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BME 123-T

04/01/2015

Senior Thesis

Dear Undergraduate Research Honors Committee,

I have prepared my thesis report for your review. Please print this report in color as many of the figures make use of various colors for explanation. I greatly appreciate your time and consideration of my credentials.

Best Regards,

Rolando Cruz Perez

High-throughput Cell-capture Device for Semi-automated Single-cell Nanobiopsy and Nanomanipulation

A THESIS

SUBMITTED TO THE DEPARTMENT OF BIOMOLECULAR ENGINEERING OF THE UNIVERSITY OF CALIFORNIA, SANTA CRUZ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE

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Abstract:

A single-cell nanomanipulation system allows the user to investigate the cellular processes of a single cell, over a period of time, without killing the cell. This has been done on cells that are stuck to the bottom of a petri dish. To investigate cells that are usually floating, requires a means of capturing the cells out of solution and holding them down in a way that allows a nanopipette to access the cells.

The focus of this research is a microfluidic cell-capture device. I characterized and integrated a cell-capture device with the single-cell nanomanipulation system. This system consists of the nanopipette biosensor with accompanying software. The nanopipette, a capillary quartz tube with a nanometer-sized pore, has shown great promise for single-cell manipulation and analysis with sub-cellular resolution. I contracted a consultant to use semiconductor processing techniques to fabricate the silicon structures of the devices and I finished the fabrication steps to complete the devices.

The device consists of a sandwich structure with a transparent membrane window perforated by a 6 X 6 array of 2-micrometer (µm) diameter through-holes. The device, coupled with a negative-pressure control system, enables the capture and release of single cells floating in cell media while keeping the cells alive. By supplying a negative pressure of 200 Pascals (Pa), the cell sifter captures cells in cell media, immobilizes the cells at the through-holes, and releases the cells on demand.

As a proof of concept, HeLa cells expressing green fluorescent protein were treated with trypsin, and captured on the device. Nanobiopsies were attempted on 12 single cells to aspirate subpopulations of mitochondria. I conducted polymerase chain-reaction to amplify mitochondrial DNA (mtDNA) from each sample. Initial results are inconclusive, but remain promising. Ongoing research will further develop and characterize the cell-capture device.

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Acknowledgments

I would like to thank Dr. Nader Pourmand for giving me the opportunity to develop this project and for guiding me toward a successful career in scientific research. I would like to thank the Pourmand Lab for all of their help, useful discussions, and training. I would like to thank Tom Yuzvinsky for his helpful discussions and training me on cleanroom procedures and equipment, Taiuk Rim for helping me design a photomask concept. I would like to thank Dr. Jim Kruger of the Stanford Nanofabrication Facility for his work in the microfabrication of the sandwich structure and useful discussions regarding the microfluidic device design. I extend gratitude to all of my peers for their helpful discussions, support, and helpful feedback. I would like to thank the Maximizing Access to Research Careers program for providing funding that enabled me to explore my full research potential. I am forever indebted to my family, for supporting me throughout my journey.

1. Nanomanipulation Of Single Cells Floating In Cell Media

Nanomanipulation, the manipulation of single cells at the nanoscale, of *non-adherent* single cells, cells that are usually floating and not stuck to a surface, is an engineering problem that presents several challenges. I was asked to produce a microfluidic device that would capture many non-adherent single cells in parallel into predetermined locations. A device that captures single cells floating in cell media must maintain cellular viability after capturing cells to preserve the benefits of the nanomanipulation system, must be optically transparent so it can be used with an inverted microscope setup, and must maintain a compact device profile to accommodate the existing nanomanipulation system setup. The device specification is as follows:

- The device must capture floating cells into predetermined locations and provide access to the cells for the nanopipette.
- The device must provide a force that is sufficient to hold the cells in place while being pierced by a nanopipette. The maximum amount of force I can apply to hold the cells down, the materials I can use to fabricate the device, and the device geometries are constrained by the need to maintain cellular *viability*, ability to continue growth.
- This device must be optically transparent so that it can be integrated with the existing single-cell nanomanipulation system that uses an inverted microscope.
- A compact device profile is crucial to maintaining a versatile single-cell nanomanipulation system. The nanomanipulation system uses piezoelectric actuators and micro-manipulators that require spatial clearance and previous attempts to integrate a device have encountered over-head clearance issues. A a low-profile cell-capture device is crucial.

1.1. Breaking Free from Adherent Cells: Extending the Capabilities of the Nanomanipulation System to Include Non-Adherent Cells

The single-cell nanomanipulation system developed by the Pourmand Lab has shown great promise for single-cell and sub-cellular analysis (7). This system has been used in studies on single cells growing on petri dishes, known as *adherent* cells. These adherent cells use proteins to stick to each other and certain surfaces. The proteins provide enough adherent force for the cell to be pierced by the nanopipette without moving out of place. To facilitate the expansion of the capabilities of the system I have pushed forward the development of a device that captures single cells floating in cell media, known as non-adherent cells. I helped design the device layout, contracted a nanofabrication consultant, and integrated the microfluidic device with the single-cell nanomanipulation system. I have used the microfluidic device to capture single cells out of cell media into a predetermined array to interrogate the captured single cells with the nanomanipulation system.

1.2. A Microfluidic Solution for Capturing and Immobilizing Single Cells Floating in Cell Media

Microfluidics, the study and design of fluid flow through micro-channels in a material, encompasses a large application space. Several groups have developed microfluidic platforms for bioparticle manipulation that can separate and immobilize particles of interest (15). Using microfluidics to capture and release single cells floating in cell media can provide a compact, fast, sensitive, and economic method for immobilizing single cells into a predetermined array for interrogation with the nanopipette. A microfluidic cell-capture platform should present a robust module readily amenable to future modifications to the single-cell nanomanipulation system. Adopting a microfluidic approach to cell capture will enable high-throughput nanomanipulation of non-adherent single cells.

1.3. High-Throughput Semi-Automated Nanobiopsy and Nanomanipulation Of Non-Adherent Single Cells

I report on the *nanobiopsy*, the removal of a minuscule amount of material from a single cell, and nanomanipulation of multiple non-adherent single cells immobilized by a microfluidic device. The system consists of a nanopipette biosensor, micro-manipulators, nano-piezoelectro actuator, software, and a microfluidic cell-capture device. The non-adherent single HeLa cells were captured out of cell media at a predetermined array of through-holes by means of *hydrodynamic trapping*, the trapping affect caused by an object blocking fluid flow through a structure, in this case a through-hole. I confirmed post-capture cellular viability via vital stain. I nanobiopsied mitochondial DNA from non-adherent single HeLa cells using the nanomanipulation system. Gel-electrophoresis confirmed the presence of amplified mitochondrial DNA from the single-cell nanobiopsies. Fluorescence microscopy confirmed the nanoinjection of mitochondrial dye post-capture.

2. Existing Methods For Single Cell Nanomanipulation and Microfluidic Handling

2.1. Biological Relevance

Single-cell and subcellular analysis has gained increased attention in recent years as evidence suggests cell heterogeneity plays an important role in disease, such as cancer and neurodegeneration (1, 2). Investigating the gene-expression profile of single cells provides data that can be hidden in population studies. In traditional genomic studies involving whole populations of cells, very low abundance transcripts are often lost in the vast number of more highly expressed transcripts. This is because expression profiles of cell populations are averages of the single-cell expression profiles. This can also be the case with genomic information. Figure 1 shows the discrepancy found in the number of single nucleotide variants (SNVs) found in a population sample and the number of SNVs found in single cells from that same population. This undetected *omic* information relating to the genome, transciptome, proteome, etc., is vital to understanding the nature of cell heterogeneity.

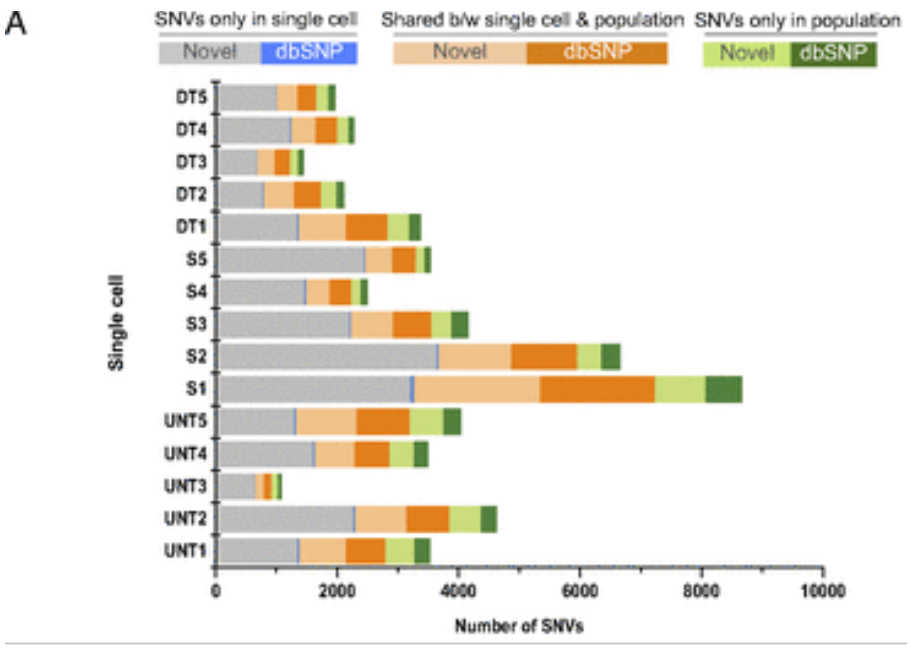


Figure 1 - Single cell analysis revealed single nucleotide variations that were not detected at the population level. This figure shows the difference between single nucleotide variants (SNV) detected in single cells and SNVs detected in the population from which those cells came. The grey bars show novel SNVs detected at the single cell level that were not detected in the population samples. This loss of novel SNV data at the population level is critical because the undetected SNVs may confer a disease state in a single cell that would go undetected if analysis was done strictly at the population level. Therefore, single-cell analysis is crucial to understanding the true nature of cellular heterogeneity. (figure from Lee et al. (1))

Cell heterogeneity arises from the temporal and innate variations in cellular state, variations in genetic profile, and variations in molecular species in single cells (3). Single-cell analysis enables a true representation of cellular activity. By interrogating many single cells in parallel, significant statistical data can be produced. This approach can potentially further understanding of underlying biological mechanisms at the single-cell level and aid in the simultaneous observation of population-wide responses. Population-wide studies can be constructed from data generated by the parallel investigation of biomechanisms at the single-cell level. Single-cell analysis has limitations such as low transcript copy numbers, higher cost to sequence single-cell samples, and loss of data in the form of lost molecules during library prep.

2.2. The Nanomanipulation System

Nanopipettes and nanogenomics have demonstrated the ability to provide *spatiotemporal*, data regarding both space and time, resolution of single-cell genomics (4, 5). The nanopipette, a capillary

quartz tube with a maximum pore diameter of 200 nanometers (nm), has shown great promise for single-cell nanomanipulation and analysis with sub-cellular resolution (6). Nanopipettes have been integrated into a semi-automated nanomanipulation system for analysis of single cells growing adhered to the bottom of a petri dish (7). Figure 2 shows the workflow of the nanobiopsy procedure developed by Actis et al.

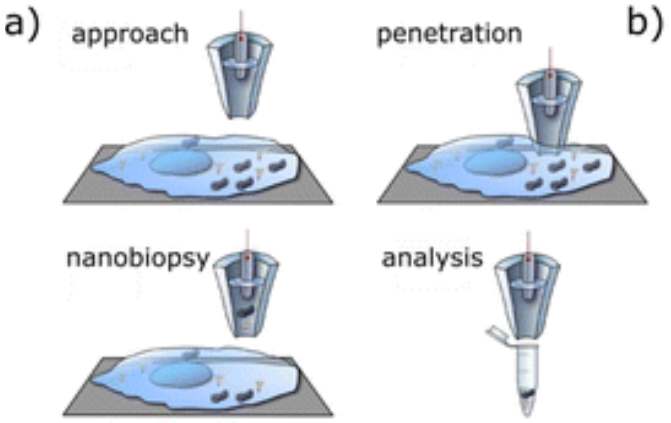


Figure 2 - Diagram of single cell nanobiopsy technique. The procedure facilitates the removal of femtoliter amounts of material from a single cell. First, an ionic current is read as the nanopipette approaches a cell's surface. The decrease in ionic current experienced by the nanopipette as it approaches the surface is used to determine the location of the cell. Once the cell surface is found the software guides the nanopipette into the cell cytoplasm. Once inside the cell, the polarity on the electrodes is reversed, causing material to be aspirated into the nanopipette. The sample is then collected into a droplet of solution for downstream analysis (figure from Actis et al. (7)).

The nanobiopsy technique presented by Actis et al. facilitates the removal of material from the same single cell over a period of time, presenting the opportunity to study cellular processes at the single-cell level. Adapting this technique to allow the high-throughput parallel interrogation of single cells growing suspended in cell media would greatly expand the capability of the single-cell nanomanipulation system, simplify the technique even further, and facilitate the application of the system to other areas of biological research. A microfluidic approach to cell immobilization would present many benefits such as requiring only small amounts of input samples and reagents, short experiment and analysis times, reduced manufacturing costs, increased automation, and the opportunity to perform massively parallel analysis on a single sample or multiple samples.

2.2.1.Nanopipette Biosensors

Nanopipettes can be made from borosilicate or theta quartz and are pulled by a capillary carbon dioxide (CO_2) laser puller to produce a tip opening less than 200 nm in diameter. Nanopipette tip geometries can be tuned by altering the parameters of the clean-room-free pulling process. A nanopipette may interrogate a single cell multiple times over a period of time due to the very small size of the tip. Electrodes and electrolytes are often used in conjunction with a nanopipette to facilitate single-cell sensing and removal or deposition of material into a single cell by exploiting electrowetting phenomena. Nanopipettes are proving to be a very versatile nanotechnology for biologists.

Conventional methods for single-cell analysis and manipulation, such as micropipette injection involved in *in vitro* fertilization, typically use pipettes with tip diameters greater than 200 nm (8, 9). These larger tips are not very invasive when interrogating cells with diameters greater than ~ 100 µm, such as zygotes, but often kill cells with much smaller diameters.

Due to the size constraints on micropipette tips, single-cell analysis over time of cells other than large zygotes has been challenging. Nanopipettes provide a solution to this challenge. Figure 3 shows the geometric characteristics of the nanopipette.

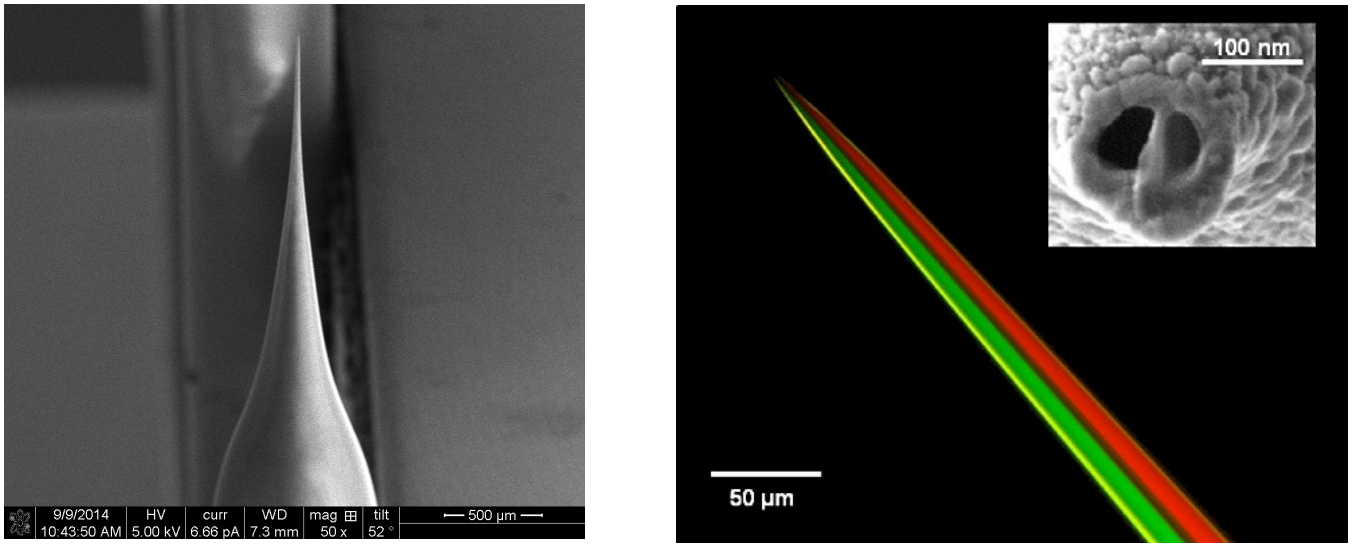


Figure 3 - Scanning electron microscope (SEM) image of nanopipette (left, taken by Wai Mak, 2014). Micrograph of a double barrel nanopipette tip and SEM image of tip (inset), the double barrel nanopipette is used for nanoinjections (right, figure from Actis et al (7)).

