters are reset back to their base value (127). For the particular example, when a particle resides at segment 1, the direction of the feedback forces generated by box1 (F_z) and box2 (F_x) are shown in Fig. 3.19(b) [green and blue arrows respectively]. The resultant feedback force pushes the particle towards the trapping center (segment 5). In a similar way, the algorithm identifies the particle position residing in any of the segments and generates feedback force to push the particle to the trapping center.

3.4.4 2D ABEL trapping demonstration

The trapping functionality is demonstrated by 2D trapping a single microbead following the above stated principle. A schematic of the whole experimental setup is illustrated in Fig. 3.20 where the 2D ABEL trapping device is outlined in the middle of the figure. At first, the laser (633nm) light was split into four different paths using 50:50 beam splitters (Thorlabs) as shown in Fig. 3.20. After that, all the four paths were carefully sent through an optical chopper wheel (Thorlabs) to achieve the devised

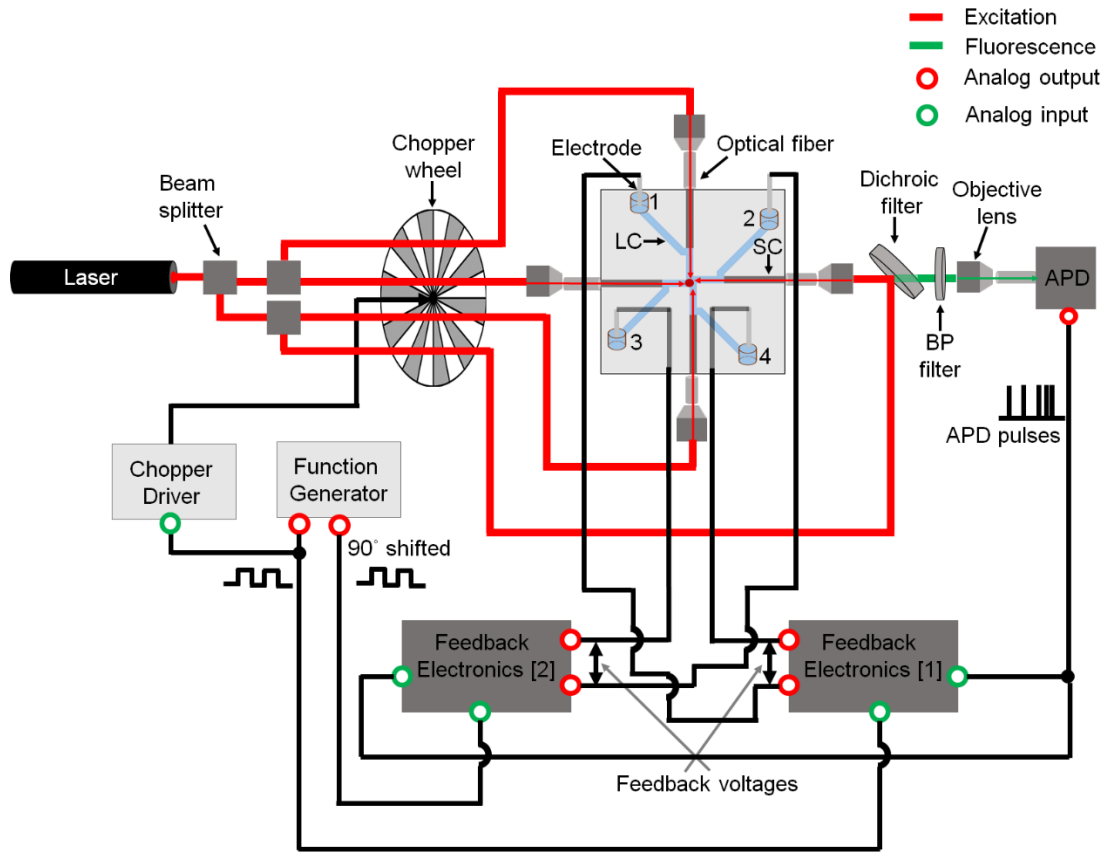


Fig. 3.20 Schematic representation of the 2D ABEL trap experimental setup.

excitation pattern as shown in Fig. 3.19. The chopper wheel was operated by a chopper driver which was externally triggered (1 kHz square wave) using a function generator (Agilent). The chopper driver adjusts the wheel rpm in a way which optically modulates (on/off) all four paths at the triggered frequency (1 kHz). Due to the relative positions of the four excitation paths at different chopper slots, Z_1 and Z_2 is alternately (180° out of phase) excited which is true for X_1 and X_2 as well. Moreover, the excitation pairs ($Z_{1/2}$ and $X_{1/2}$) are 90° shifted relative to each other. All four modulated excitation light beams were then coupled into single mode fibers (Newport) to launch light into the device using objective lenses (Newport). One of the SC waveguides was used for both

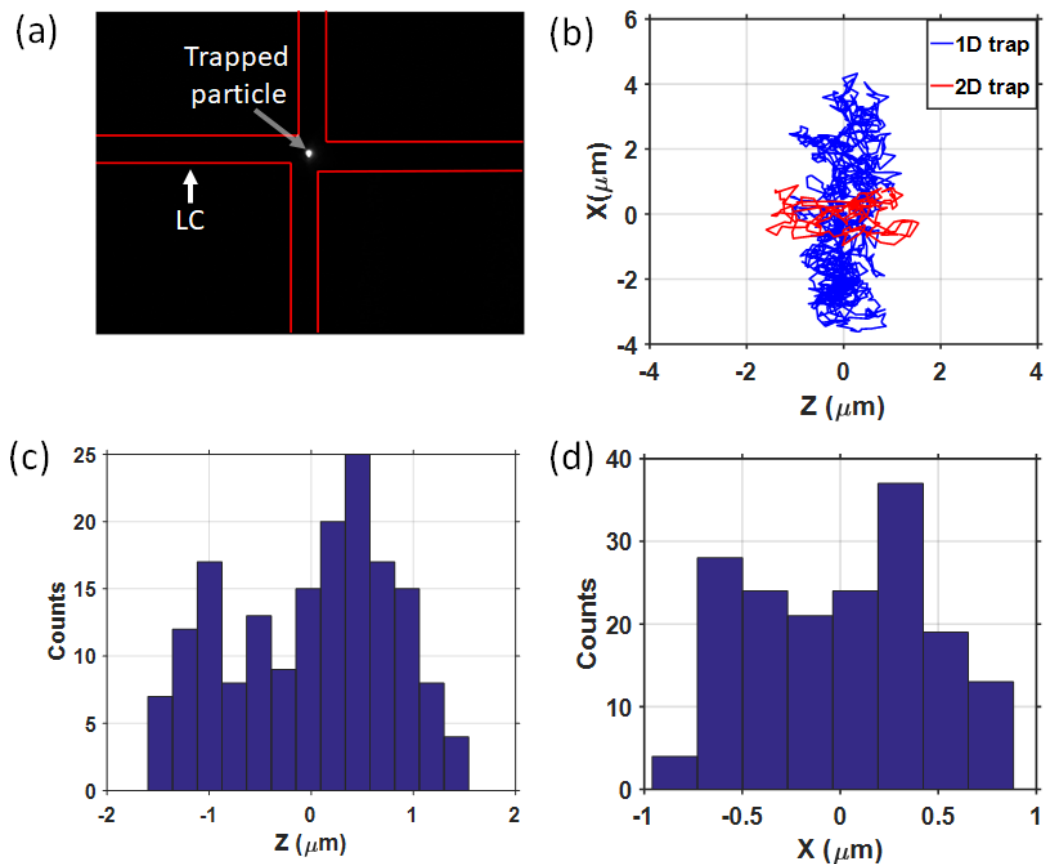


Fig. 3.21 Trapping analysis; (a) still picture of the trapped particle; (b) trajectories of 2D (red) and 1D (blue) trapped particle during the trapping period; (c) extracted position histogram along the Z direction; (d) extracted position histogram along the X direction.

excitation and fluorescence collection using a dichroic filter as shown in Fig. 3.20. The fluorescence signal was further filtered using a bandpass filter (Omega Optical) and sent to the APD using connectorized multi-mode fiber (Thorlabs). Fluidic reservoirs are attached at the end of each channel to introduce target samples into the device. The fluidic level of the reservoirs was tuned to adjust the fluidic flow through the channels via hydrostatic pressure. Ag-AgCl electrodes were immersed into the reservoirs to apply an electrokinetic feedback force on the particles.

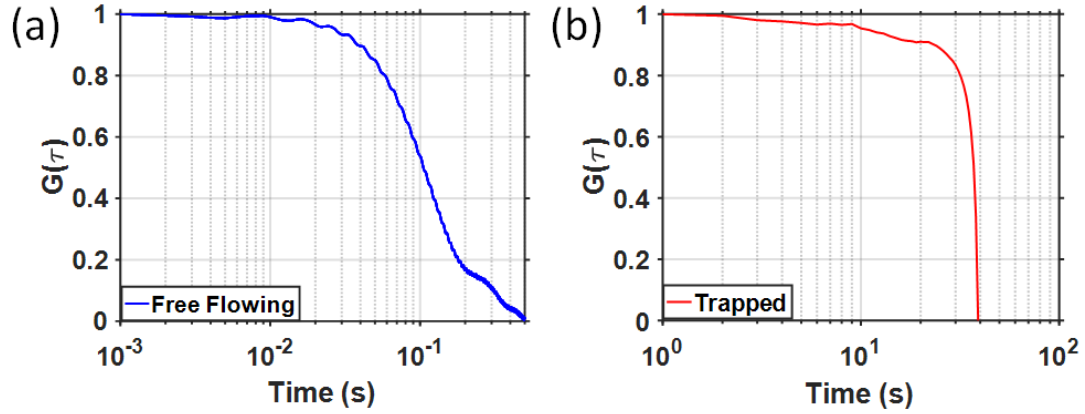


Fig. 3.22 (a) FCS curve of a free-flowing particle; (b) FCS curve of a trapped particle.

The trapping functionality is validated by 2D trapping a single microbead. Out of plane imaging and recording was carried out using the same custom-built camera stated earlier. A still picture of the trapped bead is shown in Fig. 3.21(a), and its trajectory is shown in red trace in Fig. 3.21(b). The blue trace in Fig. 3.21(b) represents a trajectory of a trapped microbead using the previously stated 1D ABEL principle. Both 1D and 2D trapped particle trajectories are plotted in the same graph for quantitative comparison. The improvement of 2D trap over the 1D is clearly visible where the red trace shows much tighter confinement than the blue trace. From the particle trajectory, corresponding Z and X position histograms were extracted which is depicted in Fig. 3.21(c) Fig. 3.21(d), respectively. The position histograms reveal that the particle mostly resided at the center of the trap with a movement of $\sim \pm 1 \mu\text{m}$ in both Z and X directions. The trap was further characterized by calculating the trap stiffness (the same way as stated earlier this chapter) for both Z and X directions and found to be 6.5 nN/m and 20.5 nN/m, respectively. Compared to 1D ABEL trap, the 2D trap boosts the lateral (X) trap stiffness by 14x as the 1D ABEL has only has poor, passive

X-confinement provided by the channel walls. Furthermore, as the trapped particle mostly resides at the center of the trap, the possibility of recording less/no fluorescence collection from the particles near the walls is significantly improved. Fig. 3.22(a) shows an FCS curve of a free-flowing particle whereas Fig. 3.22(b) shows an FCS curve of the trapped particle. The residence time increases more than two orders of magnitude for the trapped particle which illustrates the efficacy of the trapping. 2D ABEL trap improves trapping performance and facilitates enhanced control over trapped particle which has the potential to develop into a prominent choice for trapping and analysis of small bioparticles.

3.5 Alternative 2D ABEL trapping platform

The 2D ABEL trapping platform stated in previous section performs well however, there is room to improve the device design. One better design would be a platform which could separate the optics and fluidic sections. In the previous design, The LC channels (the LC channel section after SC-LC intersection) were implemented for both optics (both excitation and fluorescence) and fluidic purpose. In the second architecture, an octagon is designed at the center of the device with interconnected SC waveguides. A schematic of the new device architecture is shown in Fig. 3.23(a) which illustrates how the two offset pairs (along Z and X direction) of SC waveguides (gray) are connected to the center octagon. The SC waveguides bring excitation lights to the central trapping octagon and one SC waveguide also collects fluorescence light from the trapping octagon. Thus, the LC channels are dedicated only for fluidic purpose

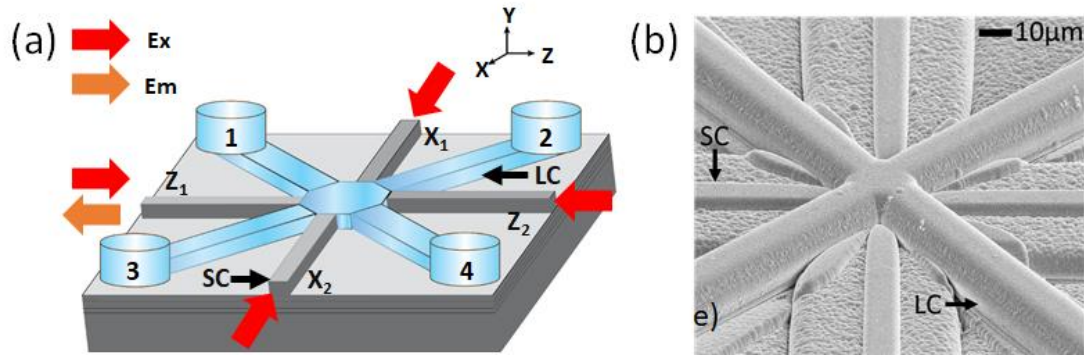


Fig. 3.23 (a) Outline of the alternative 2D ABEL trapping architecture; (b) SEM image of the central region in a fabricated device.

separating the optics and fluidics region. The new device was fabricated using similar method stated for the 2D ABEL device fabrication stated in previous section. Fig. 3.23(b) depicts an SEM image which shows how the SC and LC waveguides are connected with the center octagon in a fabricated device.