Nanopipettes have been used as a label-free biosensor (10). Nanopipettes decorated with interactive biomolecules enable biorecognition of analytes and have been used as protein sensors and metal ion sensors (11, 12). Nanopipettes integrated into a nanomanipulation system have been used to find single mammalian cells growing on a petri dish and inject them with fluorescent dye (13). This system uses scanning ion-conductance microscopy (SICM) to sense the surface of a cell with a nanopipette, allowing the semi-automated interrogation of that single cell. Figure 4 shows a schematic representation of a typical nanopipette biosensor setup, and Figure 5 shows how the nanopipette can be used as a biosensor.

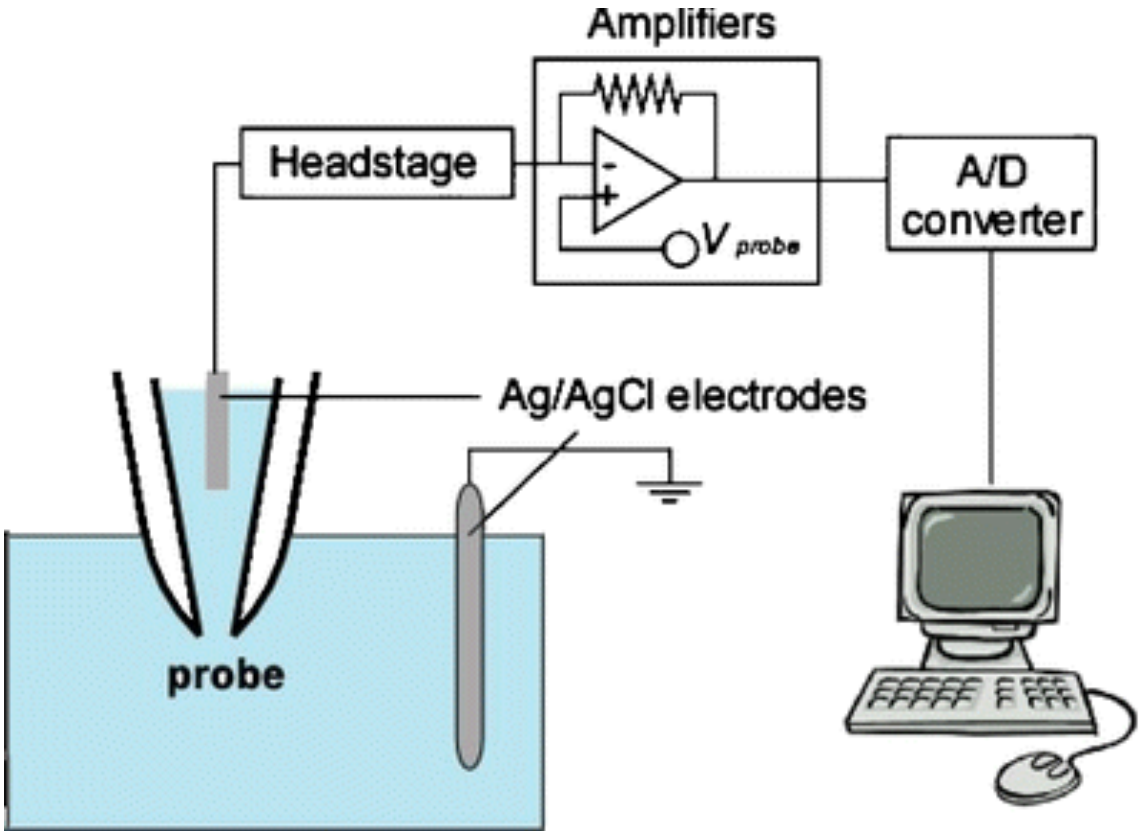


Figure 4 - Schematic of a typical nanopipette biosensor setup. A potential is applied across two electrodes. The movement of a nanopipette is controlled through a feedback loop and software controls the actuation of the movement. (figure from Actis et al (10)).

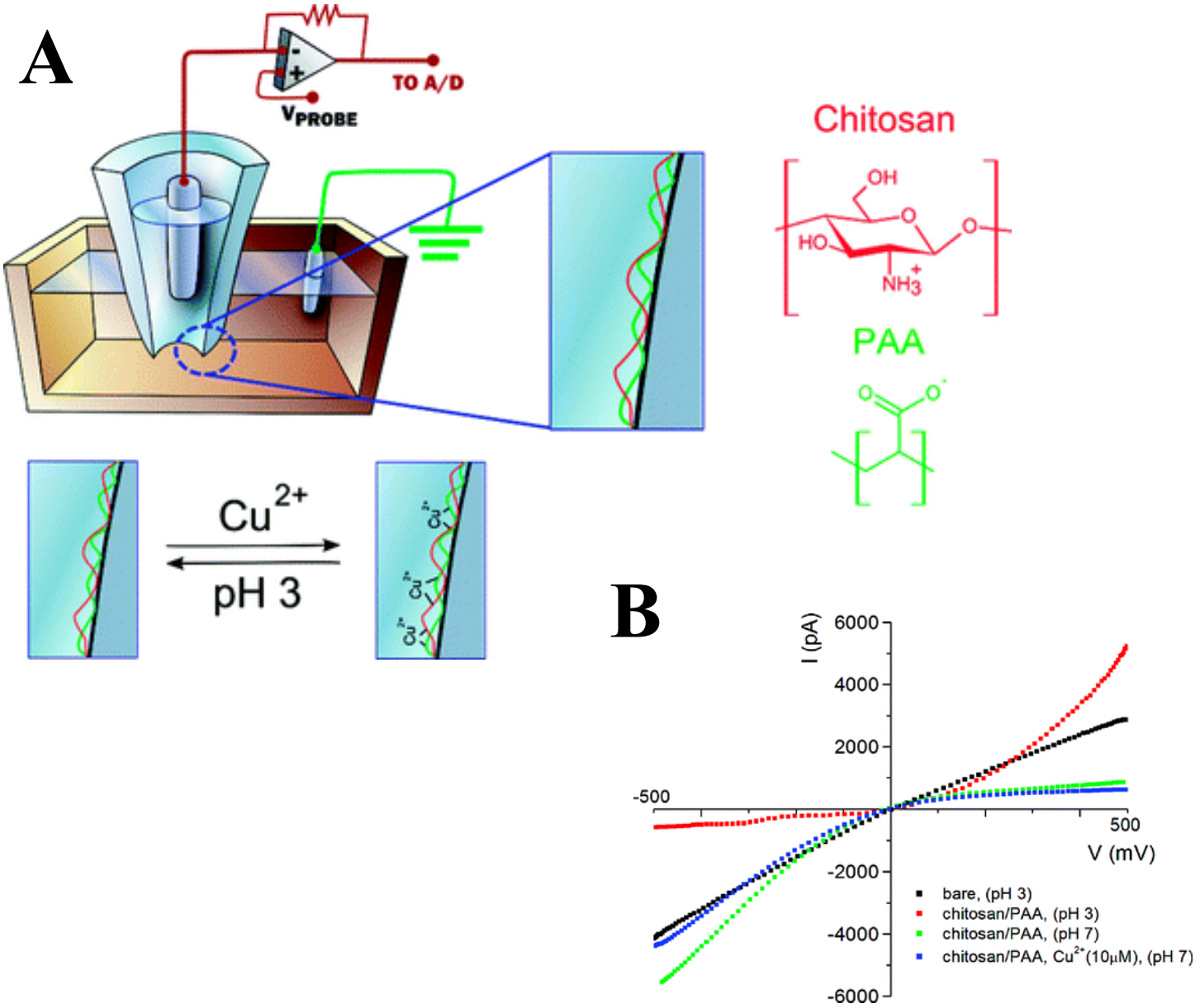


Figure 5 - Diagram of nanopipette biosensor setup for metal sensing. The inside surface of the nanopipette is decorated with functional groups that can interact with molecules of interest. This surface chemistry is the basis of the nanopipette biosensor. (figure A, figure from Actis et al. (12)). A layer of poly acrylic acid (PAA) was deposited onto the inner surface of the nanopipette tip. The biopolymer chitosan was also deposited on the inside of the nanopipette tip. The PAA and chitosan coatings bond with cupric ions. This changes the surface properties of the nanopipette tip in a way that we can observe. Once the cupric ions are added into the solution we can sense a change in the current profile of the nanopipette. Changing the pH of the solution effects the ability of the chitosan/PAA layers to bind cupric ions, which makes the reaction reversible. Figure B shows a graph of current readings showing sensing of cupric ions in single cell. The black line shows the current reading of a bare nanopipette. The red line shows the current reading of the chitosan/PAA modified nanopipette at a pH of 3, where there are more hydrogen ions present in solution and therefore the chitosan/PAA layer is protonated, resulting in a more positively charged surface that produces a positive current rectification signal. The green line is the same nanopipette setup in pH 7, where there is a lack of ions in solution and the chitosan/PAA layer is deprotonated, producing a negatively charged surface and a negative current rectification signal. The blue line indicates the functionalized nano pipette's response to cupric ions at pH 7, as you can see the current signal is slightly positively rectified, due to the cupric ions binding to the chitosan/PAA coated inner surface of the nanopipette. (figure B, figure from Actis et al. (12)). Together, these characteristics allow the nanopipette to be used as a biosensor capable of interrogating a single cell multiple times.

2.2.2.Scanning Ion-Conductance Microscopy (SICM)

Scanning ion-conductance microscopy of living cells is a method of detection based on a nanopipette scanning the surface of a cell of interest, producing the topography of the cell (14). As a nanopipette is brought closer to the surface of a cell, the ionic current the electrode senses experiences a decrease. This phenomena is used to find the surface of a single cell. Figure 6 shows how SICM is used to find the surface of a single cell.

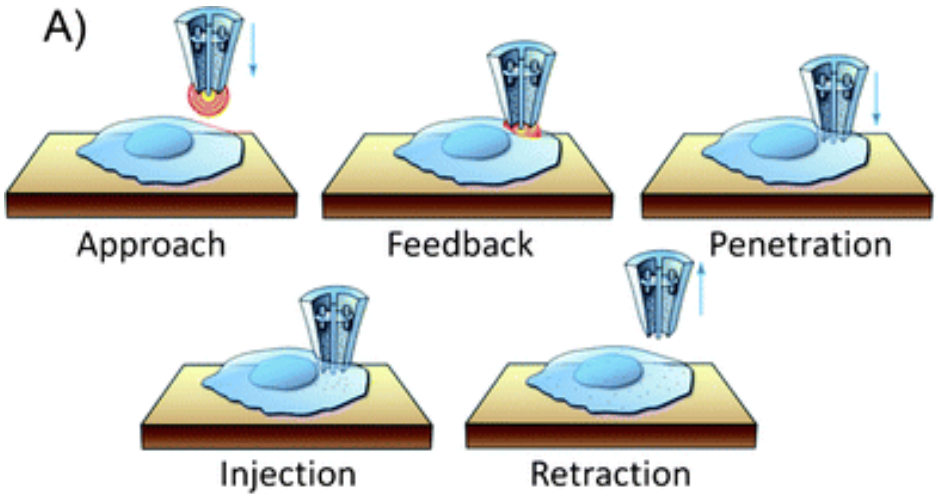


Figure 6 - Diagram showing the use of scanning ion conductance microscopy (SICM) to aid in single cell nanoinjection (left, figure from Seeger et al.(13)).

2.3. Microfluidic Cell Sorting, Trapping, and Immobilization

I conducted a literature survey of microfluidic techniques that can hold down floating cells. I was specifically looking for methods that would allow me to capture single cells into an array while providing the nanopipette access to the cells and enough force to keep the cells from moving when being pierced by the nanopipette.. Several microfluidic systems are employed to sort, trap, and immobilize single cells floating in solution (15).

Many of these techniques are *active,* using acoustic, optic, magnetic, or electric force fields exerted on the cells of interest to manipulate the cells. Many of these active techniques are *label-based*, where the cells are pre-labeled with a molecule that interacts with the applied force field, or chemically

treated surface in the case of the passive chemical immobilization, and aids in the identification and sorting of the cells of interest (16). These methods are not suitable for the nanomanipulation system because they require sealed microfluidics that are pressurized and cannot be open to the ambient atmosphere to provide the nanopipette access to the cells. Furthermore, these methods do not immobilize the cells into predetermined locations with the precision required by the nanomanipulation system.

Hydrodynamic trapping is a form of passive immobilization where a cell is immobilized by blocking fluid flow through a micro-structure. Hydrodynamic trapping can simultaneously immobilize many single cells into predetermined locations while providing access to the cells for the nanopipette. Hydrodynamic trapping also provides the necessary force to make sure the cells do not move when pierced by the nanopipette. Methods such as magnetic, dielectrophoretic, and chemical require some kind of preprocessing step that involves labeling the cells. Hydrodynamic trapping does not require these laborious and time consuming preprocessing techniques and equipment, and are therefore preferable for the nanomanipulation system. Figure 7 shows the different methods of single cell trapping in microfluidic platforms. Figure 8 shows the differences in throughput amongst the different microfluidic methods for cell trapping.

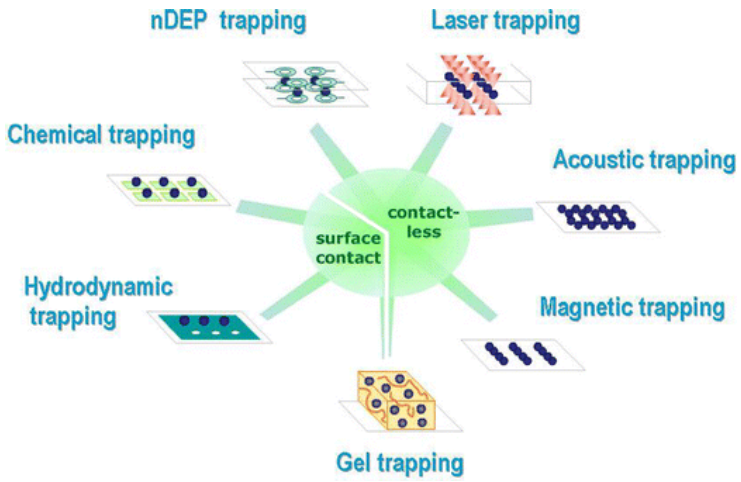
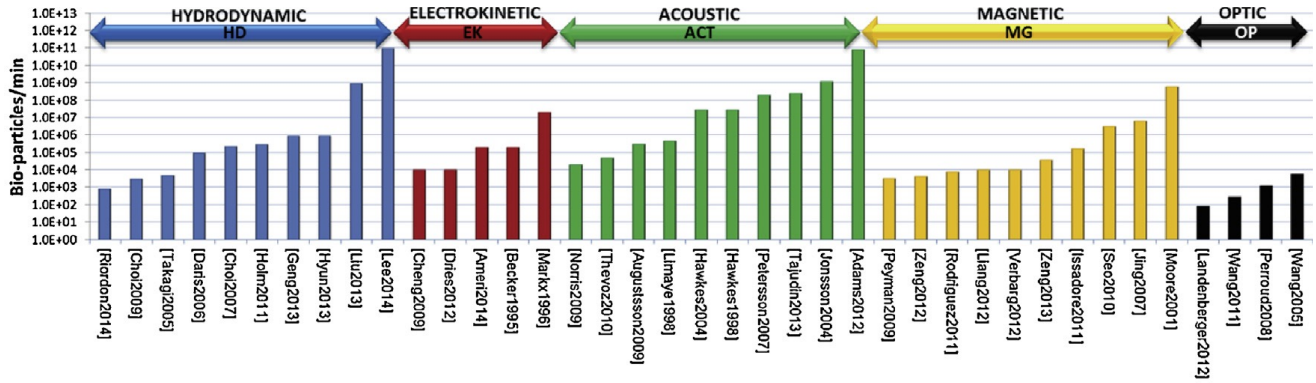
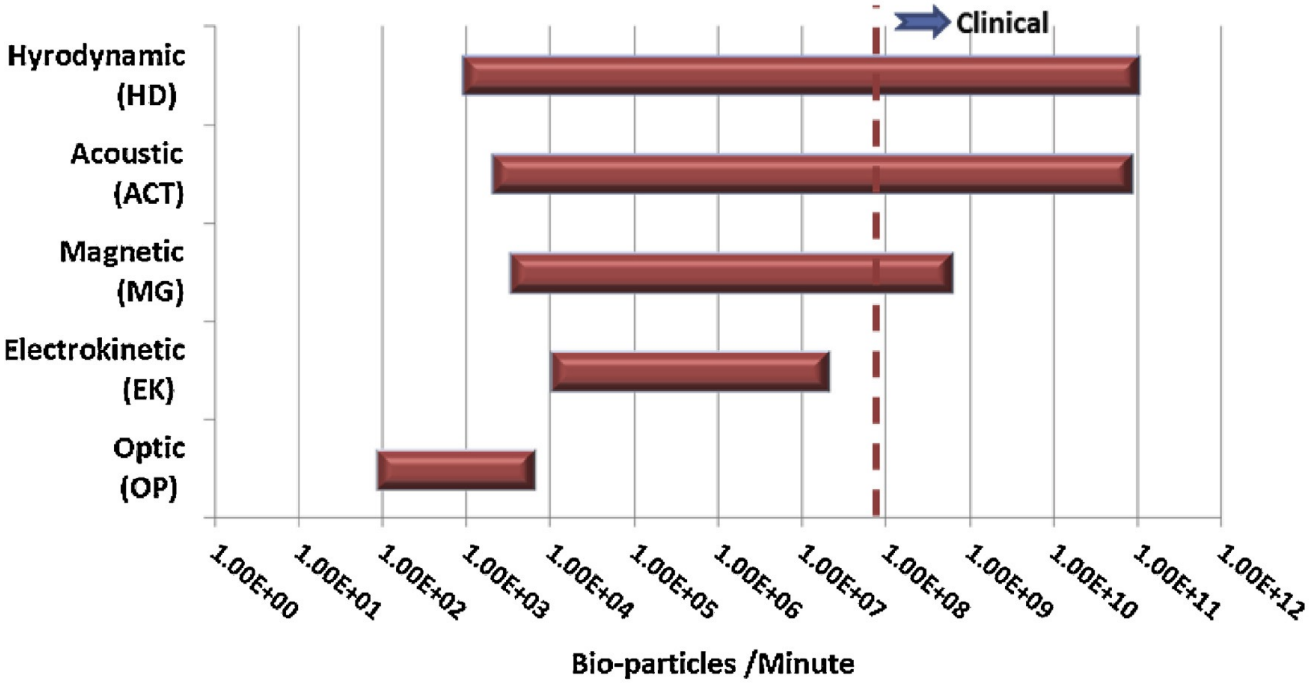


Figure 7 - Methods of single-cell sorting, trapping, and immobilization can be grouped into passive and active, label-based and label-free, and surface contact versus contact-less (figure from Johan et al. (16)). In surface-contact methods, the cells come into contact with the surface of the substrate utilized to accomplish the specific cell-manipulation method. This may elicit a stress response from the cell, often an undesirable outcome. In contactless, this surface contact is avoided and cells of interest will not elicit the undesired stress response. Alternatively, contactless methods often suffer from lower throughput, control, and specificity than their surface-contact counterparts.



(a)



(b)

Figure 8 - The different methods for single-cell sorting, trapping, and immobilization vary in their throughput (figure from Çetin et al. (15)). A high-throughput method is desirable for integration with the nanomanipulation system. The ability to immobilize many cells at once will provide access to rare cells that exhibit interesting biology and may provide a basis for large scale cellular engineering.

Previous devices that use microsieve structures have been constructed to facilitate the hydrodynamic filtration of circulating tumor cells (CTC) from blood. These devices allow fluid to pass through micropores in a separating membrane, while the cells of interest are captured at the micropores (17). Figure 9 shows a device constructed by Lim et al. that uses hydrodynamic trapping to sort a blood sample and immobilize CTCs.

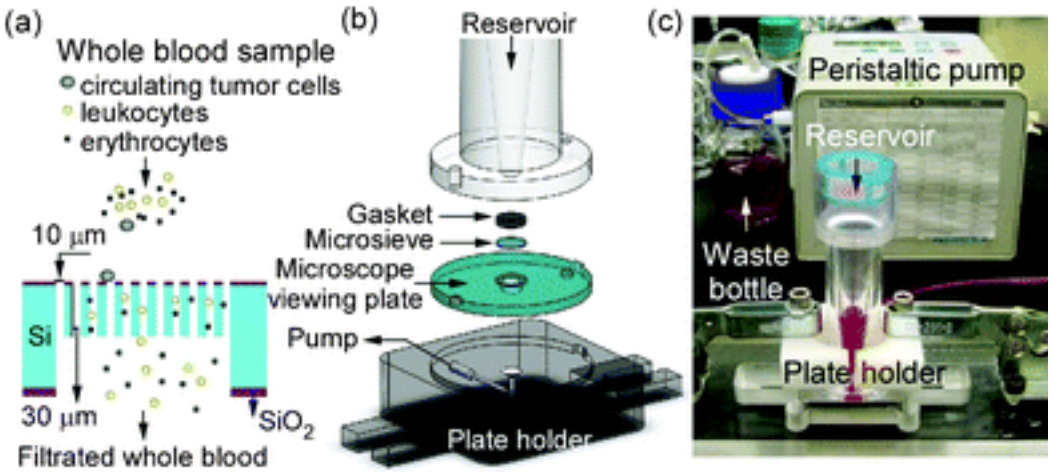


Figure 9 - Microsieve structures have been used to sift through a sample and isolate cells of interest by size separation (figure from Lim, et al. (17)).

Patch-clamp analysis devices also provide examples of microfluidic structures capable of capture and release of single cells for analysis where two compartments are separated by a thin membrane of material through which micro through-holes capture single cells at an array of these through-holes by means of electrophoresis or hydrodynamic trapping (18, 19, 20). Figure 10 shows the approach taken to fabricate structures that use hydrodynamic trapping to immobilize single cells in a microfluidic device.

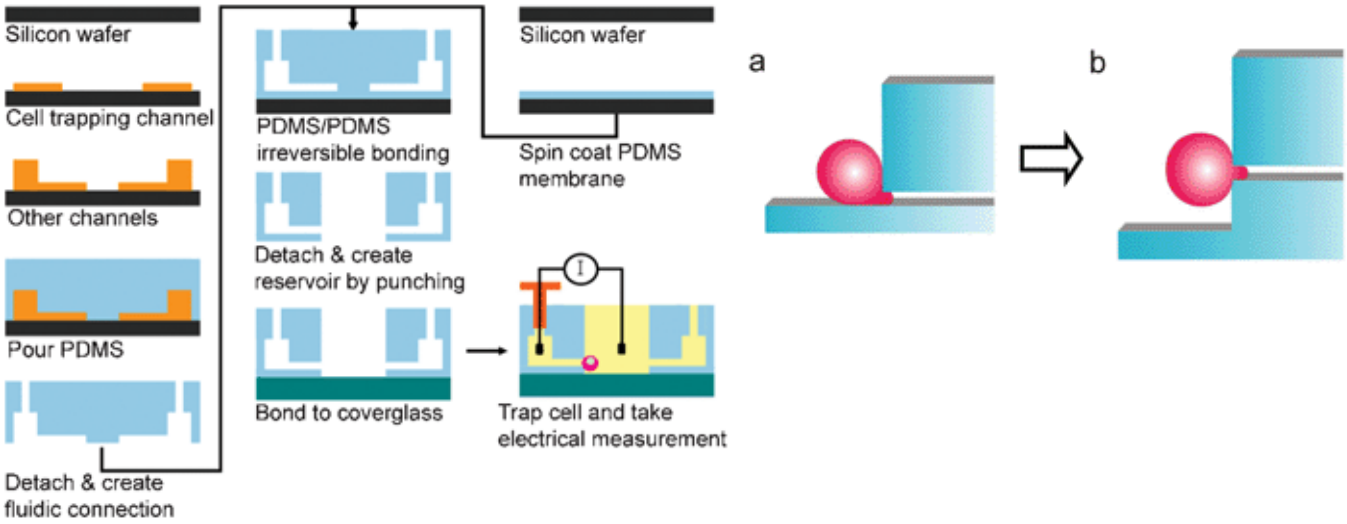


Figure 10 - Methods for single-cell immobilization in microfluidic systems are used in patch-clamp analysis. These techniques provide examples of mechanical single cell immobilization (figure from Seo et al. (19)).

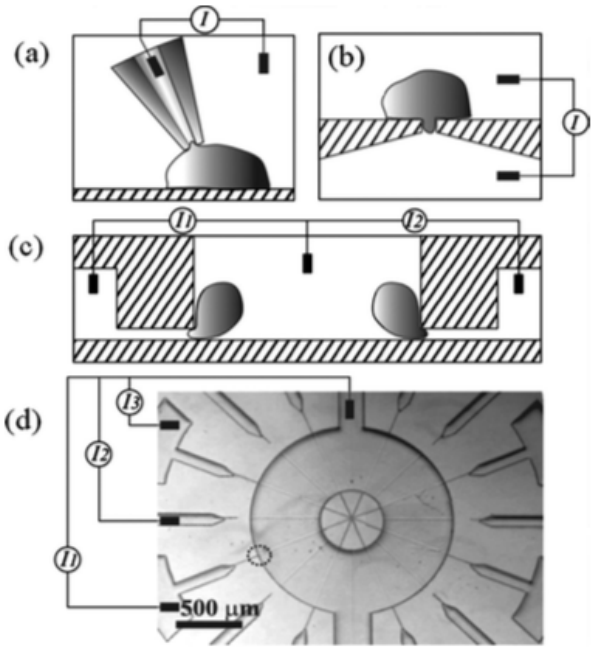


Figure 11 - Arrays of single cell immobilization sites within microfluidic platforms have been developed for patch-clamp analysis. The progression from the pipette patch-clamp (a), to the current arrayed method (d), is shown in the figure. The ability to perform this kind of patch-clamp analysis can be preserved in the design of cell capture device for the nanomanipulation system. Using dielectric materials can provide a basis for other electrode fabrication. Along with the nanomanipulation system, a full suite of other biosensors can be implemented on the cell capture device substrate to perform a combinatorial parallel analysis of single cells (figure from Lau et al. (20))

Mechanical immobilization via vacuum-assisted hydrodynamic trapping is a suitable label-free method for real-time identification, selection, and access to cells of interest for interrogation with a nanomanipulation system. Immobilizing the cells at an array of predetermined micro through-holes in an optically transparent surface allows the device to be used with an inverted microscope. Hydrodynamic trapping provides the force necessary to hold single cells for manipulation with micropipettes and is amenable to nanomanipulations.

2.4. High-Throughput Automated Microinjection

Current techniques for automated high-throughput microinjection of zygotes and other cells of interest present examples for the integration of a microfluidic cell-trapping device with the single-cell nanomanipulation system (21, 22, 23, 24). These techniques use hydrodynamic trapping of single cells at a predetermined array of through-holes and have been shown to maintain cellular viability post-capture. Glass substrates are used to maintain the optical transparency required by inverted microscopes

typically used with microinjection systems. A thin silicon nitride membrane provides optical transparency and a versatile substrate for further micro fabrication techniques such as thin metal-film deposition for electrode fabrication.

Figure 12 - Tools for microinjection of larger single cells have led to the development of sophisticated automated injection systems, such as this Fujitsu prototype. (figure from Sakai et al. (21)). Some of these early systems were very large requiring ample space, limiting applications.

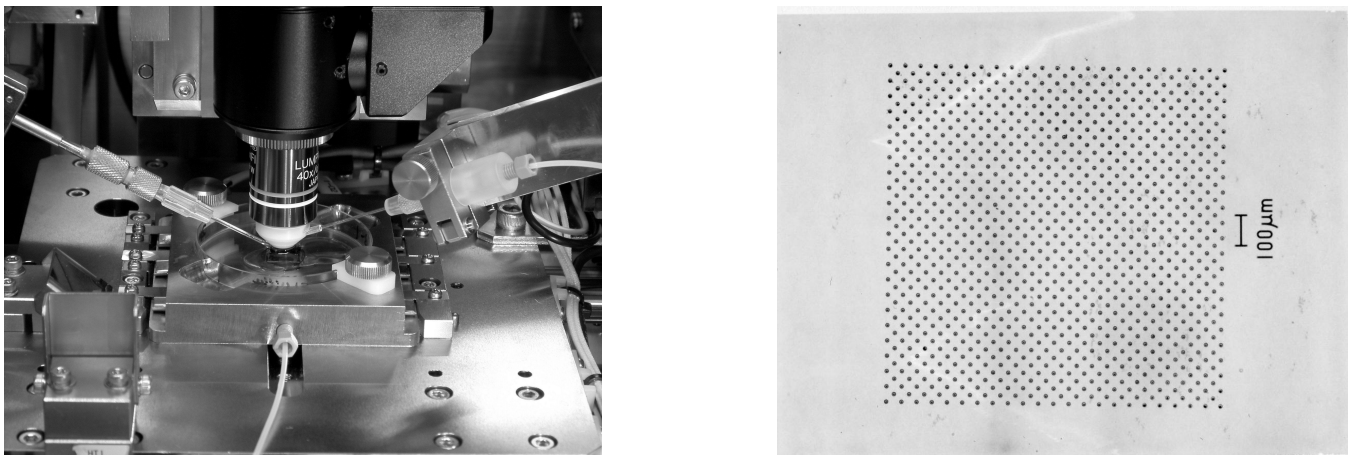


Figure 13 - Micromanipulators direct the movement of a micropipette into position for microinjection of a single cell (left). An array of through-holes for single cell capture and immobilization (right, (figure from Sakai et al. (21)).