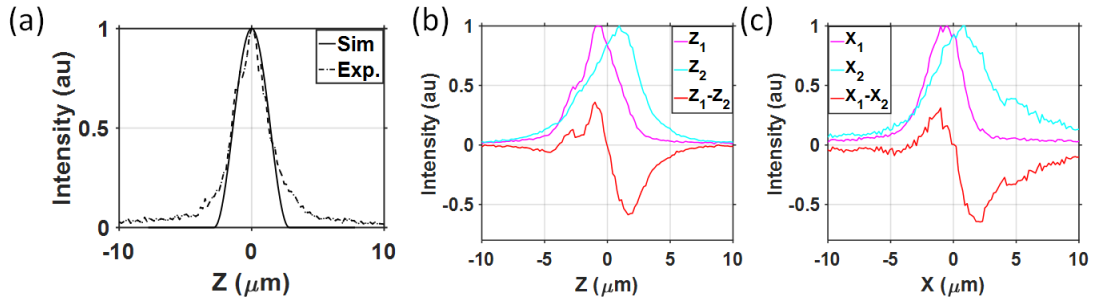


Fig. 3.24 (a) Optical mode profiles of SC waveguide (solid line simulation, dotted line experiment); (b) optical intensity profiles at central trapping region with Z_1 and Z_2 excited; (c) optical intensity profiles at central trapping region with X_1 and X_2 excited.

Fig. 3.24(a-c) show the optical characterizations of a new device where Fig. 3.24(a) shows a comparison of the simulated (solid line) and measured (dotted line) mode profiles of a SC waveguide. The profiles show excellent matching with a FWHM of $2.83\mu\text{m}$ for both experiment and simulation. Fig. 3.24(b-c) illustrates the optical intensity profiles (magenta and cyan) at the center octagon when Z pair SC waveguides

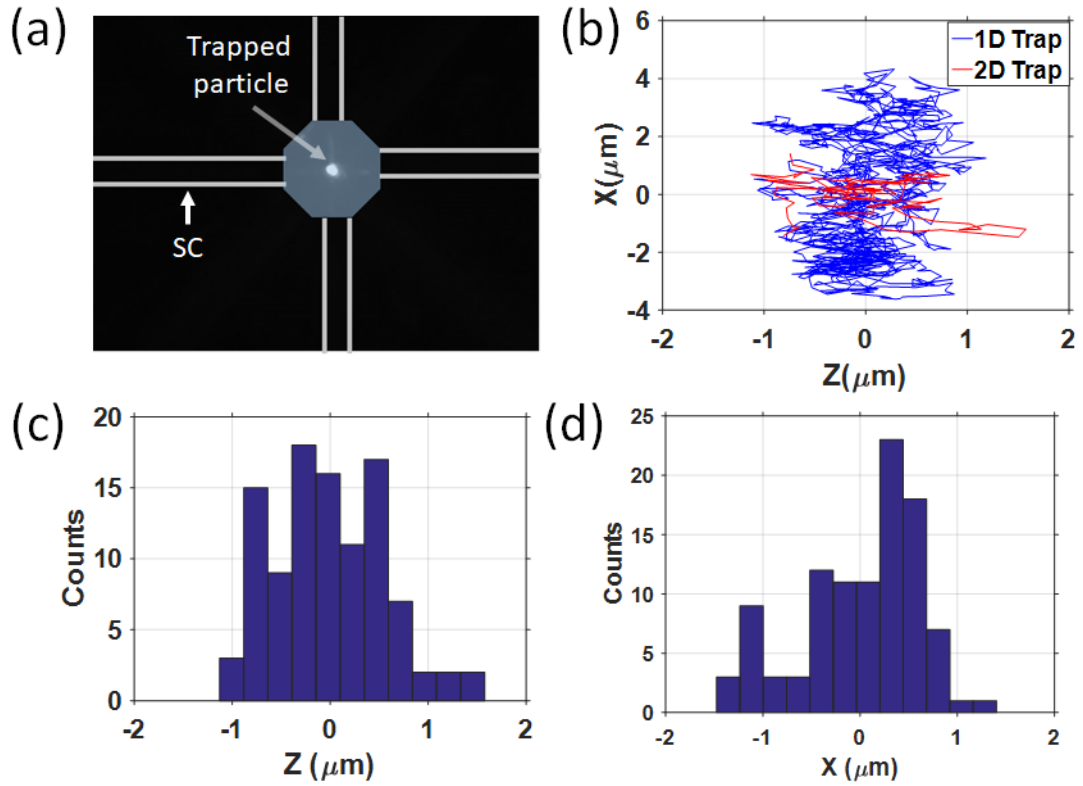


Fig. 3.25 (a) still picture of the trapped particle; (b) trajectories of 2D (red) and 1D (blue) trapped particle during the trapping period; (c) extracted position histogram along the Z direction; (d) extracted position histogram along the X direction.

[Fig. 3.24(b)] and X pair SC waveguides [Fig. 3.24(c)] were excited and the red traces in both figures show the difference curve. The excitation profiles were measured in the same way as stated earlier.

Using the same algorithm and principle as the 2D ABEL trap stated earlier, the new octagon devices were also employed for microbead trapping. Fig. 3.25(a-d) show the trapping results where Fig. 3.25(a) shows the still picture of the trapped microbead. Fig. 3.25(b) show a comparison between 1D (blue) and 2D (red) trapped particles where the improvement is again visually clear. The Z and X position histograms are plotted in Fig. 3.25(c) and Fig. 3.25(d) respectively which illustrates tighter

confinement of the trapped particle. The trap stiffness is calculated for both Z and X directions and found to be 12.6 nN/m and 10.5 nN/m, respectively and an improvement of 8x trap stiffness (X direction) was achieved compared to the 1D trap.

The current ABEL trapping principle works to some extent for the new 2D ABEL architecture. However, further modification is necessary for better trapping performance by taking the slanted LC channels angles into account. Nonetheless, the new 2D ABEL trapping provides better control over the trapped particle. They provide sufficient insight for developing a better particle trapping and analysis platform on a chip. Furthermore, the trapping platform can be integrated with other technologies (eg. feedback gating) for on chip trapping and manipulation of single particles.

Chapter 4 Optical trapping assisted detection rate enhancement of single molecules on a nanopore optofluidic device

Nanopores have already proven its capability and stablished itself as a potential tool for label free detection of single molecules. Over a dozen “nanopore” companies have now sprung up, focusing mainly on *de-novo* sequencing, but also seeking to apply this principle to other molecular targets such as proteins and small molecules [99], [160]. While great strides in nanopore-based analysis have been made, this is still a young field, and several challenges remain to be solved in order to maximize its potential. One major limitation is the delivery of a sufficient number of analytes close enough to the pore to enable electrophoretic capture and detection. This severely limits the throughput (analysis time) and the limit of detection of the assay, in particular for clinical biomarker detection at ultralow (femto- to atto-molar) concentrations [107], [161]–[164].

In this chapter, an elegant solution to this challenge is proposed and demonstration is shown with high-throughput analysis of molecular targets with enhanced detection rate that paves the way to detect targets at ultralow concentrations on a nanopore-based optofluidic system [165]. Specifically, the molecular targets are pre-concentrated on microscale carrier beads, followed by delivery and trapping of these carrier beads at the vicinity of the capture radius (discussed in detail in the following section) of a nanopore. Targets are thermally released from the carrier beads

and detected using nanopore current modulation. Almost two orders of magnitude in capture rate improvement is experimentally demonstrated while detecting 100-mer DNAs corresponding to a melanoma cancer gene (BRA^FV600E) [165]. Furthermore, this technique is applied to detect Zika ns1 (ZIKV) proteins at clinically relevant concentration and demonstration of successful detection is shown as low as 2ng/mL concentration. This approach facilitates a significant increase in local analyte concentration, thus enabling quantum leaps in throughput and limits of detection to enable disease diagnostics using label-free single molecule analysis on a chip-based system.

4.1 Necessity of nanopore capture rate enhancement

A nanopore is an electrical single molecule sensor where voltage is applied across the pore which drives the charged molecules from one side of the pore to the other side. As described in chapter 2, the particle movement through the pore temporarily modulates the ionic current that creates a current spike which is considered as the translocation signature of molecules. A high-throughput SMA platform demands targets to be detected at a high rate (high translocation rate in case of nanopore) to maximize the performance and minimize the analysis time. Thus, it is essential to improve nanopore translocation rate or particle capture rate for high-throughput SMA platform based on nanopore. Due to the pressing demand, researchers have already made several attempts to improve nanopore capture rate by enhancing electric field using salt gradient [163], manipulating internal charge [162], pressure control [164], trapping molecules near the

nanopore [166] etc. which have seen mixed results. However, the most elegant solution to nanopore capture rate enhancement lies within increasing the local concentration in close proximity of the nanopore. To understand the implication of the statement, it is necessary to understand the details of usual target capture process of a nanopore which is discussed in the following section.

The electric field responsible for particle translocation is largest near the pore, creating a finite volume (referred as capture volume) where capture of molecules is likely. This fact has been observed and both theoretically and experimentally demonstrated by different researchers [161], [167]–[169]. The capture volume is usually characterized by a hemisphere with a radius generally referred as “capture radius”. A typical schematic of nanopore capture details is shown in Fig. 4.1 where the

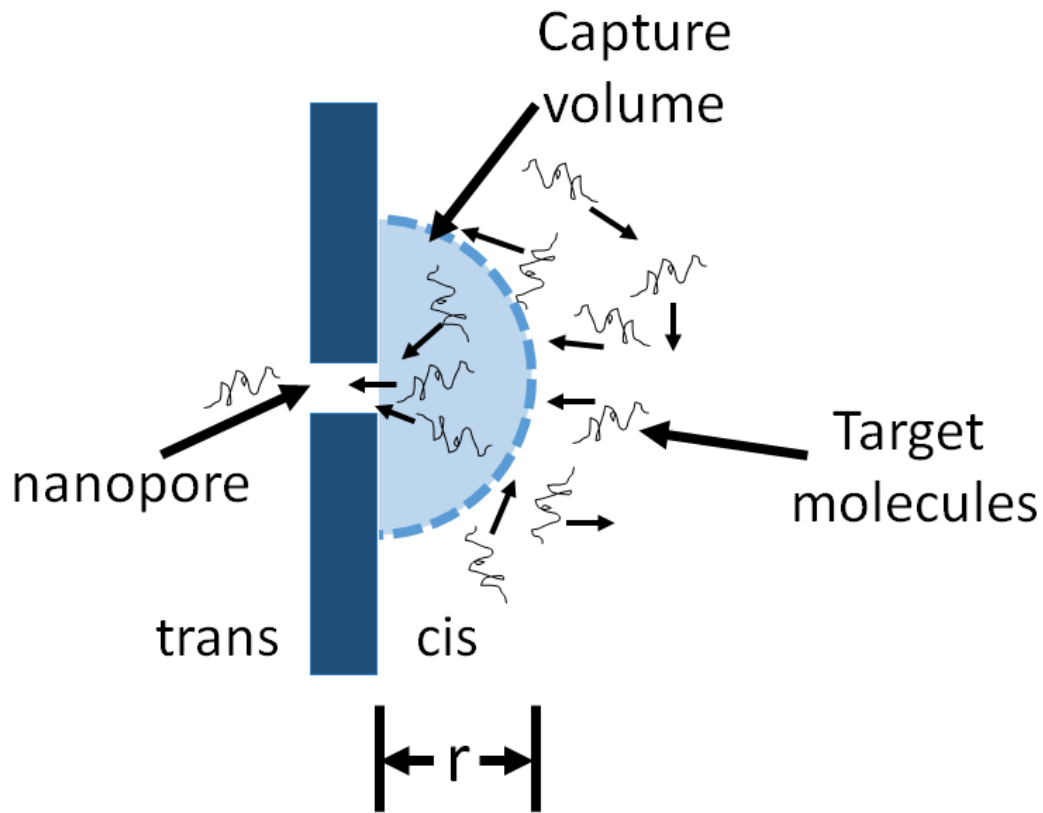


Fig. 4.1 Definition of nanopore capture radius and capture process.

capture radius is shown with dotted line. In a diffusion limited process, the capture rate (R) can be expressed as follows [60], [170], [171] -

$$R = 2\pi CDr \quad (4.1)$$

Where C is the concentration of the molecule, D is the diffusion coefficient of the molecule and r is the capture radius of the nanopore. The molecules outside the capture volume usually diffuse around whereas the molecules inside the capture volume are electrophoretically captured and translocate through the pore. Thus, the capture rate/event frequency in fact depends on the local analyte concentration (at the proximity of capture volume) rather than the bulk analyte concentration. Unfortunately, the usual

dimension of nanopore capture radius falls within a few micron [60], [170], [171]. Therefore, the capture rate is limited by the availability of molecules within that small volume, hence on the local concentration. The importance of local concentration enhancement can be well illustrated with a numerical example. Let's assume that the target molecules are loaded in the LC channel [5 μ m in height, 12 μ m in width and 3mm long] of our ARROW device and the nanopore capture radius is 3 μ m which is consistent with previously reported value [60]. Considering those values, it yields that the nanopore capture volume (56.55 μ m³) is in fact more than 3500 times lower than the total volume of the LC channel (1.8 x 10⁵ μ m³). Thus, there is a huge room to improve nanopore capture rate by increasing the local concentration.