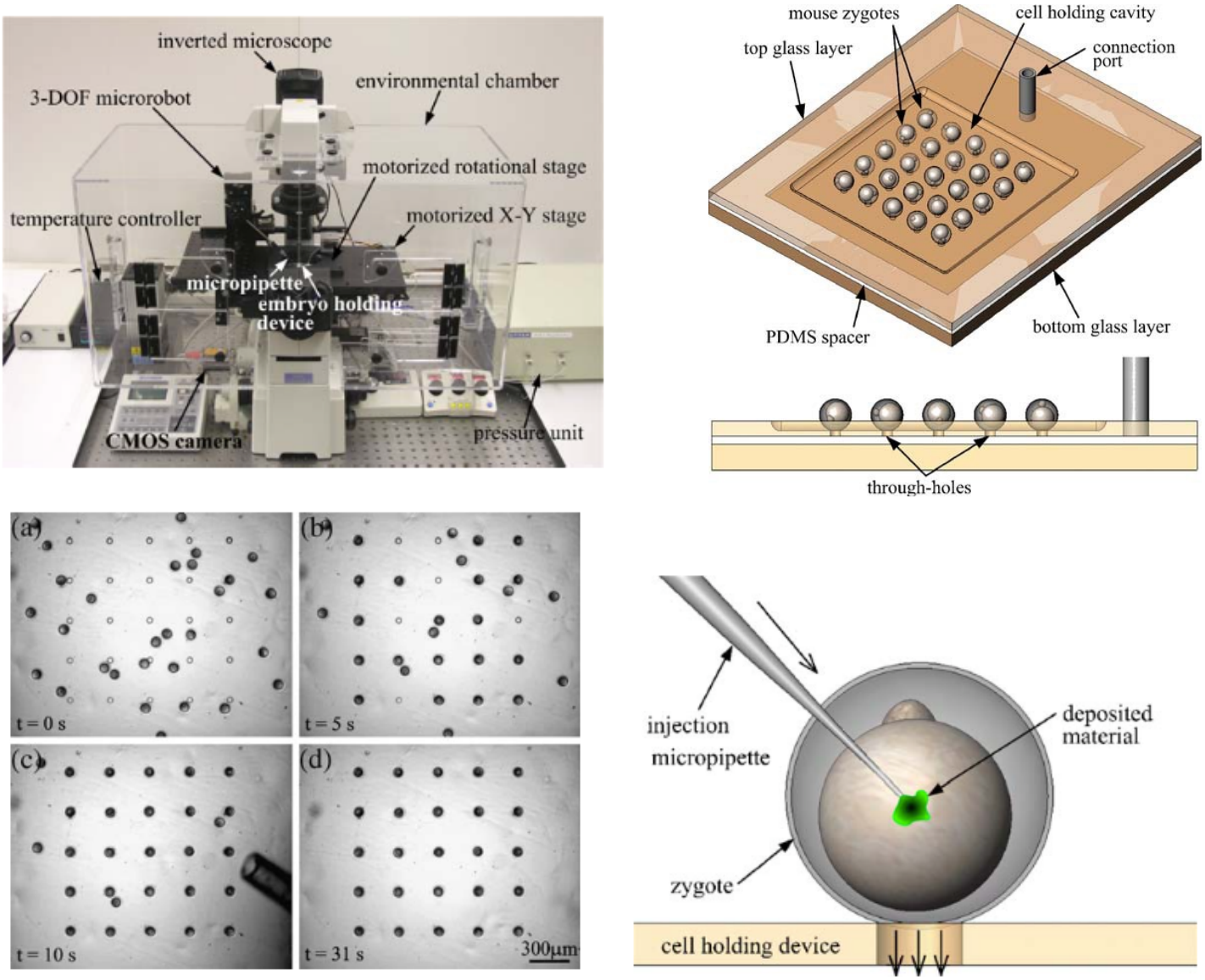


Figure 14 - Some automated high-throughput microinjection systems use devices that immobilize single cells into arrays. These devices use negative pressure to suction single cells down onto holes in a surface material. These devices show that you can hold cells down with low negative pressure and the cells will stay viable. Since microinjection and single cell nanomanipulation share similarities in their system layout and operation it is reasonable to assume that a similar device would work for the nanomanipulation system. The top left image shows the inverted microscope and micro manipulator setup used for the high-throughput single cell microinjection.The top right image shows the device layout. The bottom left image shows a micrograph of the array of holes and cells captured at the holes. The bottom right image shows a cartoon of the microinjection of a single cell immobilized on the device. (images from Liu,X. et al. (22))

2.5. Cell Sifter History

Former lab members Adam Seeger and Michelle Malouf started the cell sifter project several years before I arrived in the Pourmand lab. They produced a device with optically transparent membrane windows, each with a 6 X 6 array of holes that were provided negative pressure by a single microfluidic

channel. The channel connected the two windows on the device. One window was broken to make a connection to an air compressor. This prototype was constructed of a silicon-nitride membrane on a silicon-oxide thin film on a silicon substrate. These devices were never used to capture cells. Many of the devices that Adam and Michelle prototyped were broken but I was able to use them to help me determine how the device would look after fabrication was complete. Figure 15 shows the kinds of data that I used to help me move forward with a new cell sifter design. Figure 16 shows the non-functional device that I used to help me specify the new design parameters.

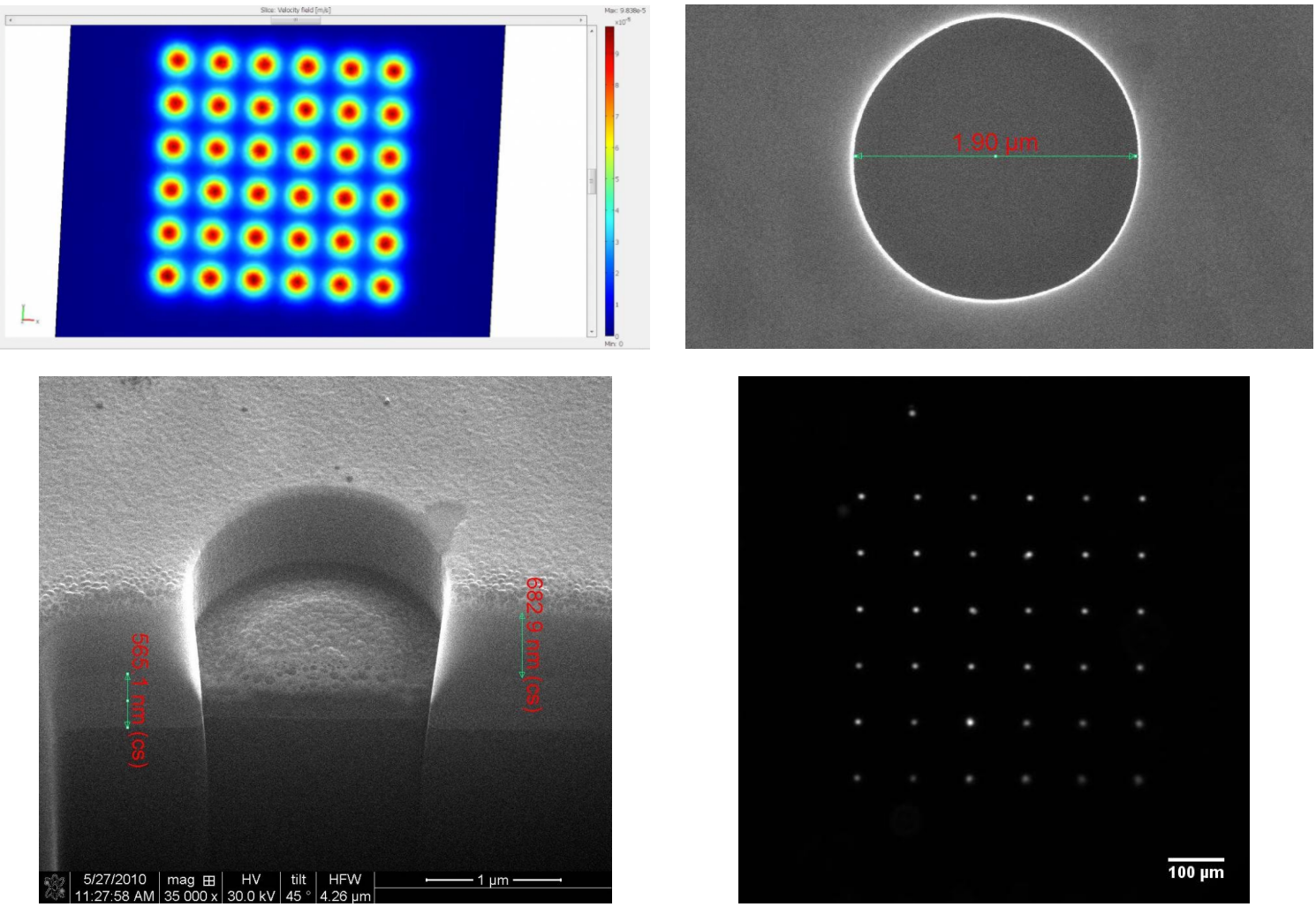


Figure 15 - When I started the project I was given access to notes and images of the previous devices that informed my approach. I used the notes and images of finite element analyses that were done by Adam Seeger. The image at top left shows the velocity of fluid, or a kind of flow field, through an array of holes in a membrane. Finite element analysis requires that users create a model of the system to be tested. Adam would have incorporated the full layout of the cell sifter device, including the channel that addresses the holes. Notes on the analyses stated that all holes experienced a uniform flow field when the channel depth was greater than 25 µm. I used these notes to inform the channel depth specification. I used SEM images such as the one at top right to specify hole diameter. The bottom left image informed layer thickness specification and the bottom right image helped me determine hole spacing. (images taken from Pourmand lab wiki)

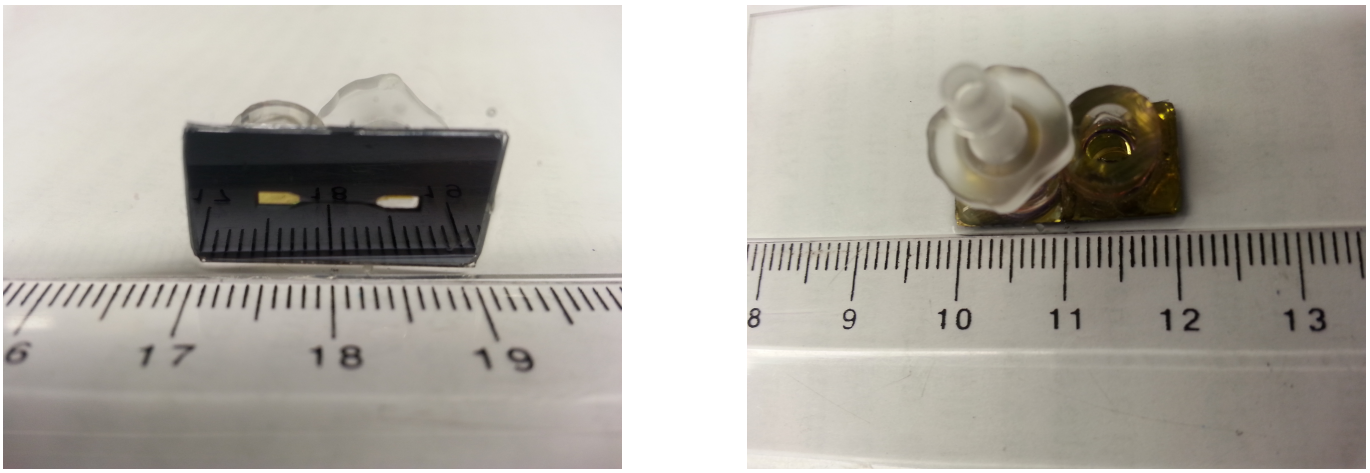


Figure 16 - This image shows the cell sifter created by Adam Seeger and Michelle Malouf. This device was constructed with a large vacuum connection (shown on the left of the device) and sample well. Epoxy was used to bond the vacuum connection and sample well to the silicon chip. The chip itself was 2 centimeters by 1 centimeter. The left image shows the bottom view of the device and the two membrane windows. The image at right shows the top view of the device (images taken from Pourmand wiki).

3. A New Cell Sifter.

3.1. Cell Sifter Fabrication and Characterization

When I interviewed for the position in Dr. Pourmand's lab I highlighted my previous research experience at the Naval Postgraduate School (NPS). At NPS, I learned micro-fabrication techniques and photolithography. I believe my previous experience was crucial to informing Dr. Pourmand's decision to assign me to the cell sifter project and task me with independently pushing forward its development. Dr. Pourmand introduced me to a graduate electrical engineering course on semiconductor processing techniques. Dr. Pourmand also introduced me to Dr. Tom Yuzvinsky, who is the manager of the UCSC clean room and knowledgeable in micro-fabrication and photolithography. I was also introduced to Taiuk Rim, a former lab member with CAD design experience. Dr. Pourmand also asked me to find and establish a connection to a semiconductor fabrication facility that would offer fee-for-service consulting so that we could contract the device fabrication and get started working with the devices sooner.

The device specification was simple, produce a device that will capture and hold down single cells out of solution into predetermined locations without killing the cells. The device had to be optically

transparent and useable with the nanomanipulation system. Figure 17 shows a diagram of the device specification.

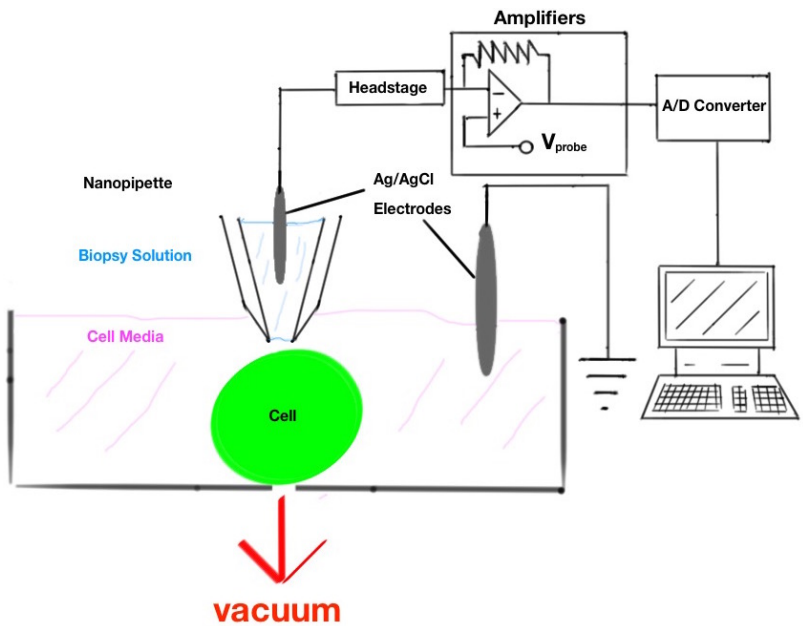


Figure 17 - Diagram showing the cell sifter specification. The device must be able to capture floating single cells and hold them down so they can be interrogated with the nanopipette. There must be access for reference electrodes and the nanopipette. The device must be optically transparent because the nanomanipulation system uses an inverted microscope that images from below the sample. A vacuum is applied to the device and the cells are suctioned down onto the device surface. The cells that are captured must remain alive after being captured and held (figure adapted from Actis et al. (10)).

I worked with Tom brainstorming ideas on how to approach the problem. I decided to refer to the previous cell sifter design for ideas. I wanted to expedite the process as my time on the project would be limited and I wanted to accomplish something meaningful during my time in the lab. I would work with Tom again on the latter end of the fabrication process.

3.1.1.Designing A New Cell Sifter

The work done by Adam Seeger, Michelle Malouf, and other former lab members served as a starting point for me. I was able to use their work to inform mine and quickly identify key design characteristics. All of the previous prototypes were no longer functional and more importantly, the previous photo mask was not available. I was asked to produce a new design. I used computer-aided-design (CAD) software to make a conceptual design that incorporated an optically transparent membrane window with a 6 X 6 array of 2 μm diameter through-holes. I chose to use an array of 36

through-holes like the previous prototype, as a proof of concept, leaving future design iterations to include many more through-holes, which would make the device truly high-throughput. I chose a through-hole diameter of 2 µm because the cells I would work with, HeLa cells, have a nominal diameter of 20 µm. Two micrometer diameter through-holes provide enough surface area to hold the cells down at the low pressures necessary to maintain the integrity of the cellular plasma membrane. The final design included a 100 µm wide microfluidic channel connecting two optically transparent membrane windows, each with a 6 X 6 array of through-holes spaced 100 µm apart.

Since I had no experience in device design or the inner workings of the semiconductor industry, it took me several months to learn about and create a CAD drawing of a photomask. A *photomask* acts as a stencil in the photolithography process. I worked with Taiuk Rim via email and Skype to create an initial photomask design. I did have limited experience with CAD design and I was able to produce a rudimentary drawing of what I thought one device would look like. Figure 18 shows the drawing I produced for the mask design. I had no knowledge of photomask design conventions and working with Taiuk allowed me to learn very basic but useful aspects of photomask design.

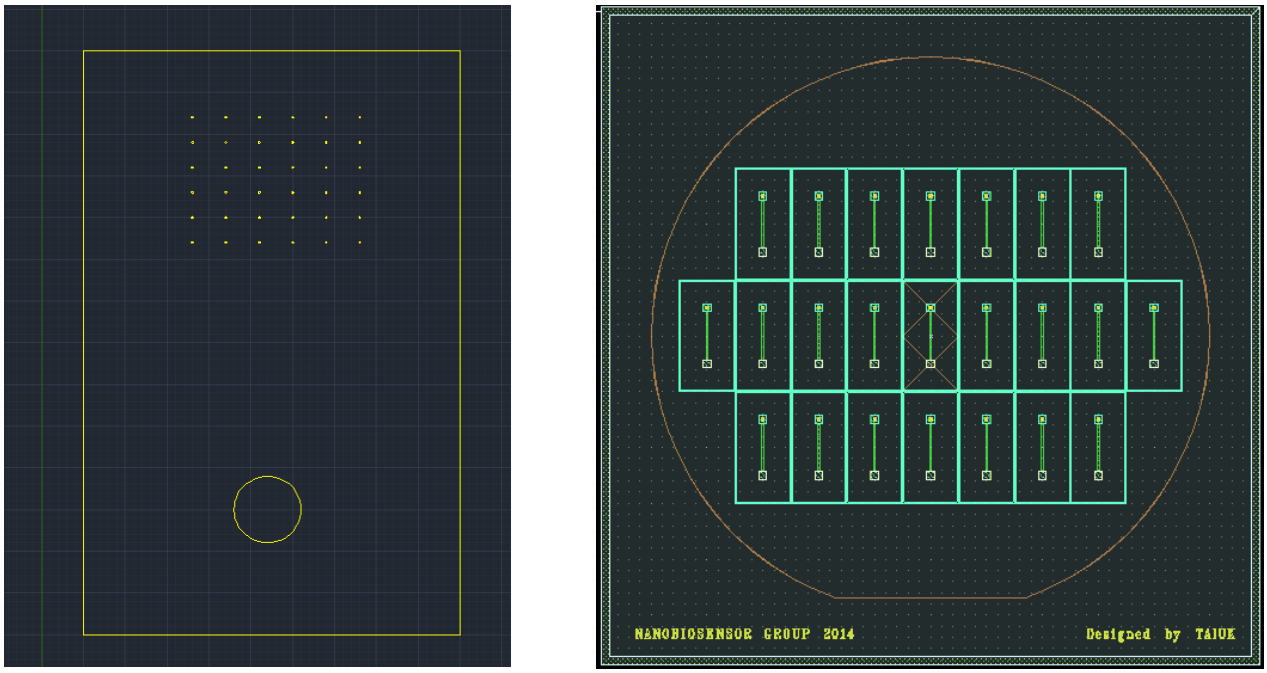


Figure 18 - I used AutoCAD 2013 to create these drawings. The left image is a drawing of a single device. I lacked knowledge of photomask conventions and how to design a photomask but I was able to send this to Taiuk Rim who then created the photomask on the right. This photomask was used to inform the design and fabrication of two pieces of photolithography equipment, a reticle that contains many different device designs on the same reticle and a traditional photomask for the backside of the devices.