4.2 Principle and methodology of nanopore capture rate enhancement

The core idea of nanopore capture rate enhancement is to accumulate and bring the target molecules in close proximity of a nanopore, thus within the capture volume to increase the local analyte concentration. In our case, the molecules of interest are DNAs and proteins which are two important biomolecules in terms of disease detection and fundamental science. The idea is to attach these target molecules with a bigger micron size carrier bead which can be carried, trapped and manipulated optically in our ARROW optofluidic device. In a subsequent step, the target carrier beads need to be trapped at the vicinity of nanopore and thermally release the targets at the proximity of the nanopore which will increase local concentration and deliver sufficient target

molecules within the nanopore capture volume. Finally, an electrical bias voltage across the nanopore will pull the targets through the pore at very high rate (enhanced capture rate) due to the huge availability of molecules within the capture volume (for details and schematic see section 4.2.3 and Fig. 4.5). To implement this, first, it is necessary to find a way to accumulate the target molecules to a carriable bead. This has been done using magnetic microbeads (MB) that allows these molecules to be attached with them. A detail of the target assay preparation scenario is discussed in the following section.

4.2.1 Magnetic bead-target assay preparation

To validate the nanopore capture rate enhancement methodology, at first, 100mer ssDNAs (sequence 5'- CTACACCTCAGATATATTTCTTCATGAAGACCTCACAGTAAAAATAGG TGATTTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCATCAGTT T-3') corresponding to a melanoma cancer gene (BRAV600E) were chosen as the target molecule. The target DNAs were attached to functionalized magnetic beads using the previously described procedure [54]. Fig. 4.2 schematically illustrates the whole assay preparation steps where Fig. (a) shows the three main building blocks of the assay. The first element is the micron size magnetic bead (Invitrogen) which has streptavidin coating in it. To functionalize the magnetic beads, 14-bp (sequence 5'- AGATTTCTCTGTAG-3') biotinylated oligomers (usually referred as pull-down) were carefully designed to match and specifically extract the target sequence as

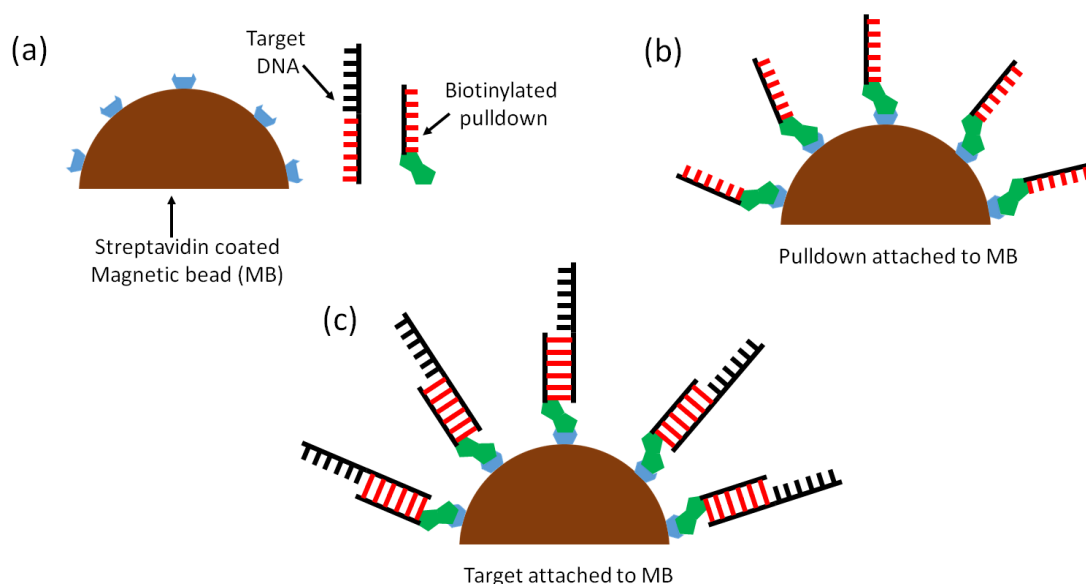


Fig. 4.2 Magnetic bead-target assay preparation steps; (a) Basic elements of the assay: starting from the left, streptavidin coated magnetic bead, target and biotinylated pull-down; (b) pull-down attachment to magnetic bead via biotin-streptavidin bond; (c) target attachment to carrier magnetic bead via pull-down-target binding.

previously reported [54]. The biotinylated pulldown is the second element and the target DNA is the final element of the assay. The whole assay was prepared in 1 x T50 (10mM Tris-HCl, 50mM NaCl) buffer which was filtered with a 20nm Whatman anotop filter at the very beginning of the solution preparation steps. After washing three times, an aliquot of magnetic beads was incubated with over saturated biotinylated pull-down oligonucleotide. The solution was kept on a rotary mixer for 2 hours which facilitates streptavidin-biotin bonding. Thus, the pull-down oligos were bound to the magnetic beads as shown in Fig. 4.2 (b). Next, the solution was washed 4x to remove excess/unbound pulldowns and resuspended in 10 μ L of 1 x T50 buffer and stored in a refrigerator for future use. After that, 9 μ L of 1 μ M target DNAs were incubated with 1 μ L of 16.6pM magnetic bead-pulldown assay which was prepared in the previous

step. The whole solution was then kept in a rotary mixer for 2 hours which allows the target DNAs to bind with the complementary sequence oligos. The whole hybridization allows the target DNAs to attach with magnetic beads via the pulldown oligos as shown in Fig. 4.2 (c). Next, the solution was again washed 4x to remove excess/unbound target DNAs and resuspended in 1xT50 buffer for experimental use. This method ensures reliable target specificity as demonstrated before which can potentially be used towards diagnostic purpose [36], [54]. The breaking temperature for the pulldown and target DNA bond is estimated to be $\sim 34^{\circ}\text{C}$ [54]. Thus, this methodology allows to thermally release target DNAs from the carrier magnetic beads.

4.2 Loss-based optical trapping

Once the targets are attached to carrier microbeads, the next necessary step is to deliver and hold the carrier beads in close proximity of nanopore. Optical methods of particle trapping and manipulation are the most popular methods due to numerous advantages including non-invasive, contact free manipulation. As ARROW devices have interconnected SC and LC waveguides to introduce light to and from the device, the carrier microbeads were optically manipulated and trapped in this case. Several optical trapping and manipulation techniques have already been reported on the ARROW optofluidic platform [40], [48]–[50].

As light has momentum, whenever light interacts with an object (reflected, refracted or absorbed), the momentum of light is changed [172] as shown in Fig. 4.3 (a). As a result of this, the particle experiences an analogous change in momentum

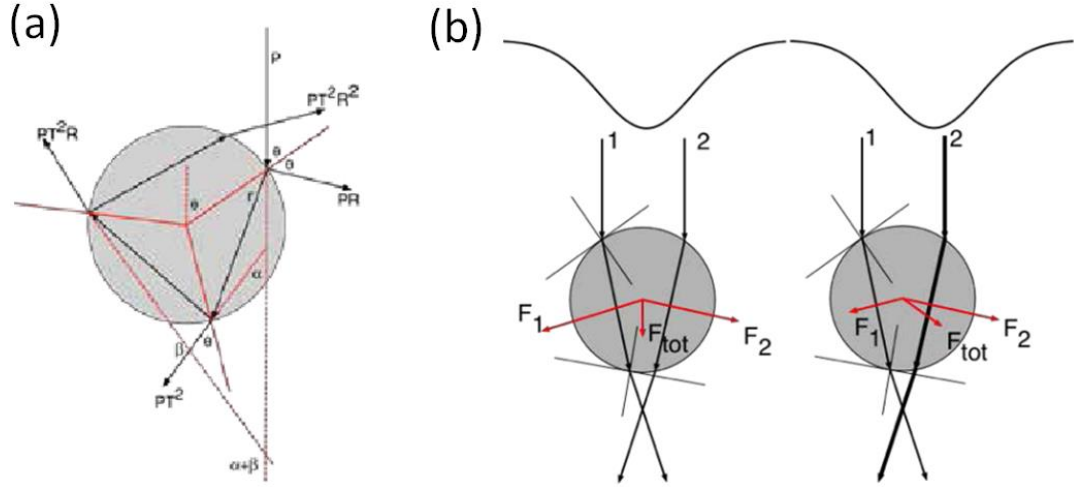


Fig. 4.3 (a) Schematics of light rays when hit a spherical particle; (b) demonstration of scattering and gradient force on a particle [from reference 172].

hence a resultant force. As described in Fig. 4.3 (b), if a particle is hit by a collimated laser beam it will be pushed along the direction of light propagation if the particle is located in the center of the beam since the momentum change is symmetric with respect to the particle's center. This component of the optical force is usually known as scattering force. However, if the particle is off center, along with the scattering force it will be pushed towards the maximum intensity point (if the refractive index of the particle is higher than the surrounding medium) of the beam due to the relatively larger number of photons in the center of the beam than that of outside of the beam. Generally, this component is called as the gradient force. Various types of optical traps have been so far reported such as optical tweezer [173], [174], levitation trap [175], dual beam trap etc. [176]. In 2009, a new form of dual beam trap [referred as loss-based (LB) trap] was invented in our lab which relies on the propagation loss along the waveguide [48]. Unlike the conventional dual beam trap where the trapping depends on the asymmetry of the beam area, the presence of waveguide loss creates the force asymmetry between

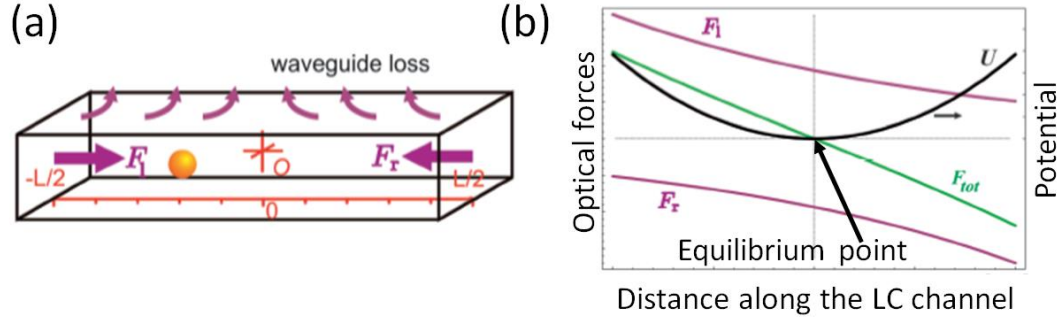


Fig. 4.4 (a) Schematic representation of LB trapping principle with optical forces due to left and right propagating optical beams in presence of waveguide loss; (b) scattering forces (F_l and F_r), total force and potential along the LC channel [from reference 48].

counterpropagating beams in the LB trap. Fig. 4.4(a) illustrates the working principle of the LB trap where, F_r and F_l represent the scattering forces due to the right and left propagating optical beams, respectively. The scattering forces can be expressed using the following equation [48]-

$$F_r^{Sca}(z) = \pm Q \frac{c}{n} P_r^0 \text{Exp}(-\alpha(\frac{L}{2} \pm z)) \quad (4.2)$$

Where, Q is the radiation pressure efficiency, c is speed of light, n is index of the embedding medium, L is waveguide length, $P_{r/l}^0$ is the input beam power at the ends of the waveguide, z is the distance from the point of equilibrium and α is the waveguide loss coefficient. The particle is trapped at the point of equilibrium with minimum potential energy where the scattering forces due to the two counter propagating beams are equal as shown in Fig. 4.4(b). One remarkable feature of the LB trap is the ability to trap particles at any point of the LC waveguide by adjusting the input optical power of the two trapping beams which is the major motivation for selecting LB trap to manipulate target carrying microbeads. Due to this flexibility, a nanopore can be drilled at any suitable location on the LC channel and target carrying microbeads can be

delivered and trapped near the nanopore using the LB trapping method. Another power feature of the LB trap is to assemble and trap multiple particles in the trapping spot which is an essential requirement for capture rate enhancement to enable assembly and trapping multiple carrier bead in close proximity of a nanopore. Previously, this feature has been demonstrated by assembling and trapping ~120 particles using an LB trap [177]. Due to these prominent features, an LB optical trap was implemented to deliver and trap target carrying magnetic beads for nanopore capture rate enhancement.

4.2.3 Experimental principle and methodology

The new concept of trapping-assisted capture rate enhancement (TACRE) on an optofluidic chip is schematically visualized in Fig. 4.5(a). The figure represents a cross-sectional view of a LC channel with a nanopore drilled on it (pointed with the black arrow). Target carrying microbeads are trapped in the vicinity of the nanopore using LB trapping as shown in the figure. Next, the target DNAs are thermally released from the carrier beads close to the nanopore capture volume. The whole process is tantamount to picking up and accumulating all target DNAs from a bulk solution then releasing them at the nanopore capture volume thus, overcomes the limitation of few/no available molecules at the nanopore capture volume. Due to the abundance of molecules at nanopore capture volume, the capture rate significantly improves compared to a scenario when the DNAs are uniformly dispersed throughout the LC channel. Fig. 4.5 (b) illustrates the full experimental layout on the optofluidic chip. The device has an ARROW microfluidic channel (blue) connected to solid-core SC

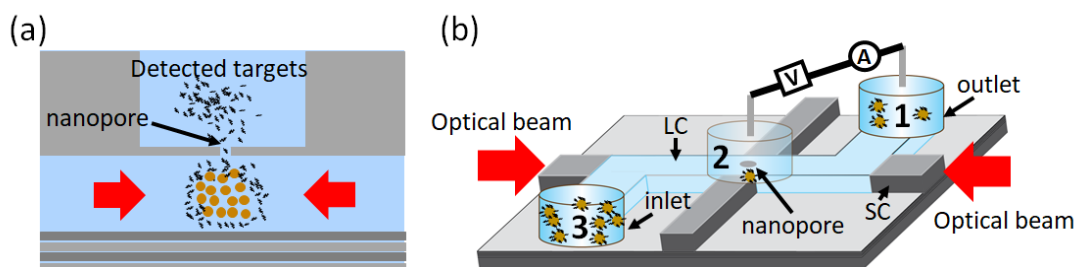


Fig. 4.5 (a) Schematic representation of trap assisted capture rate enhancement principle; (b) experimental setup on a nanopore optofluidic device showing how target carrying microbead(s) are trapped and voltage applied across the nanopore.

waveguides (gray). As before, fluidic reservoirs 1 and 3 are attached at the ends of the LC channel to introduce sample solutions and to apply electrical bias. Nanopores with suitable dimension are fabricated on the LC channel using the method described in chapter 2. An additional reservoir (#2) was attached on the nanopore and an electrical bias voltage was applied between reservoirs 1 and 2 for the electrical detection of target molecules as shown in the figure. Target carrying microbead solution was loaded in the inlet reservoir (#3) whereas the nanopore reservoir were filled with 1xT50 buffer. The outlet reservoir was filled with 1xT50 buffer where the volume was maintained in such a way that the beads were moving towards the outlet almost with a diffusion limited speed. Fiber coupled laser light from a 532 nm diode pumped solid state laser (Light House Photonics) was coupled to the left and right SC waveguides as shown in Fig. 4.5(b). Fig. 4.6(a) and Fig. 4.6(b) show still pictures of single and multiple beads trapped in close proximity of a nanopore following the LB trapping principle. For thermal heating and release of targets from carrier beads, the whole device was heated to $\sim 50^{\circ}\text{C}$ for ~ 2.5 min using a ceramic heater (HT9 Laird TEC) whereas the temperature was controlled using a laser diode controller (Newport 3724B). As before, an electrical

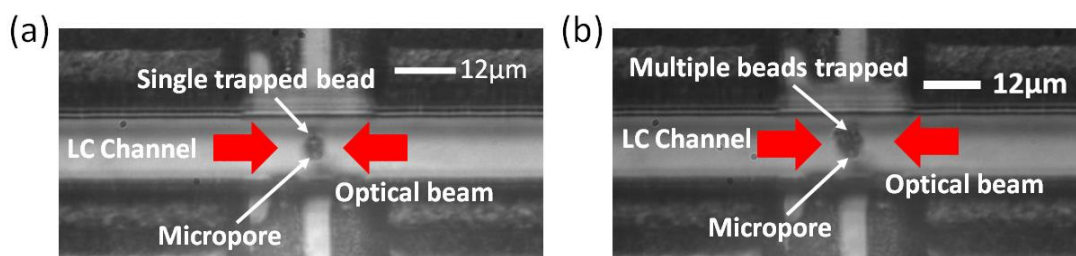


Fig. 4.6 (a) Still picture of a single microbead trapped under the pore; (b) still picture of multiple microbeads trapped under the pore using the LB trapping principle.

bias voltage with proper polarity and magnitude was applied and the corresponding nanopore current was measured using the Digidata 1440A and Axopatch 200B respectively.

4.3 Results and discussion

At the very first step, the target DNAs (just a bulk solution of DNAs without any magnetic bead assay, suspended in 1xT50) were loaded on the nanopore optofluidic device to see if it is possible to electrically detect the targets. The concentration of the DNAs was carefully chosen to be 2.25nM which is the equivalent concentration if maximum possible bonded DNAs in a single magnetic bead ($\sim 240 \times 10^3$, as per the data sheet) were uniformly distributed in the LC channel. This concentration and experimental results were used as the reference of comparison for future TACRE measurements. When an electrical bias voltage was applied across the pore, translocations of the target DNAs were observed which is shown as red trace in Fig. 4.7(a). Several translocations were observed with multiple sub-peaks arising from

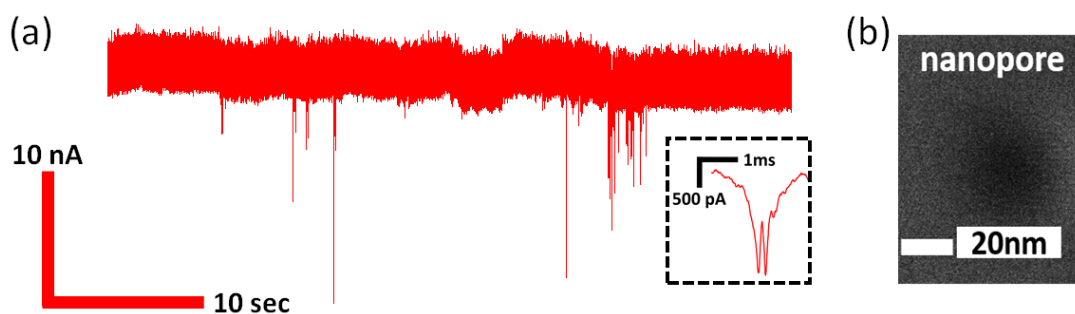


Fig. 4.7 (a) Current trace through nanopore with translocation spikes when target DNAs move through the pore, inset: example of a multi sub-peak translocation signal due to multiple DNAs moving through the pore simultaneously; (b) SEM image of the nanopore used to detect target DNAs.

multiple DNAs moving through the pore at the same time as shown in the inset of Fig. 4.7(a). A conservative threshold of $1/6^{\text{th}}$ of the maximum translocation amplitude for the dip depth between sub-peaks was used to identify a second molecule. This criterion was applied consistently throughout the whole TACRE experiments for DNA targets. Fig. 4.7(b) shows an SEM image of the nanopore used for DNA detection. The nanopore was fabricated using the procedure described in chapter 2. The event capture rate for the reference trace is calculated and found to be $0.0945 \text{ s}^{-1}\text{V}^{-1}$. Next, it is intended to follow the TACRE principle, trap different number of target carrying bead(s) and observe if it is possible to improve the event capture rate compared to the reference case where DNAs were uniformly distributed throughout the LC channel.

To validate the TACRE principle, first, a single target carrying microbead was trapped in close proximity of the nanopore, then the device was heated to 50°C for ~ 2.5 min using the methodology described earlier. After that, an electrical bias voltage (7V) was applied across the pore to pull the targets through the pore at a high rate due to their proximity to the nanopore. The top blue trace in Fig. 4.8(a) shows the observed

translocations of DNA molecules modulating the current through nanopore. This time, the event capture rate is calculated and found to be $0.974 \text{ s}^{-1}\text{V}^{-1}$. The event capture rate is improved over an order of magnitude compared to the reference trace which in turn demonstrates the efficacy and practicality of the TACRE methodology. In the present configuration, the TACRE improvement factor was limited by the time delay between thermal release and applying the nanopore voltage needed to avoid electrical noise interference. During this delay, the DNA targets started to diffuse away from the pore which lowered the effective local concentration during detection. Considering DNA diffusion, an estimated capture rate improvement factor is calculated to verify if the experimental result matches the predicted improvement factor. The DNA diffusion coefficient (D) is calculated using equation 3.1 and found to be $79.35 \text{ } \mu\text{m}^2\text{s}^{-1}$ which is equivalent to the diffusion co-efficient of a sphere which has an equal volume as the DNA. The distance (x) diffused by DNAs within a time t can be expressed as follows-

$$x = \sqrt{2Dt} \quad (4.3)$$

Accordingly, the DNAs will travel a distance of $60\mu\text{m}$ - $220\mu\text{m}$ within a time of 20s – 300s respectively. Assuming a linear relationship between capture rate (R) and target concentration (C) as stated in equation 4.1, the capture rate improvement factor can be calculated to be within 7x – 25x respectively. The measured improvement factor (10.3x) falls within the calculated range and decently agrees with the diffusion-induced concentration reduction. In addition to the single bead trapping 3, 6, 12 and 14 target carrying microbeads were trapped following the TACRE methodology and respective capture rate improvement factors were determined. Fig. 4.8(a) shows a representative

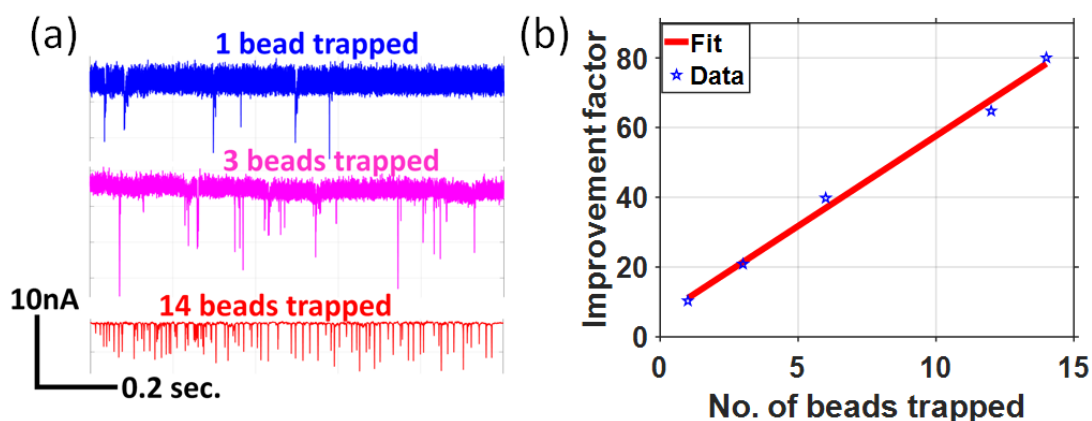


Fig. 4.8 (a) Nanopore current trace after thermal release and detection of DNAs showing monotonic increase in event frequency with number of beads trapped; (b) detection rate improvement with number of trapped beads relative to the reference unconcentrated bulk solution (symbols: data; line: linear fit).

plot of the actual current traces observed when different number of beads were trapped.

It is visually clear that the capture rate monotonically increases with the number of beads as more targets are present near the pore. An improvement of almost two orders of magnitude ($\sim 80\times$) in the detection rate is observed (in case of 14 beads trapped) with a nearly linear dependence on the bead number [as shown in the linear fit in Fig. 4.8(b)]. This result demonstrates the effectiveness of optically assisted target concentration for nanopore analysis and validates the TACRE principle.

This demonstration represents a crucial step towards fully integrated nanopore-based analysis of single molecules with broad applications. An increase of almost two orders of magnitude in molecular detection rate shows the power and ease of optical trapping to expand the applicability of nanopore analysis. In the case of 14 beads trapped experiment, over 5,600 individual DNAs were detected in three minutes. This target number corresponds to a concentration of ~ 1 attomol when contained in a typical blood draw of 10mL. This corresponds to the low concentration end of infectious

diseases [37] and lies well below the current limit of detection for protein-based immunoassays. Furthermore, the time delay (between thermal release and voltage application) of the current process can be eliminated in the future by implementing alternative target release methods (e.g. optical release with UV light). Then, much larger enhancements of at least 50,000x are feasible. Furthermore, TACRE is compatible with integration of all fluidic sample handling steps on a single chip[178] to create a complete sample-to-answer molecular analysis system on a chip. As bead capture ensures target specificity[54] and TACRE enables low concentration target detection with reduced analysis time this methodology has the potential to find a place in diagnostics applications.