3.1.2.Semiconductor Fabrication

To aid in cost-effectiveness and expedite device fabrication, Dr. Pourmand and I elected to contract a fabrication consultant working at the Stanford Nanofabrication Facility. After a few months of research into the semiconductor contract fabrication industry, I contracted Dr. Jim Kruger of the Stanford Nanofabrication Facility in a fee-for-service capacity to fabricate the cell sifter semiconductor sandwich structure. I provided the photomask design and discussed the device specification with Dr. Kruger who then provided a quote for the project. I handled all of the paperwork and correspondence between our groups. In addition to fabrication, Dr. Kruger aided in finding a photomask design and fabrication firm, Compugraphics USA. I produced what I thought a suitable fabrication process flow would look like, shown in figure 19.

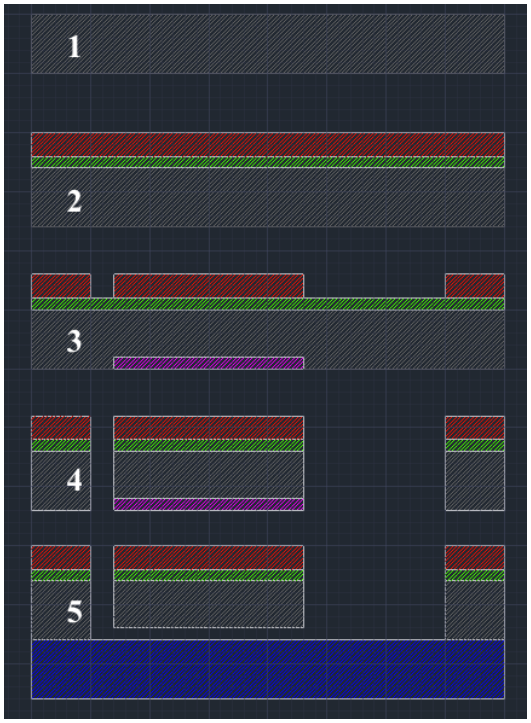


Figure 19 - The image is a the cross section view of the proposed process flow, from top to bottom, I provided to Jim Kruger. The first step, number 1 begins with cleaning the surface of a 4 inch silicon wafer (grey). Step two is the growth of silicon dioxide (green) and silicon nitride (red) layers onto the silicon substrate (actual process included thin films on both sides of the wafer). Step three includes the etch of the two layers and deposition of the photoactive material known as *photoresist* (magenta) to mask the microfluidic channel on the backside of the device. Step four includes the etch through of the holes where cells will be captured and the vacuum connection. Step five is the bonding of a glass wafer to seal the microfluidic channel. The final process flow differed from this proposal in details but the basic idea was similar to the final process.

Jim Kruger used semiconductor processing techniques to fabricate a sandwich structure with two optically transparent membrane windows connected by a single microchannel. A silicon-supported composite silicon-nitride (Si_3N_4) and silicon-dioxide (SiO_2) membrane, provides a device that is optically transparent, durable, and able to support larger pressure differences than other chemical formulas of silicon-nitride. Each window is perforated by an array of micro through-holes. Each device is approximately 10 X 20 millimeters (mm) with two approximately 1 mm windows etched through an approximately 350 µm thick two-side polished silicon wafer. The critical area is an array of 6 X 6 through-holes, 2 µm diameter with a 100 µm *pitch*, or spacing between holes. The *die*, the singularized devices after being separated from each other on the wafer, were not bonded to a glass wafer to seal the backside fluidic channel as were the previous prototypes because it would add cost and complexity. Instead, I opted to use another method I will discuss later in this section, because it reduced the amount of work I had to outsource and allowed me more flexibility in the final fabrication steps. The fabrication process included the use of many semiconductor fabrication tools and equipment. I worked closely with Jim Kruger and gained valuable knowledge of the process.

A stepper reticle is used for the hole-array and backside alignment marks. A *stepper reticle* is a photomask made up of many individual subunits, each of which may incorporate a different design and one specific subunit can be chosen for a job. In this way a single stepper reticle may have several different designs. The chosen subunit is exposed to ultra-violet light many times over the area of the wafer, producing many die of the same design. The reticle Jim Kruger used was designed with many different design variations and provides greater device yield. A secondary, more traditional photomask was used for the backside of the device that includes the scribe lines, windows, and microfluidic channel.

Thicknesses of the oxide and nitride layers were calculated by Dr. Kruger to balance and retain net tenseness for flat membrane windows. An approximately 600 nm thick wet-thermal-oxide-film was

grown on a two-side polished silicon wafer. Approximately 300 nm of stoichiometric silicon-nitride (Si_3N_4) was deposited on both sides by chemical vapor deposition.

An ASML stepper was used for exposure of the 2 µm hole arrays in both positions of the two windows as well as the alignment crosses in the scribe lines. PlasmaTherm Deep Silicon Etch (PT-DSE), a reactive-ion etch process that can produce high-aspect ratio features, was used to create the 2 µm holes through the silicon-nitride and -oxide, stopping at the silicon substrate. A Karl Suss contact mask aligner was used for backside alignment for patterning of the window openings, for the scribe lines to provide device separation, and as well as the microfluidic channel to a depth of 100 µm connecting the two windows. The backside window pattern was PT-DSE etched through the silicon-nitride and -oxide.

To avoid clogging of the hole array and damage to the nitride membrane during device separation the devices were separated as part of the final potassium hydroxide (KOH) wet etch. A final KOH etch was used to etch the scribe lines and windows in the silicon substrate.

After the microfabrication process was completed by Jim Kruger, I finalized the first cell sifter devices. I worked with Tom Yuzvinsky to investigate a suitable way to seal the microfluidic channel on the backside of the dices. The new design did not include a glass wafer to seal the fluidic channel in the semiconductor processing. I knew polydimethysiloxane (PDMS) was a versatile prototyping material that would be strong enough to seal the microfluidic channel and opted to use this material in the final fabrication. I looked to the literature to find methods to bond PDMS slabs to the silicon-nitride thin-film to seal the microfluidic channel. Tom trained me on cleanroom safety protocols and the use of the plasma etch tool in the UCSC cleanroom. Tom helped me tailor existing recipes for my purposes.

I worked in the cleanroom experimenting with different PDMS slab thicknesses for the device. After many tries I decided to go with a thin slab 1.5 mm thick. This let me go to the maximum magnification on the microscope setup without crashing the microscope objective into the bottom glass

slide of the cell sifter device. Whenever the objective crashed into the glass slide the silicon-nitride membrane would be pushed up into the nanopipette, shattering the window and losing the captured cells.

I used oxygen plasma treatments to bond PDMS slabs to the silicon-nitride sandwich-structure to seal the microfluidic channel. I used the same process to bond the bottom side of the PDMS slabs to the surface of a glass slide to provide a support structure for the whole device. The oxygen plasma deposits hydroxyl (OH) groups on the surface of the PDMS, silicon-nitride, and glass slides promoting a covalent bond and permanent adhesion (25).

Initially, I used epoxy to fix a plastic barb as a vacuum connection and a 4 mm long cross section of plastic tubing as a sample well. I then moved to a pipette tip but continued using epoxy to bond the connection and sample well to the devices. This proved to be problematic during initial attempts to clean the devices after use. The epoxy is degraded by acids and therefore limits cleaning. I decide to use PDMS, and oxygen (O_2) plasma for bonding, to make the vacuum connection and sample well. This would allow me to clean the device more efficiently, fabricate the devices faster, simplify the fabrication, and make devices with more clearance between the top of the device and the micromanipulators used to maneuver the nanopipette, an issue I identified during later experiments. Figure 20 shows the first prototype integrated with the nanomanipulation system.

Figure 20 - First prototype cell sifter device integrated with nanomanipulation system. This design presented issues with overhead clearance. As you can see, the plastic nanopipette holder on the left comes into close proximity to the vacuum line on the right. The two structures would crash when moving the nanopipette tip to the micro holes on the right edge of the hole array in the window. I iterated through several designs to solve this issue.

3.1.3.Cell Sifter Characterization

After I had a completed device, I set out to characterize the structure and function. I worked with Tom Yuzvinsky to image the device with a scanning electron microscope (SEM). I investigated the wetting properties of the material. This had implications in the fluid flow through the device, as bubble formation in the device that could prevent fluid flow, and the susceptibility of cells to adhere to the surface of the device when performing cell capture experiments. I investigated ways to clean the devices after using them to capture cells. I also used protein-coated micrometer-sized polystyrene beads to prove that the device could capture micro-sized particles out of solution before using live cells.

3.1.3.1.Geometry and Structure

To properly characterize the device structure I worked with Tom at the W. M. Keck Center For Nanoscale Optofluidics. Tom helped me image the device with the SEM and then annotated geometries of interest. Some issues arose with the quality of the initial images due to possible charging of the silicon-nitride surface. Figure 21 shows photographs taken with a digital cell phone camera of the whole device and optical microscope images of the hole array. Figure 22 shows the SEM images.

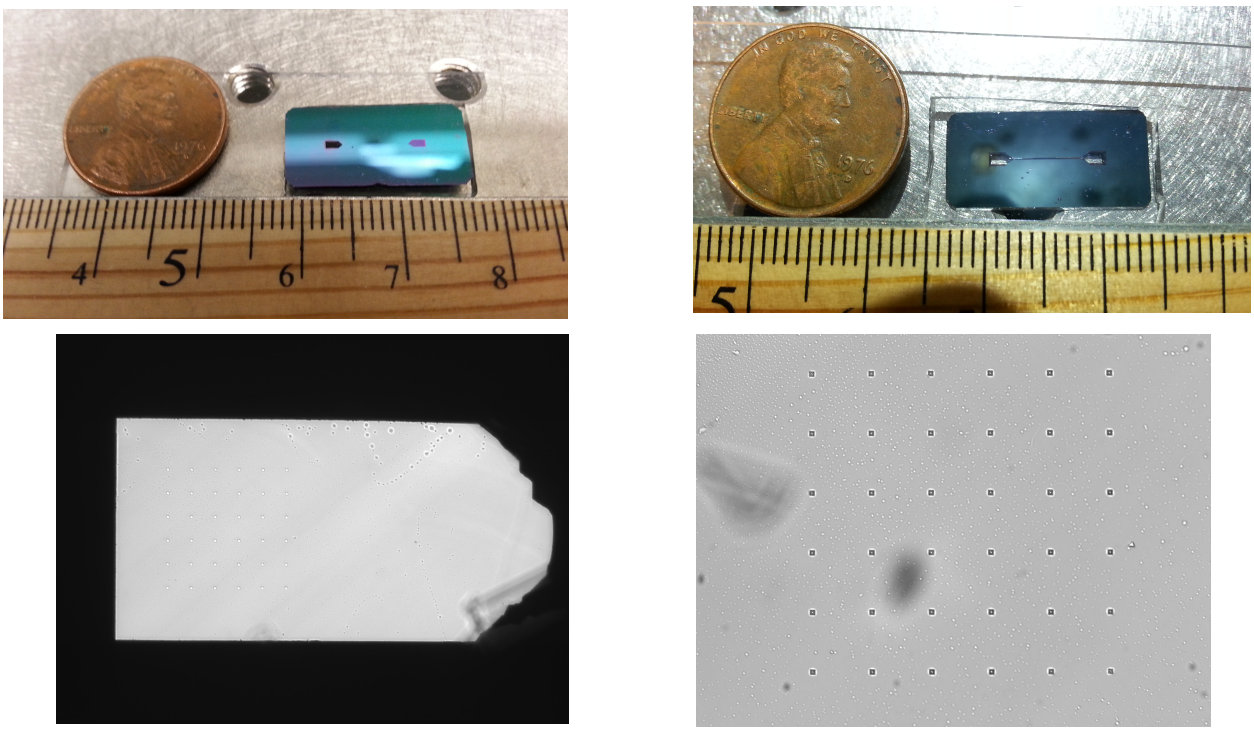


Figure 21 - Figure shows the device prior to fixing the vacuum connection and sample well. Top left image shows the top side of the device, top right shows the bottom side and microfluidic channel connecting windows. Bottom left image is optical micrograph of one window at 4X magnification, bottom right shows micro-hole array at 10X magnification.

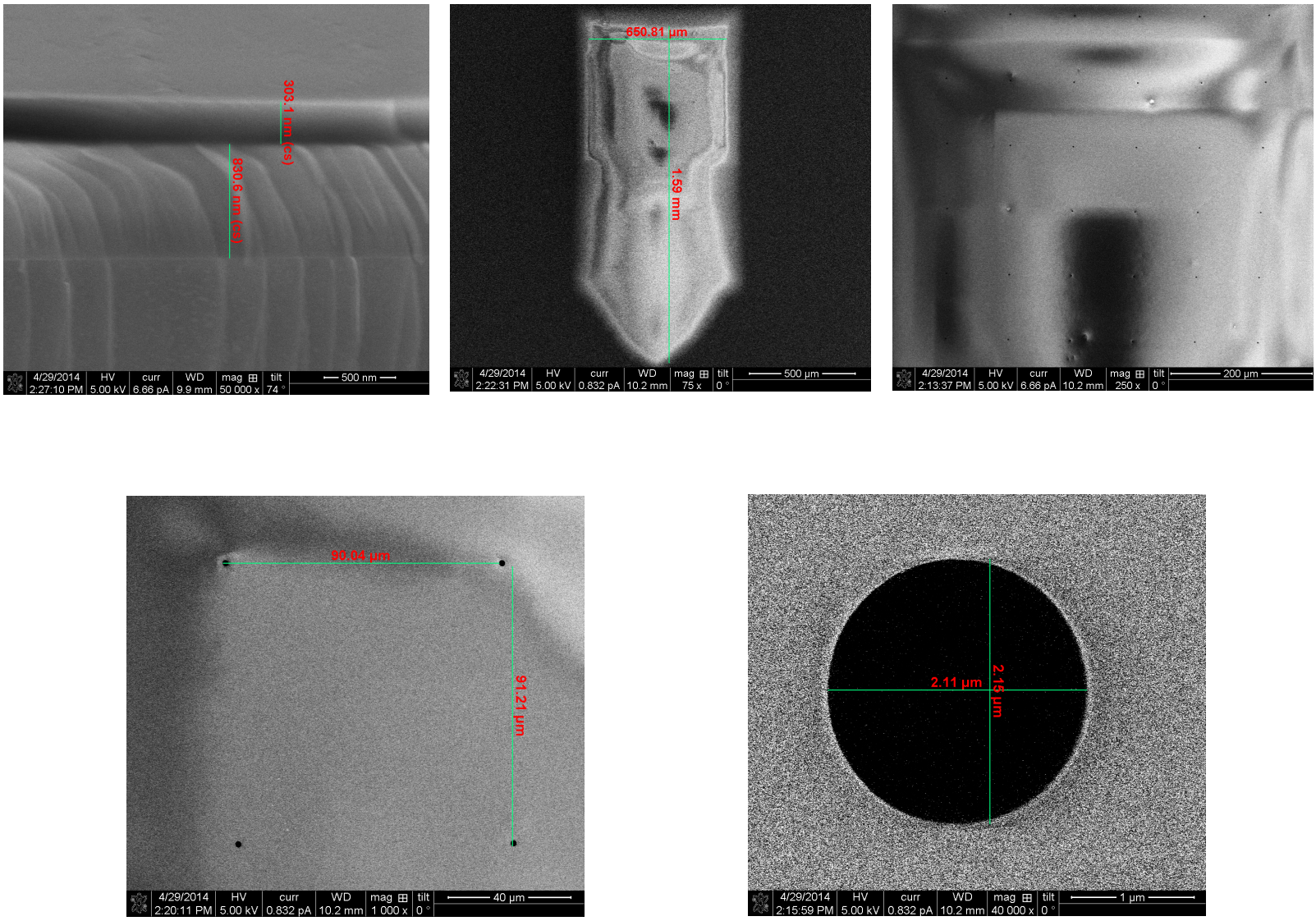


Figure 22 - Figure shows SEM image of device layers with thicknesses annotated (top left). The dimensions deviate from the design specifications because of minor variations in the fabrication process. Different semiconductor technologies offer higher levels of resolution but that precision was not necessary for this proof of concept. SEM image of membrane window (top center). The silicon-nitride membrane exhibited some charging induced by the electron microscope's electron beam. SEM image of through-hole array (top right). SEM image of through-hole array with hole spacing annotated (bottom left). The spacing between each hole is approximately 90 µm. SEM image of through-hole diameter with dimension annotated (bottom right). Each hole has a diameter of approximately 2 µm.