4.4 Enhanced Zika (ns1) detection at clinically relevant concentration

The TACRE methodology is analogous to collecting and accumulating targets from a bulk solution (where targets are dispersed throughout the solution) and releasing the accumulated targets at the proximity of nanopore. This concept can be deployed to detect targets at low concentrations, especially detection of infectious diseases at clinically relevant concentrations such as Zika ns1 detection. If feasible, this concept has the potential to be used for diagnostic purpose with high throughput target detection. To verify the practicality of the concept, Zika nonstructural 1 (ns1) proteins were selected as a target of interest which is the major antigenic marker for viral infection [179]. Zika virus (ZIKV) is a Flaviviridae family member which was first identified in monkeys in 1947 and later identified in humans in 1952 usually

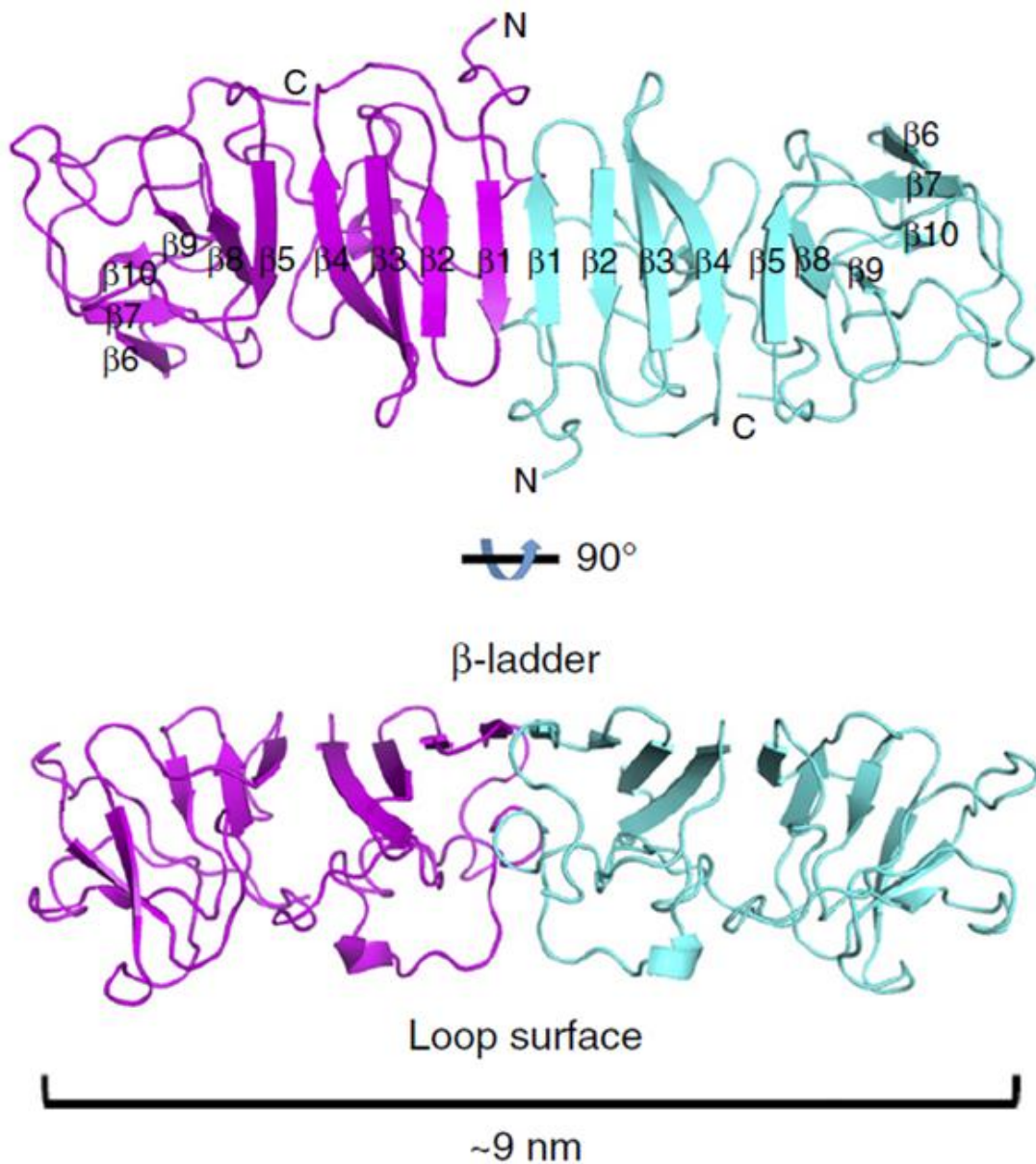


Fig. 4.9 Overall structure of ZIKV ns1₁₇₂₋₃₅₂ with head-to-head dimer [from reference 179].

transmitted to humans by mosquitoes of the genus *Aedes*. The 2015 outbreak of Zika virus infection has drawn massive attention to this disease. While the acute epidemic has subsided, many concerns remain due to the virus' ability to cause severe birth defects [180], [181]. Some research groups have recently shown that ZIKV infection

in marmosets closely resembles human illness [182]. Zika ns1 structure has been studied by researchers which reveals that these ns1 proteins can form dimers and hexamers [179], [183]. Fig. 4.9 shows a schematic representation of a head-to-head ns1 dimer with estimated dimensions and structural details. To detect Zika ns1 proteins using TACRE principle, it is essential to carry out a step by step investigation to determine the feasibility of the overall experimental implementation. First, it is necessary to know if it is possible to detect the Zika ns1 proteins using our nanopore optofluidic device. After that, it is necessary to devise a way to construct an assay that allows the Zika ns1 proteins to attach with the carrier magnetic beads. Next, it is necessary to determine if it is possible to thermally release the Zika ns1 proteins from the magnetic beads. Once the individual steps are verified, they need to be integrated on chip for rapid detection of Zika ns1s following TACRE methodology. Experimental results show that all the individual steps are feasible including the implementation of TACRE Zika ns1 detection. Following TACRE methodology, Zika detection down to 2ng/mL concentration that is clinically relevant for Zika infection has been experimentally demonstrated which is very inefficient/impossible to detect with usual nanopore detection techniques.

4.4.1 Zika detection using nanopore

In our lab, electrical detection of different bioparticles has already been demonstrated using the nanopore optofluidic device including nucleic acids [60], ribosomes [59], viruses [46]etc. However, electrical detection of proteins hasn't been previously done

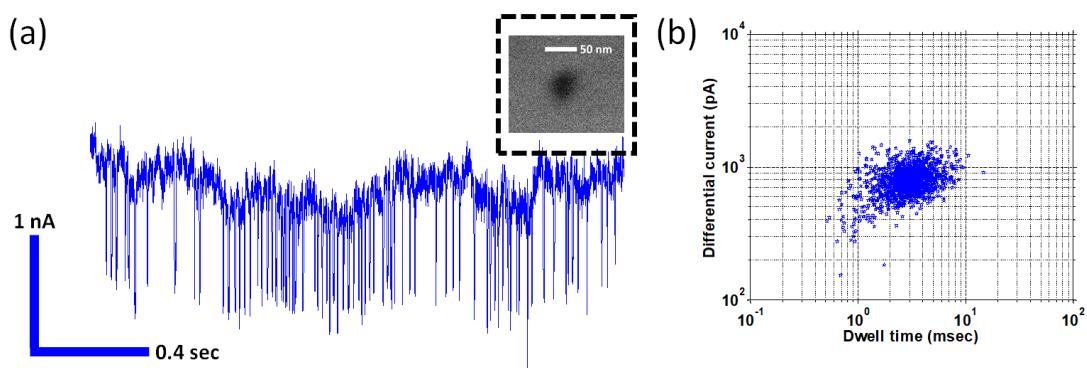


Fig. 4.10 (a) Electrical detection trace of Zika ns1 protein, inset: SEM image of the nanopore used to detect Zika ns1 protein; (b) scatter plot of dwell time vs differential current of translocations found in electrical detection.

using our device. Different research groups have reported protein detection in their device [184]–[186] which insinuates that protein detection should also be possible in our nanopore optofluidic device. For this purpose, first, a nanopore was drilled on the ARROW device using the usual procedure. An SEM image of the drilled nanopore is shown in the inset of Fig. 4.10(a). An aliquot of 34 $\mu\text{g/mL}$ Zika ns1 proteins (EastCoast Bio) were loaded in the device and a bias voltage with proper polarity was applied across the nanopore. The blue trace in Fig. 4.10(a) represents the current through nanopore with Zika ns1 detection spikes which proves that proteins can also be detected in our device along with other biomolecules. Fig. 4.10(b) represents a scatter plot of dwell time vs differential current of individual translocations. In this case, the capture rate for Zika ns1 detection was calculated and found to be 68.11s^{-1} . Once the device was electrically tested and verified, the next conceptual step is to construct a magnetic bead protein assay that can carry the Zika ns1 proteins to the vicinity of nanopore to allow Zika detection at enhanced rate following the TACRE principle.

4.4.2 Magnetic bead-target assay preparation

The idea to detect Zika ns1 proteins at low concentration with enhanced detection rate follows the same principle followed for TACRE DNA detection. Thus, at first, it is necessary to attach the Zika ns1 proteins with magnetic beads that can be carried and released close to the nanopore. The magnetic bead Zika ns1 assay was prepared following similar principle as illustrated in Fig. 4.2. However, this time, biotinylated antibodies (HM333, EastCoast Bio) were used as a pull-down instead of the short DNA segments used in the earlier case. The HM333 antibody selectively binds with Zika ns1 proteins which ensures specificity which is one of the critical features of the assay preparation procedure.

As before, the whole assay was prepared in two steps. First, the HM333 pull-down antibody was attached with the magnetic beads. For this purpose, 2 μ L of 1mg/mL biotinylated HM333 was mixed with 16 μ L of 1mg/mL of magnetic beads (MBs were washed 4x before using). The mixed solution was then kept in a rotary mixer for 2 hours which allows the HM333 antibodies to attach with magnetic beads via biotin-streptavidin bonding. After that, the solution was washed 4x following standard procedure to remove excess/unbound HM333 antibodies.

Zika ns1 antigens were mixed with magnetic bead and pull-down assay in two different ways. In the first case, the magnetic bead and pull-down assay was oversaturated with high concentration Zika ns1 proteins (3.4mg/mL) which is referred to as “saturation” case. In the second case, Zika ns1s were diluted to clinically relevant concentration (4ng/mL and 2ng/mL to be exact) and mixed with the magnetic bead and

pull-down assay to see if it is possible to detect Zika ns1s at these clinically relevant concentrations using the TACRE principle. For the saturation case, 5 μ L of 3.4 mg/mL Zika ns1 proteins were mixed with 16 μ L of 1mg/mL magnetic bead and pull-down assay. The solution was then kept for 2 hours maintaining a temperature of 37°C. Finally, the solution was 4x washed to remove unbound/excess Zika ns1 proteins for experimental use.

To prepare the whole assay aiming for 4ng/mL Zika ns1, 500 μ L of 8ng/mL Zika ns1 proteins were mixed with 500 μ L of 10 μ g/mL magnetic bead and pull-down assay. The whole volume of the solution effectively brought the Zika ns1 concentration to 4ng/mL. The solution was incubated for 2 hours at a temperature of 37°C. Finally, the solution was 4x washed to remove unbound/excess Zika ns1 proteins for experimental use.

Next, for whole assay preparation at 2ng/mL Zika ns1 concentration, 1mL of 4ng/mL Zika ns1 proteins were mixed with 1mL of 10 μ g/mL magnetic bead and pull-down assay. The whole volume of the solution effectively brought the Zika ns1 concentration to 2ng/mL. As before, the solution was incubated for 2 hours at a temperature of 37°C and 4x washed before final experimental use.