3.1.3.2.Fluid Flow

To ensure that the device exhibited proper fluid flow prior to capturing live cells I conducted several experiments to characterize the flow. The first thing I did with the first device was deposite a drop of pure water over the sample well. I fixed the vacuum connection and applied a negative pressure to the device after the bonding was complete. I expected to see some form of fluid flow when I viewed

the device under a microscope but to my disappointment, I observed no fluid flow. This was obviously a mistake as I later realized. If the device was clean and or free of particles large enough to see under the optical microscope I would not be able to observe fluid flow given the pure water. I wanted to confirm that the holes were actually open. I thought to use a dye next, but after conversations with my lab-mate Robert Shelansky, I opted to use current flow to confirm the holes were open.

I applied a potential across the silicon-nitride membrane and observed a current. I positioned a nanopipette over the membrane and deposited drops of water over the sample well side and the vacuum connection side. Because the vacuum connection side window was purposely broken the microfluidic channel filled with water. I confirmed this by a change in the color of the channel as the water was drawn into the channel by capillary action. I brought the nanopipette into contact with the drop on the well side and inserted a reference electrode into the opposite side. I applied a 500 millivolt potential across the electrodes and observed a current on the patch-clamp amplifier. Figure 23 shows my experimental setup.

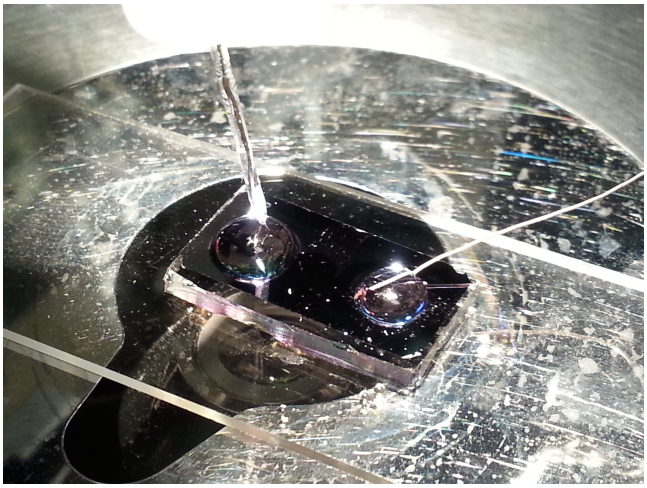


Figure 23 - Applying a potential across the silicon nitride membrane to confirm open through-holes. A nanopipette with a silver-chloride electrode inserted inside is inserted into a droplet of pure water on the silicon-nitride membrane (left). A droplet of water is placed over the broken membrane on the vacuum connection side and water is allowed to flow into the sealed microfluidic channel. A separate silver-chloride electrode is inserted into the droplet over the the vacuum connection and a 500 millivolt potential is applied across the electrodes. The observed current confirms the holes in the membrane are not blocked.

To confirm that fluid would flow through the holes I deposited dye into the sample well and applied a negative pressure of approximately 33 kPa. I observed a change in the color of the fluid in the vacuum connection side, confirming fluid was able to flow across the membrane. During these experiments it was not crucial for the chamber under the membrane to be free of bubbles but performing cell capture and nanomanipulation experiments would require that the device be free of bubbles. Bubbles are a major issue in microfluidic systems and surface wetting plays a major role in removing bubbles from the system. Figure 24 shows a bubble inside of the chamber under the membrane window.

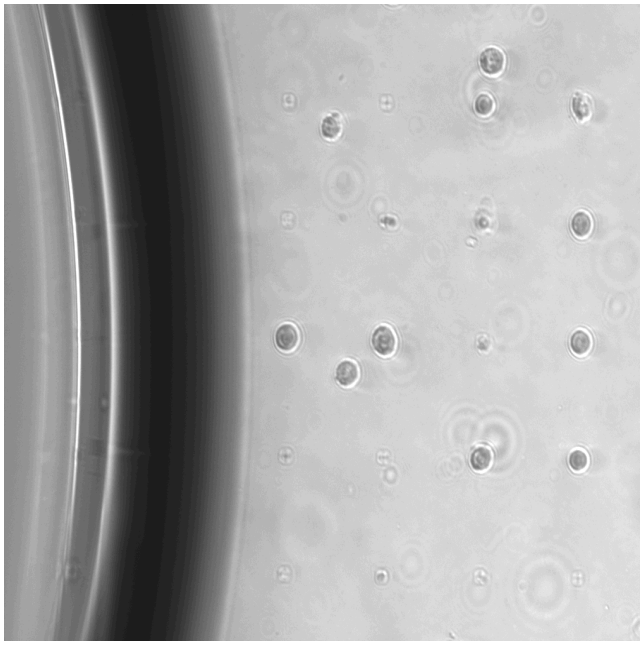


Figure 24 - The large bubble on the left consistently formed on the left side of the device directly under the array of holes. As you can see in this optical micrograph it makes it very difficult to see all of the holes and reduces the fluid flow at the holes that sit above the bubble (10x). The captured single cells can be interrogated but any cells captured on the far left holes could not be seen or interrogated. This issue needed to be addressed before continuing with further cell-capture and nanomanipulation experiments. Due to the cost of each device at such low production quantities and the single-use status of each device pending further work involving cleaning.

It was a challenge to overcome the degraded fluid flow caused by improper surface wetting and bubble formation. I tried many different methods for addressing surface wetting issues. Silicon-nitride is used for various reasons, one of which is it's inert properties. The material is very robust and resists degradation by many acids. Silicon-nitride is slightly hydrophilic but organic contaminants in the

ambient atmosphere can cause carbon-rich molecules to render the surface hydrophobic (26, 27, 28). For this reason I first tried using toluene, a non-polar solvent. Bubble formation remained an issue. I tried polar solvents such as isopropyl alcohol, ethanol, acetone, and of course water.

In the end I found that treating the devices with oxygen plasma, immediately immersing them in pure water, and degassing the whole setup in a desiccator overnight worked well. The next day I removed the devices from the water and either let them come to room temperature for an hour or warmed them slightly over a hot plate, taking great care not to heat them above approximately 100ºC which degrades the PDMS-silicon-nitride bond. This treatment left the surface of the silicon-nitride and surface of the PDMS in the microchannel hydrophilic. This was great for fluid flow, but disastrous for cell viability. When cells are captured on the hydrophilic surface, and then released by applying a slight back-pressure, a residue is observed around the rim of the hole and surrounding area. I assume this is portions of the cells lipid bilayer or cell surface proteins. The result is clogged holes.

Clogging of the 36 holes is a major challenge that must be further investigated prior to establishing reusability of each device. Clogging can be caused by particulate in the fluid used to suspend the cells, the large proteins present in the cell media, and the aforementioned issues when capturing cells. To address particulate contamination I work with purified and filtered water for any work with the devices. To address the *protein fouling*, clogging of the holes by the aggregation of proteins at the hole openings, I established a protocol to suspend the live cells in serum-free media prior to depositing the sample into the sample well. The serum in cell media is the source of the large proteins and clogging because of this type of protein aggregation is no longer an issue. I am currently investigating ways to prevent the adhesion of the cells to the silicon-nitride surface. I am proposing to *silanize* the membrane surface, a type of surface chemistry that renders the surface hydrophobic. This should prevent the cells from sticking to the surface and clogging the holes when they are captured and released. Consideration must be given to bubble formation when performing this surface treatment.

Cleaning the devices prior to use is essential. I have established a protocol to clean the devices and rid them of any residues left over from the fabrication process. To clean the devices I soak them in isopropyl alcohol for 5 minutes and rinse them with purified and filtered water three times, I then air dry them with nitrogen. Figure 25 shows the membrane prior to being cleaned and post-cleaning.

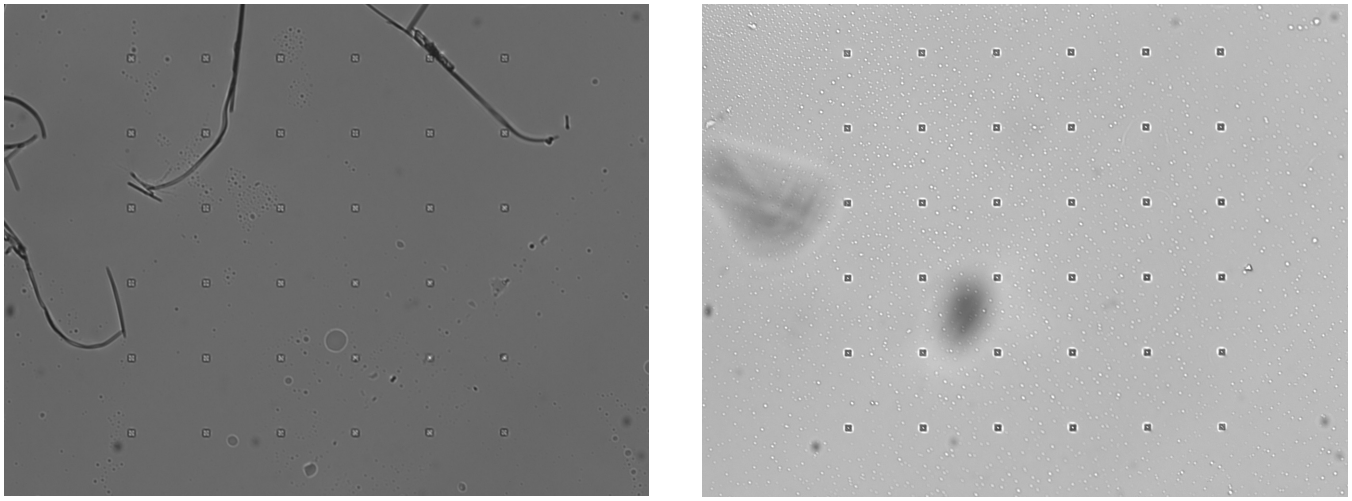


Figure 25 - Image showing a contaminated silicon nitride membrane (left). Cleaning proved to be challenging due to the fragile nature of the membrane. Ultrasonic cleaning was not suitable for the devices as the force would rupture the membranes, I suspect due to the resonant frequency of the membrane. Once clean, the holes are clearly visible and the membrane is free from contaminants (right, not same device as image on the left).

I learned quickly that sonicating the devices would not work after attempting to clean several devices with an *ultrasonicator*, a piece of equipment often used to clean jewelry and other delicate items. Every time I tried to sonicate the devices the membranes shattered. I suspect that the ultrasonic frequencies and cavitation on the membrane's surface cause the window to resonate and ultimately shatter. Ultrasonic cleaning is attractive because it would allow the devices to be easily cleaned and reused. Further investigation is needed to establish a quick and easy post-experiment cleaning protocol.

3.1.3.3.Applied Pressure and Pressure Tolerances

The negative pressure applied to the device is a crucial parameter in the cell capture experiments. A literature review revealed that typically a negative pressure of 1-3 kPa was used to immobilize single zygote cells and keep them alive (22). Sakai et al. used 200 Pascals to immobilize cells with a diameter

of approximately 10-20 µm, roughly the diameter of the cells I am using and plan to use in the future (21). I decided to make the 200 Pa my target pressure. I collected discarded tubing and an unused air-compressor from the Pourmand lab. I used these items to construct plumbing to apply pressure to the device. I incorporated a waste-fluid reservoir and a paper filter to prevent fluid from entering the vacuum pump. Figure 26 shows the progression of the tubing and valving setup.

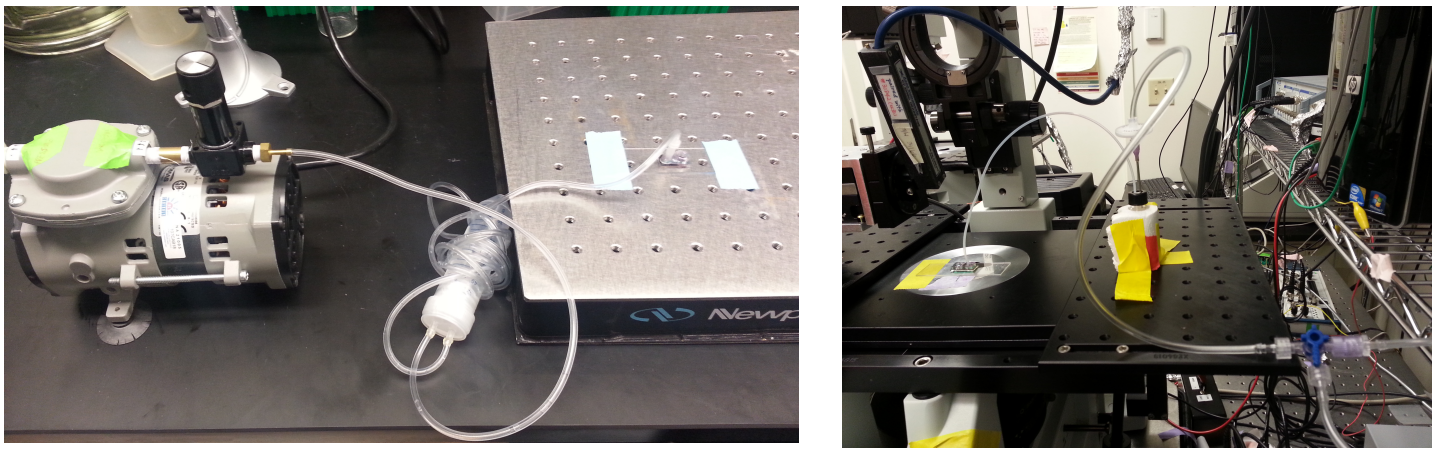


Figure 26 - In the image at left you can see the air-compressor (left side of image) with the precision microregulator. The tubing system incorporates a waste-fluid catch to prevent fluid from entering the compressor. Notice the large tubing connecting the catch (center of image) to the device itself (far right of image), it is unnecessarily large at approximately 4 mm inner diameter. In the image at right you can see the current setup. The tubing connecting the catch (center of image) to the device (left side of image) is much smaller, approximately 1 mm inner diameter. At far right of the image you can see the type of valves used to control the applied pressure.

Using these common lab materials allowed me reduce the cost of the project but had tradeoffs. The compressor is equipped with a precision microregulator that allows me to apply a pressure range from 0-35 kPa and control pressure down to the tens of Pascals, but the regulator is operated by hand turns. This lacks the response time required for future experiments and should be replaced with an electronic computer-controlled pressure-controller. This would allow the operator to set a capture pressure and then transition to a holding pressure once the cells are captured. This is important because I have observed a bias in the size of cells being captured. At low pressures such as 200 Pa the smallest cells are captured first because they have less mass and require less force to manipulate them. A pressure

controller would allow the user to set a higher pressure to capture larger cells and then bring it down quickly to a pressure that will maintain the cell wall integrity for the duration of the experiment. I also used a vacuum pump to help with flushing free-floating cells prior to performing the cell nanomanipulation.