4.4.3 Experimental results and discussion

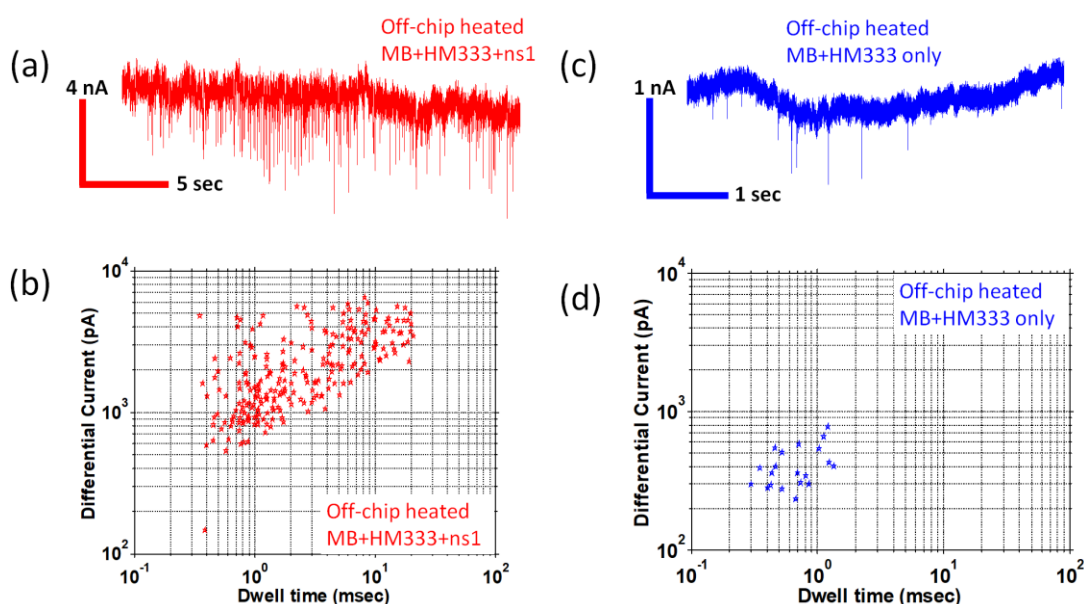


Fig. 4.11 (a) Particle detection trace arise from thermally released (off-chip) supernatant extracted from MB+HM333+Zika ns1 assay; (b) scatter plot of dwell time vs differential current of the events arise from thermally released (off-chip) supernatant extracted from MB+HM333+Zika ns1 assay; (c) particle detection trace arise from thermally released (off-chip) supernatant extracted from MB+HM333 assay; (d) scatter plot of dwell time vs differential current of the events arise from thermally released (off-chip) supernatant extracted from MB+HM333 only.

The idea to detect Zika ns1 proteins using TACRE was envisioned in a similar way as the detection of DNAs. The Zika ns1 carrying magnetic beads will be carried close to the nanopore and thermally released targets will be electrically detected at a high rate due to much more availability near nanopore. However, to do so, it is necessary to verify if it is possible to thermally release (at 50°C) the ns1 proteins from the carrier beads. To verify this, an aliquot of Zika ns1 magnetic bead assay (MB+HM333+Zika ns1, saturation case) was taken in a vial and heated to 50°C (off-chip) for 2.5 min and the supernatant was immediately taken out from the vial and stored in a separate vial. It should be noted that during this process, the magnetic beads were held at the bottom of the vial using a strong permanent magnet that allows to take out only the supernatant

leaving the magnetic beads at the vial. Next, the extracted solution was loaded into the nanopore optofluidic device and electrical bias voltage was applied across the nanopore where translocations of particles were observed as shown in Fig. 4.11 (a). A scatter plot of the dwell times vs the differential amplitudes of each translocation events are shown in Fig. 4.11 (b). This demonstration proves that it is possible to thermally release the proteins from the whole assay. However, it is also necessary to examine whether only Zika ns1 proteins were thermally released from the whole assay or both HM333 and Zika ns1 proteins were thermally released from the whole assay or both HM333 and

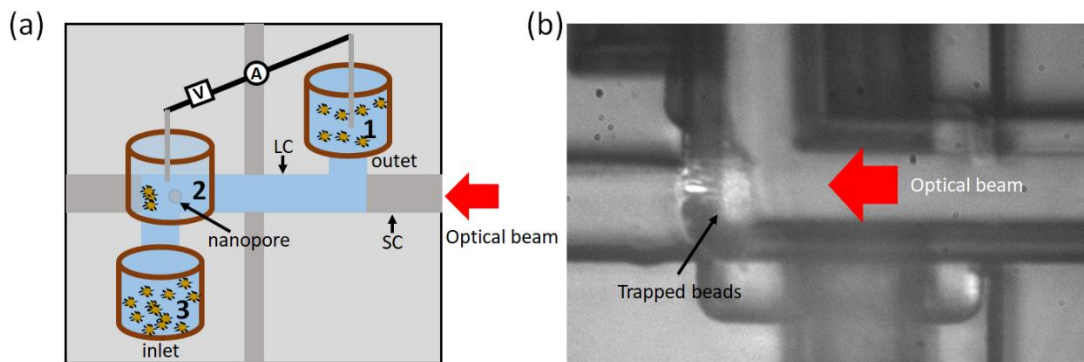


Fig. 4.12 (a) Schematic of low concentration Zika ns1 detection principle using TACRE methodology; (b) still picture of multiple trapped microbeads against the LC channel wall using a single optical beam.

Zika ns1s both released from the whole assay. To find out the answer, another separate experiment was carried out where an aliquot of magnetic bead and HM333 pull-down assay (just MB+HM333), was off chip heated to 50°C for 2.5 min. Again, the supernatant was taken out and stored in a separate vial using the procedure described above. As before, this solution was also loaded in the same nanopore device and the current trace was observed. Some translocations were seen in this case as shown in Fig. 4.11 (c). Fig. 4.11 (d) shows a scatter plot of dwell time vs differential current of the translocation events. This demonstration states that though significantly small but some

HM333 does some out during the thermal heating process. As it is experimentally verified that it is possible to thermally release the Zika ns1 proteins from the beads, the next conceptual step is to proceed towards the TACRE experiment. The estimated capture rate improvement factor based on the target diffusion decently matches with the previous experimental observation. As the targets may diffuse within a range of $\sim 220\mu\text{m}$ during time delay between the thermal release and voltage application it suggests that a nanopore within that range should still work for TACRE and can detect targets at high rates. For Zika ns1 detection, this consideration has been taken into account to simplify the optical trapping scenario. This time, the target carrying magnetic beads were pushed and held against the LC channel wall using a single optical beam as shown in Fig. 4.12 (a). It is comparatively easy to trap the beads in this way and a still picture of multiple trapped beads against the side wall is shown in Fig. 4.12 (b). The nanopore to detect Zika proteins was drilled $\sim 150\mu\text{m}$ away from the LC channel wall where the target carrying microbeads are trapped and held using a single optical beam.

Next, an aliquot of the whole magnetic bead Zika ns1 assay (MB+HM333+Zika ns1, saturation case) was loaded into the nanopore optofluidic device. 12 target carrying microbeads were trapped using the methodology described above. The whole device was heated to 50°C using the ceramic heater and an electrical bias voltage was applied across the nanopore. The red trace in Fig. 4.13(a) shows a part of the current through the nanopore with Zika ns1 detection pulses. Fig. 4.13(b) shows a scatter plot of dwell time vs differential current of 4725 detected individual translocations. In a separate

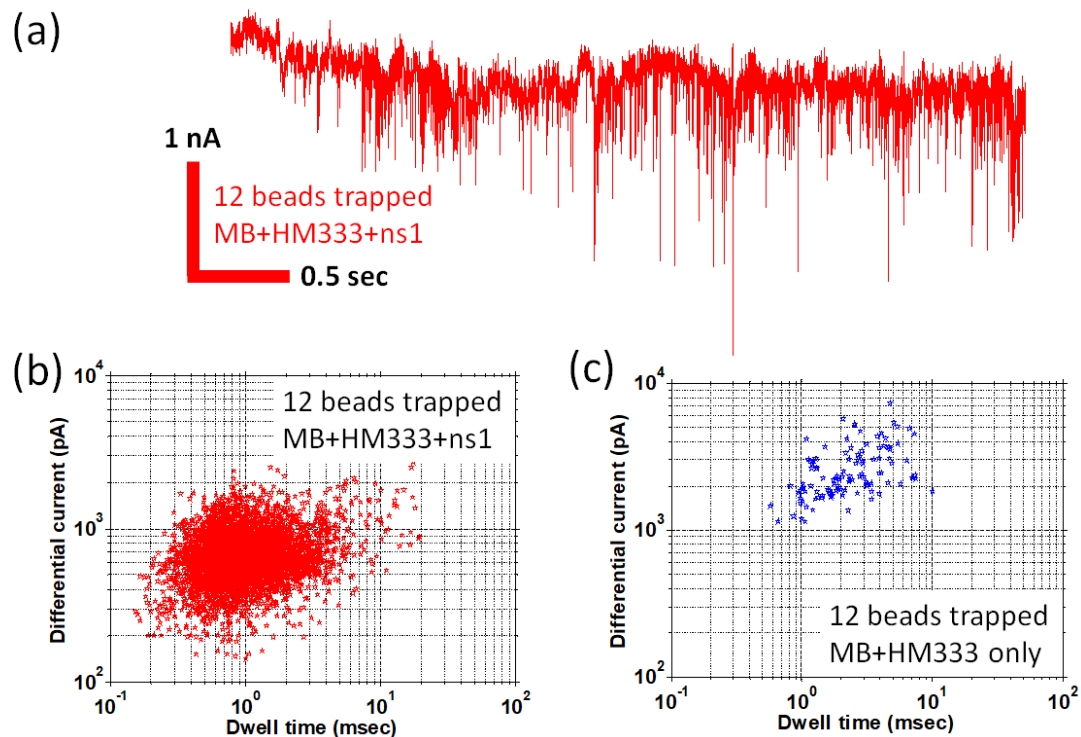


Fig. 4.13 (a) Nanopore detection trace after thermal release of target proteins from 12 trapped microbeads (MB+HM333+Zika ns1, saturation case) using TACRE methodology; (b) scatter plot of dwell time vs differential current of translocations found in the electrical trace (MB+HM333+Zika ns1, saturation case); (c) scatter plot of dwell time vs differential current of translocations found in the electrical trace arise from 12 trapped microbeads where the assay was prepared without Zika ns1 proteins (MB+HM333 only).

experiment, an aliquot of magnetic bead and HM333 assay (just MB+HM333) was loaded into the nanopore device and 12 beads were optically trapped. Like before, the device was heated to 50°C and an electrical bias voltage was applied across the pore. This time, a total of 114 translocations were seen as shown in the scatter plot in Fig. 4.13(c). As there were no ns1 present in the assay, the detected events correspond to the HM333 that came out during the heating process which is consistent with the previous observation (see Fig. 4.11). However, the contribution of the thermally released HM333 antibodies is substantially smaller and almost negligible compared to

the contribution of thermally released Zika ns1s. Therefore, this demonstration proves the practicality of TACRE and its applicability for different biomolecules.

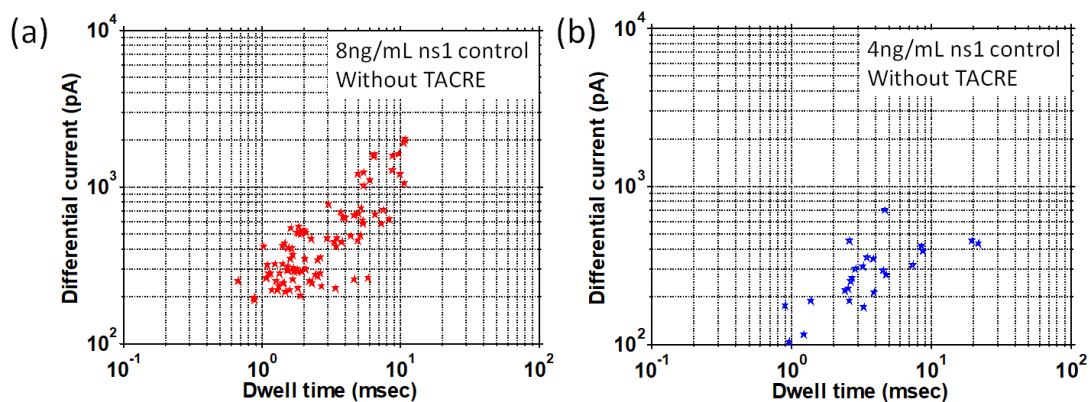


Fig. 4.14 (a) Scatter plot of dwell time vs differential current of translocations found in the electrical trace when just 8ng/mL Zika ns1 (without any MB assay) were loaded in the nanopore optofluidic device (without TACRE); (b) scatter plot of dwell time vs differential current of translocations found in the electrical trace when just 4ng/mL Zika ns1 (without any MB assay) were loaded in the nanopore optofluidic device (without TACRE).

To this end, an attempt has been taken to detect Zika ns1s at clinically relevant concentration. At first, the nanopore capture rate was estimated and calculated using a linear relationship between the analyte concentration and nanopore capture rate (equation 4.1). As a capture rate of 68.11s^{-1} was observed for a Zika ns1 concentration of $34\mu\text{g/mL}$, the capture rate for 8ng/mL and 4ng/mL Zika ns1 concentration can be calculated and found to be 0.016s^{-1} and 0.008s^{-1} respectively. Next, an aliquot of 8ng/mL Zika ns1 (without any magnetic bead assay) was loaded into the device and an electrical voltage was applied across the nanopore. Only 96 translocations were seen at a capture rate of 0.1613s^{-1} which are shown in the scatter plot of Fig. 4.14(a). When the device was loaded with 4ng/mL Zika ns1 (without any magnetic bead assay) only 24 translocations were seen at a detection rate of 0.0708s^{-1} which are shown in the scatter

plot of Fig. 4.14(b). Though the experimental capture rates are found to be 8-10x higher than the estimated capture rate, still they demonstrate poor detection rates thus, requires significantly large amount of time to detect sufficient number of events which increases the analysis time. In principle, TACRE methodology should be applicable to detect targets at low concentration as it accumulates the targets and increases local concentration.

To experimentally verify this, an aliquot of magnetic bead Zika ns1 assay prepared with 4ng/mL Zika ns1 (see solution preparation for details) was loaded into the nanopore device. After trapping 6 target carrying beads, the device was heated to 50°C and an electrical bias voltage was applied across the nanopore. Fig. 4.15(a) shows the scatter plot of the 622 translocations with a capture rate of 1.989s^{-1} . This capture rate is ~28x higher than the usual nanopore detection rate (without TACRE) which indicates that the analysis time can be reduced 28x using TACRE.

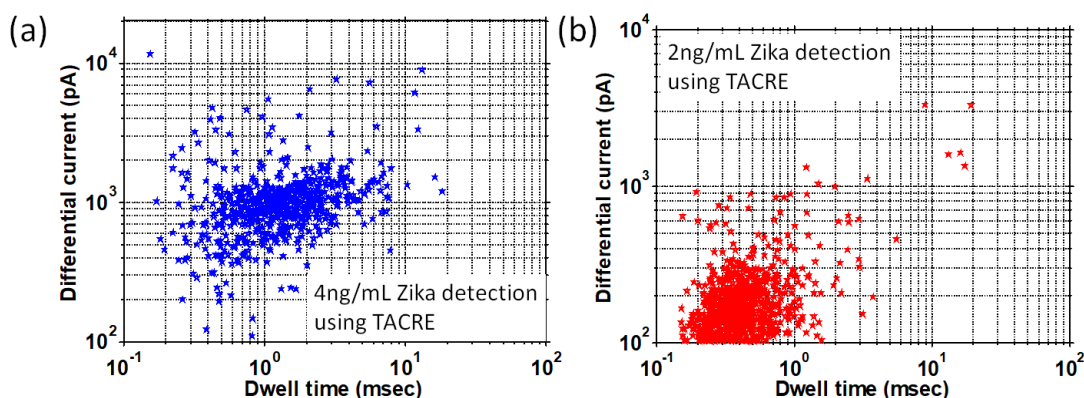


Fig. 4.15 (a) Scatter plot of dwell time vs differential current of translocations found in the electrical trace after thermal release of target proteins from 6 trapped microbeads (MB+HM333+Zika ns1, 4ng/mL case) using TACRE methodology; (b) scatter plot of dwell time vs differential current of translocations found in the electrical trace after thermal release of target proteins from 42 trapped microbeads (MB+HM333+Zika ns1, 2ng/mL case) using TACRE methodology.

Next, an aliquot of Zika ns1 assay prepared with 2ng/mL Zika ns1 (see solution preparation for details) was introduced into the device. Using the same procedure, 42 target carrying microbeads were trapped. Thermally released Zika ns1 proteins were detected when an electrical voltage was applied across the nanopore. This time, a total of 951 translocations were seen at a capture rate of $1.6s^{-1}$ which is shown in the scatter plot of Fig. 4.15(b). This capture rate is $\sim 400\times$ higher than the estimated ns1 capture rate at 2ng/mL ($0.004s^{-1}$) which demonstrates the true power of TACRE. This dramatic improvement in capture rate enables target detection at very low concentrations with significantly reduced analysis time. Furthermore, the capture rate can even be improved (thus reduction in analysis time) by trapping more beads which in turn illustrates the efficacy and true power of TACRE methodology.

These demonstrations pave the way towards a full lab-on-chip device for disease detection with high throughput. As TACRE enables target detection at low and

clinically relevant concentration, it has tremendous potential to find its application in early stage disease detection. As discussed in chapter 2, different target particles usually produce different translocation signatures (depth and dwell time) as they pass through the pore. Depending on individual particle properties (size, charge, shape etc.) it is possible to distinguish different particles if they produce separable translocation patterns. As TACRE works for different bio molecules, this can also possibly be employed for multiplexed detection of different target molecules simultaneously. However, cares should be taken in such applications as there may potentially be cross-reactivity among different biomolecules. Moreover, different molecular assay may require different temperature for thermal release which may become an issue in such applications. Nonetheless, with proper design and selection of biomolecules, it may still be possible to implement TACRE for multiplexed detection of biomolecules simultaneously.

Chapter 5 Summary and outlook

In this work, a nanopore based single molecule analysis platform has been expanded with precise controllable features that provide users to reconfigure the platform upon experimental necessity towards on demand, enabling user friendly, high throughput, configurable single molecule analysis on a chip with rapid succession. Moreover, a new platform for particle trapping and prolonged analysis has been developed that overcomes the drawbacks of previous platform and a sophisticated integration of two very powerful SMA techniques - feedback gating and particle trapping - has been demonstrated towards on-demand prolonged analysis of single particles. Furthermore, a novel technique has been developed to overcome one of the very fundamental drawbacks of nanopore based particle detection with breakthrough results that can significantly improve the particle detection rate with potential application in early stage, low concentration disease detection.

The first advancement has been made by adding a feedback control mechanism over the nanopore detection technology. The feedback control system monitors particle translocation in real time and can turn on/off the pore according to user defined instructions. This precise feedback control over nanopore has meaningfully impactful implications on nanopore based single molecule analysis techniques with several remarkable features. The feedback control system is versatile with applicability to a broad range of biomolecules. The feedback control system allows the user to analyze biomolecules on a demand basis with programmable settings that can be adjusted upon user's instruction and even allows the system to be automated for high throughput

single molecule analysis on a chip. The feedback control system can also be integrated with on chip particle analysis techniques such as ABEL trap which fuses two very powerful single molecule analysis techniques on a single platform. This allows on demand prolonged analysis of single molecules on a chip with precise control over particle delivery. The performance of the current feedback control system can be improved by replacing the current slower ADC with a faster one. Moreover, a faster and dedicated platform such as FPGA is expected to perform better than the current microcontroller. The faster and dedicated feedback control system can possibly be employed for automated multimodal detection and even automated particle trapping and analysis platform which can begin a new era on nanopore based single molecule analysis. With sufficient insight, many exciting breakthrough results are imminent to come in near future.

The second advancement is the development of a new 2D ABEL trapping platform which overcome the drawbacks of previous platform. This new platform enables particle trapping with better control over the trapped particles with improved trap stiffness. The current trapping platform can further be upgraded by incorporating automatic adjustment of feedback gain. Moreover, if inequality of the optical excitation power in an excitation waveguide pair can be predicted and determined that can eliminate another manual adjustment knob. FPGA can be a suitable candidate to overcome the challenges. With upgraded 2D ABEL platform, there are opportunities awaiting to enable trapping smaller bioparticles with prolonged time periods.

The third advancement is the development of an elegant technique to improve nanopore detection rate using an effective integration of on chip optical trapping with nanopore called as “trap assisted capture rate enhancement (TACRE)”. Nanopore event detection rate is improved by pre-concentrating molecular targets on microscale carrier beads, followed by delivery and trapping of these carrier beads at the vicinity of the capture radius of a nanopore. The target-bead assay is prepared in a way that ensures specificity of proper target capture. Thermally released targets are detected using nanopore at a very high rate due to their availability within the capture volume. An improvement of almost two orders of magnitude in event detection rate illustrates the efficacy and supremacy of the technique which enables the quantum leaps in throughput and limits of detection. Another powerful application of TACRE is to enable target detection at low/ clinically relevant concentrations with improved detection rate and reduced analysis time. Zika ns1 proteins have been detected at their clinically relevant concentrations which demonstrates the practicality and power of TACRE. The current TACRE method is limited to the time delay between the thermal release of targets and voltage application for electrical detection of targets to avoid electrical interference. Alternative target release methodology (such as photocleaving) can be adopted for simultaneous target release and detection which can dramatically improve the capture rate since the targets will not diffuse as it does in the current configuration. TACRE can potentially be employed towards early stage disease detection which can make significant breakthrough in the bio-world. These TACRE demonstrations are just the beginning of a series of exciting results and many more to come in near future.

Overall, the whole work is a foundation research guideline based on which many building blocks can be added to explore new avenues that will bring more exciting breakthroughs.

Appendix

MATLAB data analysis code

```
clear;
clc;

data=importdata('File_name.txt');
sampleRate = 'Sample_rate';
maxTime = 'Max_time';
m = 'Sample_per_iteration';

maxdata= max(data(:,2));
mindata=min(data(:,2));

initSample = 'Initial_sample';
SD = std(data(1:initSample,2));

Threshold= 'Up_threshold'*SD;
lowBound = 'Low_threshold';
maxLength = sampleRate*maxTime/m;
minSharpness = 'Sharpness'*SD;

refMob = 'Reference_mobility';

acceptablePulse = 'Pulse_catagory';

lowThresh = lowBound*SD;
pulseType = 0;

Ndata= floor((length(data)-initSample)/m);

NdataUsed = Ndata;
Ref = zeros(NdataUsed,1);
Ref(1)= mean(data(1:initSample,2));

x=initSample+1;

Time=data(1:(NdataUsed*m+initSample),1);
```

```

Sample=data(1:(NdataUsed*m+initSample),2);
Pulse_detection(1:NdataUsed)=mindata;
peakDetect(1:NdataUsed) = 0;
peakData = zeros(1,3);

EdgeSeeking=1;
PulseMaxSeeking=0;

PulseMax=Ref(1);
peakTime = 1;
peakHight = 0;
lowThreshCross = 0;
quartPeak = 0;
tallPulse = 0;

peakFound = 0;

pulseTime=0;
pulseFound = 0;
pulseVerified = 0;
verifiedCount = 0;

timeref = zeros(NdataUsed,1);
Avg = zeros(NdataUsed,1);

for n=1:NdataUsed
    timeref(n)=Time(x);
    Avg(n)= mean(data(x-(m-1):x,2));

    if EdgeSeeking==1
        if abs(Ref(n)-Avg(n))<(Threshold)
            Ref(n+1)=Ref(n)+refMob*(Avg(n)-Ref(n));
        else
            if (Avg(n)-Ref(n))<0
                pulseType = -1;
                Ref(n+1)=data(x+(m-1),2);
            else
                pulseType = 1;
                PulseMax = Ref(n);
                EdgeSeeking=0;
                PulseMaxSeeking=1;
            end
        end
    end
end

```

```

        end
    end
end

if EdgeSeeking==0
    pulseTime=pulseTime+1;

    if pulseTime<maxLength
        Ref(n+1)=Ref(n);
        lowThresh = lowThresh-(lowBound*SD/maxLength);

        if PulseMaxSeeking==1
            if abs(Avg(n)-Ref(n)) > abs(PulseMax-Ref(n))
                PulseMax = Avg(n);
                peakHight = abs(Avg(n)-Ref(n));
                peakTime = n;
            end
            if abs(Avg(n)-Ref(n)) < (peakHight/'Peak_height_fraction')
                PulseMaxSeeking=0;
                peakDetect(peakTime) = PulseMax;
                if peakHight/'Peak_height_fraction' > lowBound*SD
                    tallPulse = 1;
                end
            end
        end
    end

    if tallPulse == 1
        if pulseTime>'Max_time_fraction'*maxLength
            if abs(Ref(n)-Avg(n))<('Up_threshold_fraction'*Threshold)
                pulseFound = 1;
                EdgeSeeking=1;
            end
        end
    end

    if abs(Ref(n)-Avg(n))<(lowThresh)
        if (PulseMaxSeeking == 1)
            peakDetect(peakTime) = PulseMax;
            PulseMaxSeeking = 0;
        end
        pulseFound = 1;
        lowThreshCross = 1;
        EdgeSeeking=1;
    end
end

```

```

else

    if tallPulse
        pulseFound = 1;
    else
        peakDetect(peakTime) = PulseMax;
        pulseFound = 0;
    end
    EdgeSeeking = 1;
end

if EdgeSeeking
    if pulseFound
        if lowThreshCross
            pulseVerified = 1;
        else
            if tallPulse
                pulseVerified = 1;
            end
        end
    end

    if (peakHight/pulseTime) < minSharpness
        pulseVerified = 0;
    end
    if (pulseType ~= acceptablePulse)&&(acceptablePulse ~= 0)
        pulseVerified = 0;
    end
    if (pulseVerified)
        peakData(verifiedCount+1,3) = x;
        peakData(verifiedCount+1,1) = pulseTime;
    end
    pulseFound = 0;
    tallPulse = 0;
    lowThreshCross = 0;
end
pulseTime=0;
Ref(n+1)=data(x+(m-1),2);
PulseMaxSeeking = 0;
PulseMax = Ref(n+1);
peakHight = 0;
lowThresh = lowBound*SD;
end