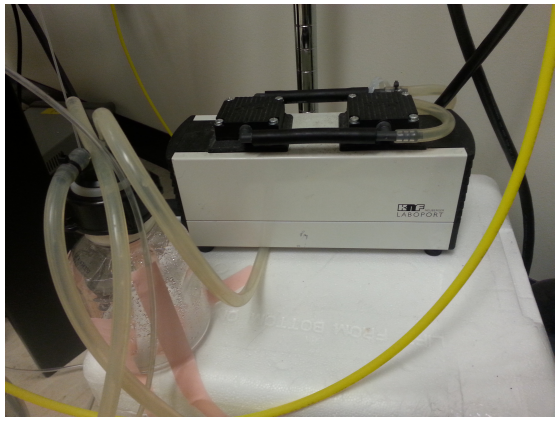


Figure 27 - Image shows the vacuum pump I use with a pasteur pipette for flushing the device of free-floating cells. I constructed a series of valves to lower the pressure the vacuum applied. This is important because applying a higher pressure to remove free-floating cells than is used to capture the cells removes the cells that have been captured at the holes. I am currently optimizing this system.

I ordered a digital pressure gauge to allow me to apply the desired negative pressure. This was essential for me to establish a consistent pressure in congruence with the cited literature. With this gauge I am able to identify the point at which the regulator hits the target pressure of 200 Pa. Figure 28 shows the digital pressure gauge.

Figure 28 - This digital pressure gauge provides the capability to apply negative pressures of 200 Pa without the use of an expensive pressure controller. I use this gauge to measure differential pressure. This device was easy to integrate and relatively cheap for the precision it provides, approximately +/- 34 Pa.

I observed limitations in the membrane's pressure tolerance, or the amount of pressure I could apply before the membrane failed and shattered. Typically, I had the capability to apply a maximum of approximately 35 kPa with the air-compressor setup I was using. When I applied the maximum amount of pressure the membrane failed. A high pressure tolerance is important because flow rate through the device is related to the hydrodynamic resistance in the system. For any given pressure you will observe a certain flow-rate. This is because the pressure in the system is equal to the flow-rate in the system multiplied by the system's hydrodynamic resistance. Also, for any given flow rate through the system a certain pressure will be produced and exerted on the walls of the microfluidic structures, bonds between materials, fluid tubing, and connections associated with the device. Further characterization of the amount of pressure needed to get a desired flow-rate through the holes is needed. More holes in the membrane decrease the hydrodynamic resistance, so with design iterations that incorporate more holes I will be able to increase the applied pressure and flow-rate, capturing cells faster.

As a proof of concept it is acceptable to use the current materials and equipment but changes must be made if the device is to be used for research purposes. Pressure control is key to making future design iterations fully capable of efficient cell capture and release. Pressure control affects both the ability for the device to capture cells and keep them alive, as well as the structural integrity of the device.

3.1.4.Final Prototype

I designed and fabricated a final prototype to overcome the nanopipette clearance challenges and cleaning issues. The changes have helped me reduce excess tubing and made the device easier to fabricate. I incorporated a new waste fluid catch and most importantly a device completely made of PDMS. A fully PDMS epoxy-free design facilitates the use of O2 plasma treatments and cleaning via

weak acid baths. This lower profile design has improved usability because it does not interfere with the travel of moving parts on the nanomanipulation system. Figure 29 shows the device progression.

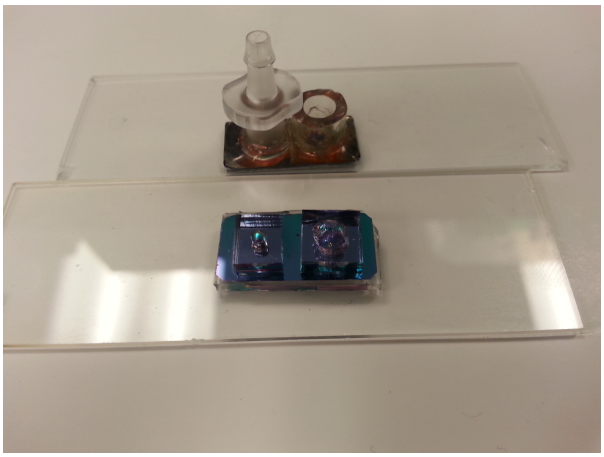


Figure 29 - The devices made by Adam Seeger and Michelle Malouf greatly informed my approach (top). The sample well and vacuum connection were bonded to the device with epoxy which hinders several processing steps. The large vacuum connection presented an overhead-clearance issue with the existing nanomanipulation system setup. The final device (lower) has a much lower profile from the original cell sifter device and the secondary structures, sample well and vacuum connection, are made entirely of PDMS.

The final prototype device (from bottom up) consists of a 1 mm thick glass microscope slide, 1.5 mm PDMS slab, the approximately 350 µm thick silicon device, two PDMS blocks (8 mm x 9mm x 3 mm) with center holes (approximately 1.25 mm diameter hole for vacuum connection side and approximately 3.5 mm diameter hole for sample well). The sample well can accept, in theory, approximately 50 microliters (µL). I used 10 µL of sample for experiments because I wanted to practice using small amounts of sample. This is important to me because smaller samples will reduce cost but also allow us to investigate rare samples or samples with limited supplies. Figure 30 shows the final prototype.

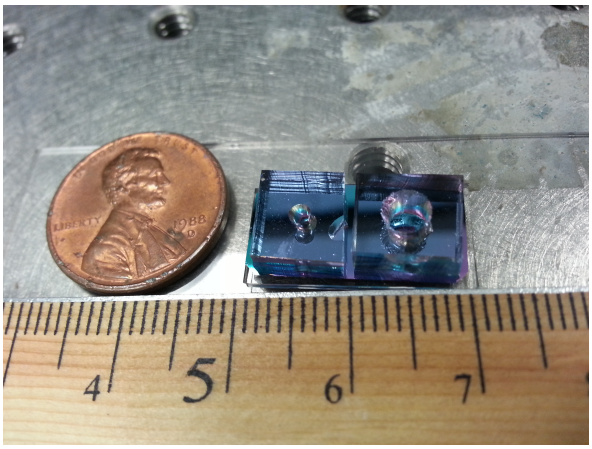


Figure 30 - The final prototype device is suitable for the proof of concept but additional design iterations may deviate from this method of final fabrication. Several design suggestions are made in later sections.

3.2. Capture Single HeLa Cells Floating In Cell Media

The most important thing the cell capture device must do is keep the cells alive after capturing them. This is important because one of the great advantages the nanopipette has over other technologies is that cells stay alive after they have been interrogated by the nanopipette. If the cell capture device killed the cells in the process of capturing them and holding them down, then one of the advantages the nanopipette offers would be negated.

My approach was simple, first capture polystyrene microbeads that have an average diameter of 34 µm (close to the diameter of the cells I will use) with the 200 Pa outlined in the literature to prove that the pressure would produce enough fluid-flow through the device and force necessary to capture and hold down the beads. These types of experiments once optimized could be used to characterize the capture efficiency of later devices with a greater number of holes. Figure 31 shows an 11x magnification of a sector of the whole array.

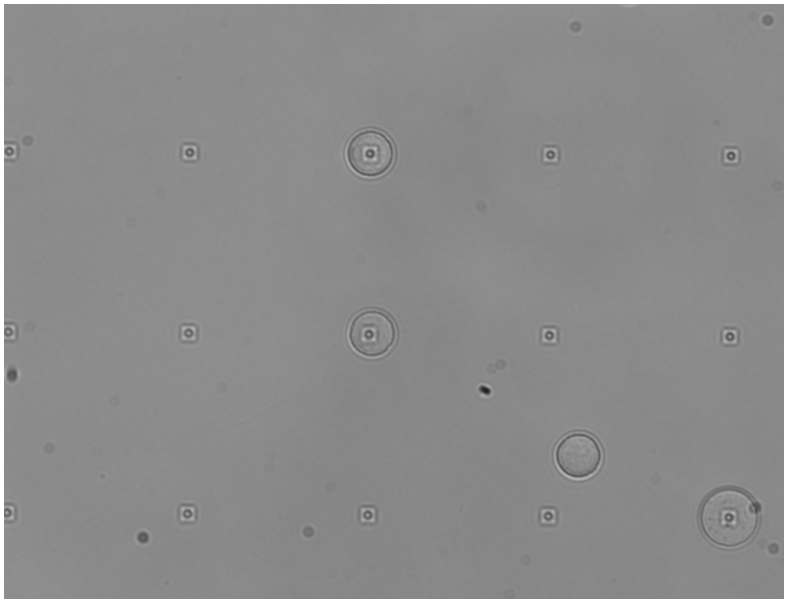


Figure 31 - Image shows optical micrograph of device hole array at 11x magnification. A 10 µL sample of microbreads were applied to the device and 200 Pa of negative pressure applied. Only four holes captured beads. I suspect this was due to the fact that the device was used for a previous experiment that could have introduced contamination into the device, clogging the holes. Subsequent experiments involving a silanization protocol produced similar or worse results (fewer holes captured beads, silanization possibly clogged holes). This process should be optimized to enable further characterization of future devices.

I followed up the microbead capture experiments with attempts to capture live cells. I opted to use HeLa cells expressing green fluorescent protein (GFP), because HeLa cells can be readily cultured and treated with the enzyme trypsin. Trypsin cleaves integrin proteins cells use to adhere to surfaces and other cells, making a single-cell suspension. When I completed the initial cell capture experiments, I had not yet established a way to deliver the very low 200 Pa negative pressure, so I was working at a higher pressure of approximately 7 kPa. I was also limited to high cell concentrations in the sample, because 36 holes in the membrane greatly limits the amount of fluid-flow across the membrane. Since the flow is low, a low concentration of cells in the 10 μL sample would take a long time to capture cells at all the holes. For instance, if a cell is sitting in one corner of the device sample area and only one hole on the opposite side of the sample area is left with fluid-flowing through it, the cell would take a long time to migrate over to the hole under the effects of the flow created by the negative pressure across that hole.

Future designs should include some means of providing continuous microfluidic flow over the surface of the device membrane. This would keep the cells moving over the holes and increase the likelihood they will be captured by a hole instead of possibly being trapped in a corner of the device. If the cells were washed over the holes and the negative pressure applied simultaneously this would decrease the time it takes to capture cells at all holes with samples with low cell concentrations and increase capture efficiency.

I was able to capture single HeLa cells expressing GFP out of a 10 μL suspension in serum-free cell media by applying an approximately 7-8 kPa negative pressure. The cell concentration was at approximately 4 X 10^6 cells per mL, resulting in approximately 4000 cells being deposited onto the device. At this concentration, cells are captured at all holes almost immediately after applying the negative pressure to the device. Figure 32 shows the results of the initial cell capture experiments.

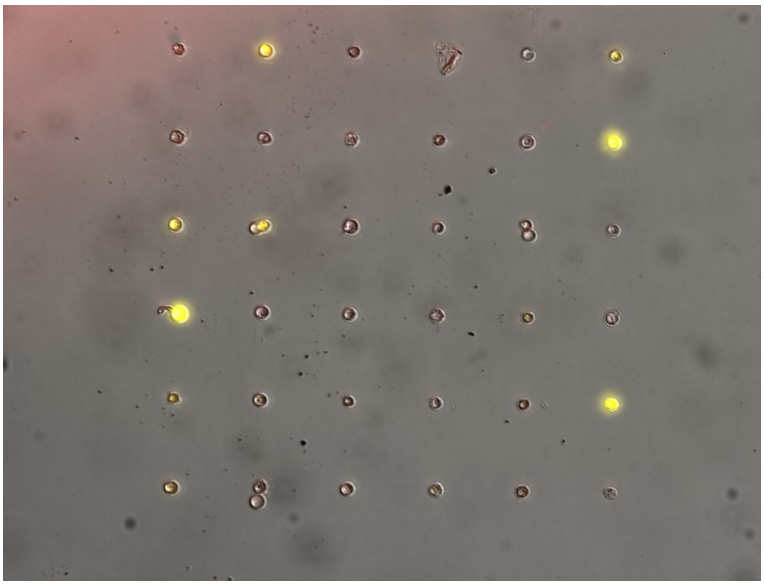


Figure 32 - ImageJ Z-stack of micrograph images of 36 single HeLa cells immobilized on the 6 X 6 array of through-holes. HeLa cells are constitutively expressing green fluorescent protein (GFP) and have been stained with DASPMI to label the mitochondria (10 X magnification). During these experiment I had no way to measure the pressure being supplied by the air-compressor, because I had not yet received the digital pressure gauge. I counted the number of turns it took to reach the full open position on the precision regulator. I then divided the maximum pressure rating of the regulator by the number of turns it took to reach the full open position. In this way I gained a very rough estimate of how much pressure the regulator was supplying the system.

Another desirable capability the cell capture device should have is the ability to release cells after they have been captured and interrogated. This is important because the nanopipette is used to study single cells multiple times over a period of time. Preferably the device should allow the user to capture cells, interrogate them with the nanopipette, release the cells, and repeat the experiment at a later time. Further work is needed to establish a suitable protocol for post-capture cell-culture, but my initial attempts to re-suspend cells after they have been captured have shown encouraging results. To re-suspend cells that I have captured I simply disconnected the fluid-catch connection to the air-compressor and connected a 50 mL syringe to the fluid-catch. I applied a back pressure of approximately 7 kPa to re-suspend the cells. The cells did leave behind residue on the holes. I have yet to try this experiment on newly silanized devices which should prevent the cells from adhering to the device surface.

The most crucial thing the device should avoid is killing the cells when capturing them and holding them down. My initial results suggest this is possible and further optimization should produce

more favorable results. I used HeLa cells free from any fluorescent protein expression to conduct cell viability experiments. I used a cell vital stain that identifies cells that are dead and cells that are alive by either binding DNA, as is the case with the dead stain that fluoresces red when the compound binds DNA, or activated when present in the cell cytosol, as is the case with the cell-permeable green fluorescent live-stain. I stained the cells prior to depositing the sample on the device and performed the capture experiment. Further optimization is needed but initial results show that the cells will remain alive while being subjected to a negative pressure of approximately 200 Pa for 5 minutes. I have conducted subsequent experiments with inconclusive results that suggest the cells are still alive after 1 hour on the device. Figure 33 shows HeLa cells that have been captured out of solution and checked for viability.

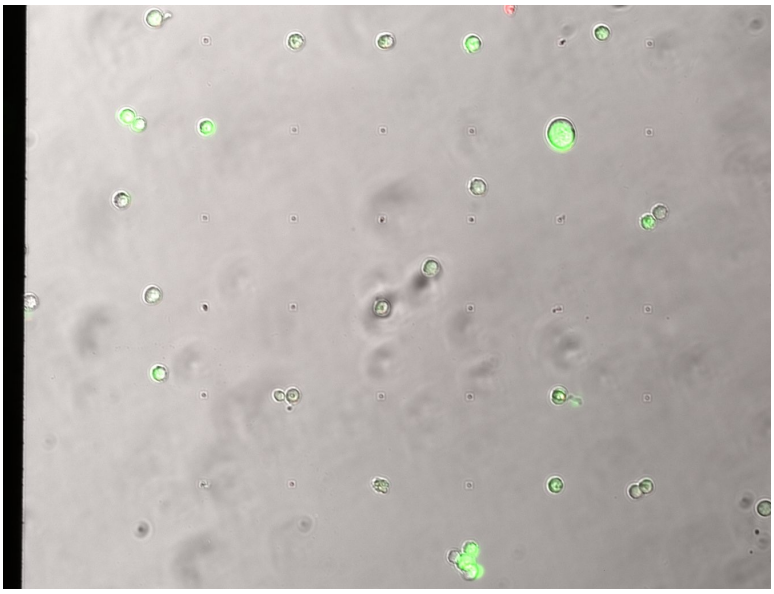


Figure 33 - ImageJ image stack shows optical micrograph of device hole array with captured HeLa cells at 10x magnification. The cells have been held on the device for 5 minutes and were stained with vital stain prior to being deposited onto the device. The vital stain includes a compound, calcein AM, that enters live cells and produces a green fluorescence. Another compound, ethidium homodimer-1, binds DNA and fluoresces red. When a cells membrane has been compromised and the cell is dead or dying, the compound is able to bind the cells DNA and produce a signal. As you can see from the image, the cells are still alive after five minutes, not all holes have captured a cell, and some cells are adhering to each other. Treating cells with trypsin does not make the cells permanently non-adherent. Further work is needed to characterize the ability of the device to maintain cell viability.