```

```

end
Thrsl1(n)= Ref(n)+Threshold;
Thrsl2(n)= Ref(n)-Threshold;

lowThrsl1(n)= Ref(n)+lowThresh;
lowThrsl2(n)= Ref(n)-lowThresh;

Pulse_detection(n)= mindata +
pulseVerified/'Pulse_detection_variable'*abs(maxdata-mindata);

if pulseVerified
    verifiedCount = verifiedCount + 1;
    pulseVerified = 0;
end

x=x+m;
end
verifiedCount

hold off;
figure(1);
plot(Time,Sample,'b');
hold on;

plot(timeref,Ref(1:end-1),'g');
plot(timeref, Avg, 'c');
plot (timeref, Thrsl1,'k');
plot (timeref, lowThrsl1,'k');
plot (timeref,Pulse_detection,'r');

for n=1:NdataUsed
    if peakDetect(n)
        plot(timeref(n),peakDetect(n),'mo');
    end
end

xlabel('Time (s)');
ylabel('Current (pA)');
legend('Data','Reference','Average','Upper threshold','Lower threshold','Translocation
found','Location','southwest');

figure(2);

```

```

plot(Time,Sample,'b');
hold on;

plot(timeref,Ref(1:end-1),'g');

plot (timeref,Pulse_detection,'r');

for n=1:NdataUsed
    if peakDetect(n)
        plot(timeref(n),peakDetect(n),'mo');
    end
end

xlabel('Time (s)');
ylabel('Current (pA)');


i = 1;
k = 0;
z = 0;
localMin = 0;
localMax = [0,0];
finalData = zeros(verifiedCount,5);
peakStart = 0;
peakStop = 0;
finIndex = 1;
zoom = 2;


startingPeak = 1;
for n=startingPeak:verifiedCount
    peakStop = peakData(n,3);
    peakStart = peakStop-peakData(n,1)*m;
    [localMin,minIndex] = min(Sample(peakStart:peakStop));
    localMax = max(Sample(peakStart:peakStop));
    peakData(n,2) = localMax;

    axis([Time(peakStart-zoom*1000),Time(peakStop+zoom*1000),localMin-
zoom*(localMax-localMin),localMax+zoom*(localMax-localMin)]);
    prompt = 'Verify Peak: 0 to ignore, 1 to confirm';
    check = input(prompt,'s');
    while check=='8' || check=='7'
        if check=='8'
            zoom = zoom+1;

```

```

else
    if zoom
        zoom = zoom-1;
    end
end
axis([Time(peakStart-zoom*1000),Time(peakStop+zoom*1000),localMin-
zoom*(localMax-localMin),localMax+zoom*(localMax-localMin)]);

prompt = 'Good?';
check = input(prompt,'s');
end

if check=='9'
    n
    break;
elseif check=='1'
    axis([Time(peakStart-1000),Time(peakStop+1000),localMin-(localMax-
localMin),localMax+(localMax-localMin)]);
    [gxi,gyi] = ginput(2);
    abs(gxi(2)-gxi(1))
    finalData(finIndex,1) = abs(gxi(2)-gxi(1));
    abs(gyi(1)-localMax)
    finalData(finIndex,2) = abs(gyi(1)-localMax);
    gxi(1)
    finalData(finIndex,3) = gxi(1);

    prompt = 'flag? (1) for yes (0) for no';
    check = input(prompt);
    finalData(finIndex,5) = check;
    finIndex = finIndex + 1;
end
end

figure(3);
scatter(finalData(:,1),finalData(:,2));
hold off;

```

PSoC code

```
/*
Detects bead or DNA translocations using ammeter output.
Sets Pin_1 (pin 4_5) low when translocation is detected pin is reset when SW_3 is
pressed
SW_2 disables detection while pressed
*/

#include <project.h>
#include <stdlib.h>
#include <math.h>
#include "utilities.h"

#define DEVICE_0 0
#define BUFFER_SIZE "set the number of samples per iteration"
#define SIZE_SD "set the number of samples to calculate the SD"
#define OUTPUT_MODE 1 //0 outputs a short pulse when translocation is
detected,
//1 ouputs sustained logic 0 on detection to shut off relay

/* DMA Configuration for DMA */
#define DMA_BYTES_PER_BURST 2
#define DMA_REQUEST_PER_BURST 1
#define DMA_SRC_BASE (CYDEV_PERIPH_BASE)
#define DMA_DST_BASE (CYDEV_SRAM_BASE)

/* Variable declarations for DMA */
uint8 DMA_Chan;
uint8 DMA_TD[1];
uint8 DMA_TD1[1];
uint8 DMA_TD_Init[1];

volatile uint8 DMA_done = 0;

uint16 ADC_sample[BUFFER_SIZE] = {0};
uint16 ADC_sample1[BUFFER_SIZE] = {0};
uint16 initialData[SIZE_SD]={0};
int main()
{
    Thress_Out_Write(0);
```

```

    Detect_Out_Write(1);
    Gate_Out_Write(1);
    CyDelay("set the delay between voltage application and SD calculation to avoid
calculation of SD in the falling edge");

    DMA_Chan = DMA_DmaInitialize(DMA_BYTES_PER_BURST,
DMA_REQUEST_PER_BURST,
                                HI16(DMA_SRC_BASE), HI16(DMA_DST_BASE));

    /* Allocate TD */
    DMA_TD[0] = CyDmaTdAllocate();
    DMA_TD1[0] = CyDmaTdAllocate();
    DMA_TD_Init[0] = CyDmaTdAllocate();

    CyDmaTdSetConfiguration(DMA_TD[0],
BUFFER_SIZE*DMA_BYTES_PER_BURST, DMA_TD1[0],
                            DMA__TD_TERMOUT_EN | TD_INC_DST_ADR);
    CyDmaTdSetConfiguration(DMA_TD1[0],
BUFFER_SIZE*DMA_BYTES_PER_BURST, DMA_TD[0],
                            DMA__TD_TERMOUT_EN | TD_INC_DST_ADR);

    CyDmaTdSetConfiguration(DMA_TD_Init[0],SIZE_SD*DMA_BYTES_PER_BUR
ST, DMA_TD[0],
                            DMA__TD_TERMOUT_EN | TD_INC_DST_ADR);

    /* Set source and destination addresses */
    CyDmaTdSetAddress(DMA_TD[0],
LO16((uint32)ADC_DelSig_DEC_SAMP_PTR),
                    LO16((uint32)ADC_sample));
    CyDmaTdSetAddress(DMA_TD1[0],
LO16((uint32)ADC_DelSig_DEC_SAMP_PTR),
                    LO16((uint32)ADC_sample1));
    CyDmaTdSetAddress(DMA_TD_Init[0],
LO16((uint32)ADC_DelSig_DEC_SAMP_PTR),
                    LO16((uint32)initialData));

    /* Set the intial TD for the channel */
    CyDmaChSetInitialTd(DMA_Chan, DMA_TD_Init[0]);

    /* Enable the DMA channel */
    CyDmaChEnable(DMA_Chan, 1);

    /* Enable global interrupt */
    CYGlobalIntEnable;

```

```

    /* Start ADC */
    ADC_DelSig_Start();

    /* Disable the ADC ISR as it is not required */
    ADC_DelSig_IRQ_Enable();

    /* Start the interrupt to identify end of DMA transfers */
    DMA_Done_isr_Start();

    /* Start ADC conversion */
    ADC_DelSig_StartConvert();

    //Start of peak detection program
    ****
    **
    uint8 newData = 0;
    uint16 SD = 0;
    int avg;
    int n;

    uint8 currentBuff = 0;

    // configurable
    settings*****
    ****
    int threshold= “set the threshold (high threshold) for target detection”;
    int lowBound = “set the low threshold”;

    int maxLength = “set the max allowable translocation length ”;

    uint8 peakFrac = “se the factor if tall pulse condition is needed to be considered”;
    uint8 PF_SC = “PF_SC is the threshold value to be considered as a tall
    pulse_peakFraction_SpecialCondition”

    int countNum = “set the number of target user want to deliver before the voltage is
    turned off”;
    float refMob = “set the reference mobility”;

    enum PulseType {positive = 0b01, negative= 0b10, all = 0b00}
    acceptablePulse,pulseType; //detector will only look for positive pulses when 1,
    acceptablePulse = positive; //set to negative for downward pulses,
    positive for upward pulses, all for all pulses

```

```

//*****
*****

pulseType = 0;
int lowThresh = lowBound;
int lowIncr;
int ref = 0;

// state vars
*****
*****

int PulseMaxSeeking=0;
enum stateVar {init, edgeSeek, endSeek} state=init;

// peak detection vars
*****
*****

int pulseMax = 0;
int peakHight = 0;
uint8 lowThreshCross = 0;
uint8 tallPulse = 0;

//uint8 pulseLength =0;
int pulseLength = 0;
uint8 pulseFound = 0;
uint8 pulseVerified = 0;
uint8 verifiedCount = 0;

for(;;)
{
    if(DMA_done)
    {
        if (state !=init) {
            DMA_done=0;
            for (n = 0; n<BUFFER_SIZE; n++) {
                if (currentBuff) {
                    avg = calcMean(ADC_sample, BUFFER_SIZE);
                }
            }
            else {

```

```

        avg = calcMean(ADC_sample1, BUFFER_SIZE);
    }
}
if (currentBuff) {
    currentBuff = 0;
}
else {
    currentBuff = 1;
}
}
newData = 1;
DMA_done=0;
}

if (newData) {
    newData = 0;

    if (pulseVerified) {
        pulseVerified = 0;
        Detect_Out_DR |= Detect_Out_MASK;
    }

    switch (state) {
        case init:
            SD = calcSD(initialData, SIZE_SD);
            threshold = threshold*SD;
            lowBound = lowBound*SD;
            lowIncr = lowBound/maxLength;
            lowThresh = lowBound;
            ref = calcMean(initialData, SIZE_SD);
            state = edgeSeek;
            break;
        case edgeSeek:
            if (abs(ref-avg)<(threshold)) {
                ref=ref+refMob*(avg-ref);
            }
            else {
                if ((avg-ref)<0) {
                    pulseType = negative;
                }
                else {
                    pulseType = positive;
                }
            }
        }
    }
}

```

```

    }
    pulseMax = avg;
    state = endSeek;
    PulseMaxSeeking=1;
}
break;

case endSeek:

Thress_Out_Write(1);
pulseLength = pulseLength + 1;

if (pulseLength < maxLength) {
    lowThresh = lowThresh-lowIncr;
    if (PulseMaxSeeking==1) {
        if (abs(avg-ref) > abs(pulseMax-ref)) {
            pulseMax = avg;
            peakHight = abs(avg-ref);
        }
        if ((abs(avg-ref)*peakFrac) < peakHight) {
            PulseMaxSeeking=0;
            if (peakHight > lowBound*PF_SC) {
                tallPulse = 1;
            }
        }
    }
}

if (tallPulse == 1) {
    if (abs(ref-avg)<(1.5*threshold*SD)) {
        pulseFound = 1;
        state = edgeSeek;
    }
}

if (abs(ref-avg)<(lowThresh)) {
    PulseMaxSeeking = 0;
    pulseFound = 1;
    lowThreshCross = 1;
    state = edgeSeek;
}

Thress_Out_Write(0);
}
else {

```

```

        state = edgeSeek;
    }
    if (state == edgeSeek) {

        if (pulseFound) {
            if (lowThreshCross) {
                pulseVerified = 1;
            }
            else {
                if (tallPulse) {
                    pulseVerified = 1;
                }
            }
            pulseFound = 0;
            tallPulse = 0;
            lowThreshCross = 0;
        }

        pulseLength=0;
        ref=avg;
        PulseMaxSeeking = 0;
        pulseMax = ref;
        peakHight = 0;
        lowThresh = lowBound;
        if ((pulseType != acceptablePulse)&&(acceptablePulse != all)) {
            pulseVerified = 0;
        }
        if (!SW_2_Read()) {
            pulseVerified = 0;        // if SW_2 is pressed, disable detection
        }
    }
    break;
}
if (pulseVerified) {
    Detect_Out_DR &= ~Detect_Out_MASK;
}
#if OUTPUT_MODE == 1
    if (pulseVerified) {
        verifiedCount++;
    }

    if (verifiedCount==countNum) {
        Gate_Out_DR &= ~Gate_Out_MASK;
    }
}

```

```

        CyDelay(1);
        Detect_Out_DR |= Detect_Out_MASK;

        // while (SW_3_Read()); // For manual re-application of voltage after
gating
        CyDelay("set the delay to automatic voltage re-application");
        Gate_Out_DR |= Gate_Out_MASK;
        CyDelay("delay so that distortions related to reconnection of power to
chip are not detected as translocations");
        verifiedCount = 0;
        pulseVerified = 0;
    }

    #endif

}
}
}
int calcMean(uint16* array, uint16 size) {
    int sum = 0;
    int i;
    for (i=0;i<size;i++) {
        sum += array[i];
    }
    return (sum/size);
}

int calcSD (uint16* array, uint16 size){
    int mean=calcMean(array, size);
    int j;
    int y=0;
    for (j=0;j<size;j++)
    {
        y+=(array[j]-mean)*(array[j]-mean);
    }
    return (sqrtf(y/size));
}
/* [] END OF FILE */

```

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