Maintaining cell viability is a crucial advantage the single-cell nanomanipulation system holds over other similar technologies and the cell capture device should maintain this advantage. I will continue to explore the limits of the cell capture device to maintain cell viability and release cells after being interrogated with the nanomanipulation system. Several factors will effect this ability such as the device surface characteristics, applied negative pressure, ambient atmosphere, and the ability to culture the cells on the device or collect them after they have been interrogated so they can be cultured in another container.

3.3. Nanomanipulate Single HeLa Cells Captured On Cell Sifter

To confirm the ability of the cell nanomanipulation system to interrogate single cells captured on the cell sifter device, I will nanobiopsy mitochondrial DNA from several captured cells. I will repeat this experiment a minimum of three times. I will confirm the presence of nanobiopsied material by gel-electrophoresis.

Challenges arise when performing nanobiopsies from captured single cells on the cell sifter device such as crashing of the nanopipette into the device membrane. Crashing results in the shattering of the silicon nitride membrane and subsequent loss of captured cells. This can be remedied by fabricating a thicker membrane.

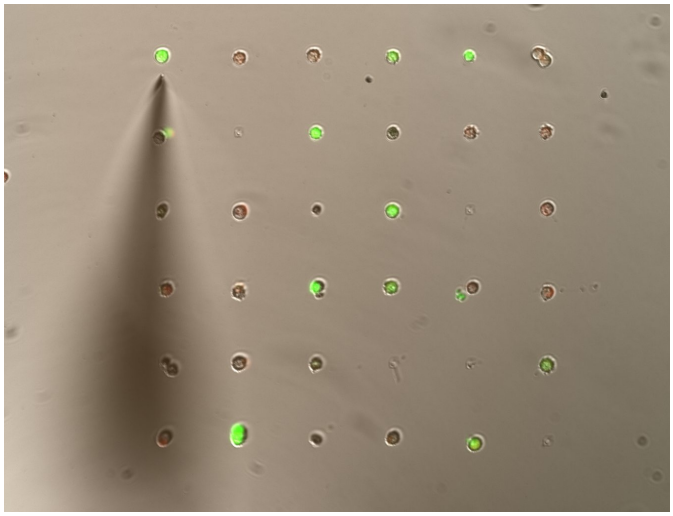


Figure 34 - Micrograph image of captured cells with nanopipette approaching for a nanobiopsy procedure (10 X magnification). HeLa cells are expressing GFP and mitochondria have been stained with DASPMI.

To confirm the nanobiopsy I will amplify the nanobiopsied mtDNA and run a gel-electrophoresis analysis. The nanopipette is inserted into the cell cytoplasm for 15 seconds and the polarity on the electrodes is switched such that cellular material is aspirated into the nanopipette tip. The material is then ejected into a droplet of buffer and collected. The buffer contains primers for the region of the mitochondrial genome. The amplification should produce an 8 kilobase (kb) fragment consistent with a targeted region in the mitochondrial genome that has been established in our lab. This will confirm that I am able to go to a single cell of interest on the device and remove a subpopulation of mitochondria from inside the cell. Figure 35 shows my expected results.

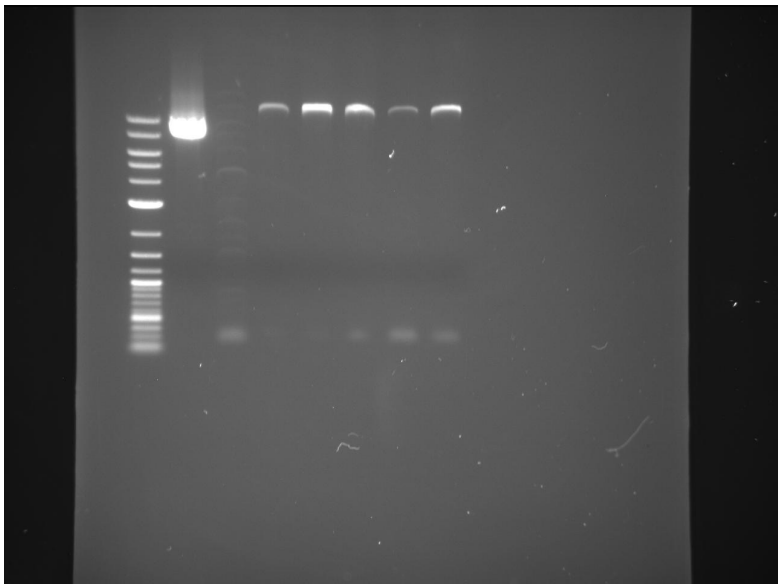


Figure 35 - Image shows gel-electrophoresis results of polymerase chain reaction (PCR) amplification of mitochondrial DNA from cells stuck to the bottom of a petri dish. From left to right, the leftmost lane is the DNA ladder, used as a reference for DNA fragment sizes. Immediately right of the ladder is the positive control, a DNA extraction from approximately 1 million cells. Lane three is the negative control. The following five lanes are single cell aspirations from cells stuck to the bottom of a petri dish. The bands are slightly higher than the 8 kb band on the ladder but should be confirmed to be 8 kb after amplification purification and gel-electrophoresis. I expect to see similar results after single cell aspirations from cells captured on the cell capture device.

I have attempted to perform nanobiopsies from single cells on the cell-capture device. I have encountered several challenges such as crashing the device into the nanopipette tip which destroys the membrane and causes the loss of the cell samples. This issue arises when maneuvering the nanopipette

into place for a nanobiopsy, because the nanopipette tip comes into very close proximity to the device membrane. Any small movement will translate into large movements at the nanometer and micrometer scale, and will cause the device to crash into the nanopipette tip. Movements should be kept to a minimum when the nanopipette is close to the device membrane.

Focusing while at high magnification must be done so very carefully as the microscope objective may crash into the bottom side of the device, the glass slide, and cause the whole device to move up toward the nanopipette tip, causing a catastrophic crash. I have introduced a thinner PDMS bonding slab to help remedy this issue but further work is necessary to correct this problem.

I have also encountered challenges in sample collection. Typically, a sample is collected into a droplet of buffer by inserting the nanopipette tip into the droplet as well as a separate reference electrode. Since the reference electrode must be removed from the device sample well and manually inserted into the droplet each time a sample is to be collected, this introduces the possibility that the device will be damaged by crashing into the nanopipette tip or reference electrode. Using separate electrodes aids in faster and safer sample collection, but introduces extra steps and still leaves the possibility for crashing the device into the nanopipette. Future design iterations should include an electrode on the silicon-nitride surface of the device itself to enable electrical switching. This modification will increase speed, efficiency, and safety of the nanobiopsy procedure.

I was able to nanobiopsy and collect mitochondrial DNA from 5 different random cells in 1 hour. These cells were growing stuck to the bottom of a petri dish, and it is time consuming to return to the same cell over time. Although I have not been successful in amplifying the mtDNA from nanobiopsies taken from cells captured on the cell capture device, I have great reason to believe that I am successfully removing material from the cells as I do observe the characteristic current readings associated with nanobiopies. For instance, I observed a drop from approximately 1 nanoamp (nA) to approximately 0.3 nA. In the experiments where I have observed these current readings, I have been able to interrogate up

to 12 different cells in under 1.5 hours, which is a significant increase in the nanobiopsy throughput. Additionally, I can identify each cell I nanobiopsied, so theoretically I could easily return to the same cell over time. Figure 36 shows the initial results from nanobiopsies performed on single-cells captured on the device.

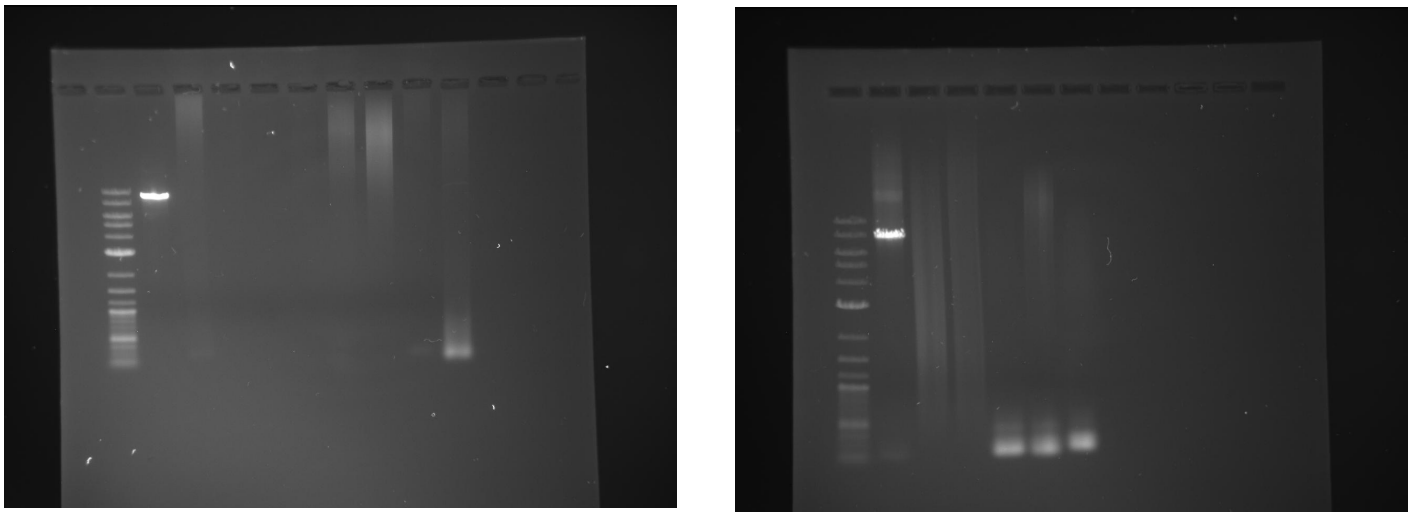


Figure 36 - One of my samples evaporated during the PCR reaction. I suspect the cap on the tube was not secure enough, so I lost one sample. I ran the remaining 11 samples through gel-electrophoresis on two different gels to confirm the presence of amplified mtDNA. As you can see from the image on the left of 7 of the 11 samples, there seems to be contamination exhibited by a streak in the negative control lane (lane 4). The following three samples seem to be empty while the remaining 4 samples contain some amplified DNA. I ran another PCR reaction and gel on the final four samples reusing the positive and negative control amplification products (image at right). Again I observed contamination in the negative control and none of the expected bands in the four samples. I suspect that there may have been contamination in the cell media, possibly fungi. I also suspect that my primer concentrations and magnesium 2+ concentration may have been miscalculated. I will address these issues in future experiments.

Using a double-barreled nanopipette, the single-cell nanomanipulation system is capable of nanoinjecting and aspirating material from the same single cell simultaneously. I plan to capture HeLa cells out of cell media, hold them down on the cell capture device, nanoinject half of the 36 cells with dye and nanobiopsy from those same cells. This is important to demonstrate the system's ability to mark and remove material from the same cell. I will nanoinject directly into the cell 2-(4-(dimethylamino)styryl)-1-methylpyridinium iodide (DASPMI), which stains the cell's mitochondria and emits red fluorescence. Usually DASPMI is added to the cell media and the cells are incubated for approximately 45 minutes. I have yet to directly nanoinject DASPMI into the cell's cytoplasm with this system. I plan to either remove material while simultaneously nanoinjecting DASPMI and then check

for fluorescence, or nanoinject the dye, check for fluorescence, and then find a suitable spot to remove material from the cell. I will perform PCR amplification on the samples and check for the presence of amplified mtDNA by gel-electrophoresis. I expect to obtain similar results to experiments where only nanobiopsies where taken. I plan to release the cells and collect them for further culture.

The eventual goal should be to capture cells, nanomanipulate them, return them to culture, and repeat the nanomanipulation. This process would prove to be very powerful if it can be done multiple times over a period of time. This would allow the user to investigate changes in gene transcription profiles of cells of the blood, for instance.

3.4. Real-Time Identification And Nanomanipulation of Specific Single T-Cells In A Heterogeneous Solution

I plan to capture single T-cells out of a heterogeneous solution of T-cells. Not all T-cells express the same surface proteins. For instance, some T-cells express a surface protein known as CD4, while other T-cells do not express CD4. These T-cells that do not express CD4, *CD4 negative* (CD4-), do express a different but related cell surface protein known as CD8. I aim to exploit this variation to show that the cell-capture device and nanomanipulaion system will allow the user to identify a certain type of T-cell in a heterogeneous solution and then go to that single cell and investigate it.

I will culture two separate populations of T-cells, some expressing CD4 cell-surface proteins and a some expressing CD8 cell-surface proteins. I will mix a heterogenous solution, as a proof-of-concept 1:1, and label them with different-colored fluorescent antibodies. The antibodies will only bind to their respective cell-surface protein. Since the fluorescent tags are different colors, I will be able to identify T-cells that are expressing the CD4 cell-surface protein versus the CD8 cell-surface protein. There will be some bias introduced regarding how many of each T-cell type will be captured, as the cell-capture device will capture the small cells first, but this will be negligible for the proof of concept. I plan to follow with nanobiopsies from cells marked with the CD8-binding fluorescent antibody. I will perform

quantitative PCR (qPCR) on the nanobiopsy samples, probing for transcripts of the CD8 cell-surface protein gene and confirming the fluorescent antibody tagging. I will perform an RNA extraction on a population sample of the CD8 expressing T-cell suspension culture. I will compare the two data sets and I expect to observe the presence of CD8 transcripts in the population sample as well as the nanobiopsies, but in lower quantities in the nanobiopsy samples.

As a proof of concept this is exciting, because you can imagine in the future being able to identify rare cells, such as circulating tumor cells (CTCs), by labeling them with a fluorescent antibody and performing capture and nanomanipulation experiments on them. CTCs are of great interest in cancer research and methods that provide this level of resolution into their cellular processes would greatly benefit the cancer research community.

IV. Applications And Limitations.

This proof of concept will demonstrate the ability to nanomanipulate single non-adherent cells. Further developing this technology will be transformative for biological studies. By integrating a microfluidic device that captures single non-adherent cells out of cell media into a predetermined array of through-holes, I have increased the throughput of the single-cell nanomanipulation system from approximately 4 cells/hr to approximately 8 cells/hr. This technique facilitates the high-throughput screening and selection of many non-adherent single cells for parallel real-time semi-automated nanomanipulation.

The promise of the microfluidic device coupled with a nanopipette and custom software lies with the ability to assay a single cell at the nanometer scale, maintaining cell viability. This technique for single-cell immobilization provides the ability to sift through a population of cells, identify single cells of interest, and interrogate those cells directly with the nanomanipulation system. Applications of this system include analysis of cellular microenvironments, various *omics* analysis, genomic, transcriptomic, metabolic, etc., with sub cellular resolution, induction of single cells for induced pluripotent stem cell

(iPSC) research, greatly increasing our understanding of these cells, and single-cell aspirations and injections that allow for transfer of biomaterial into and out of a single cell, known as single-cell *nanosurgery*. Further developing the technology will require solutions to the current limitations.

There remain several challenges that should be addressed in future design iterations. The most important improvement to be made is to incorporate a thicker silicon nitride membrane. Future designs should use the thickest membrane that will not interfere with fluorescent microscopy. Silicon-nitride has optical properties that change as the material becomes thicker or thinner. A thicker membrane will provide a more robust membrane, and the device will be less susceptible to shattering caused by contact with the nanopipette or other objects. Nanopipettes are much less expensive than the cell-capture devices, therefore breaking the nanopipette tip on a sturdier membrane is acceptable. A thicker membrane will allow the user to apply a greater negative pressure, aiding in capture efficiency and cleaning. The current device is limited to single use because of cleaning issues. A thicker membrane will allow the user to supply a pulsating negative and positive pressure to the device aiding in cleaning the through-holes when they are clogged. A thicker membrane will allow the user to clean the devices in an ultrasonicator.

Another limitation to address involves the way cells are introduced into the device. Currently a 10 μL sample is pipetted into the sample well over the device's hole array. Once the cells settle, it is difficult to capture a cell that has settled at a location far from any hole. By adding a way to introduce a continuous flow of cells over the holes the user will be able to expedite experiments with samples with low cell concentration. *Soft-lithography*, usually referring to microfabrication techniques that use PDMS, can be used to make microfluidics in PDMS structures that can be bonded to the topside of the existing cell-capture device. Increasing the number of holes in the device membrane will also improve flow through the device, subsequently increasing capture efficiency and throughput. The increased fluid-flow will also make the device easier to clean.

Increasing the number of holes in the silicon-nitride membrane should be included in any further design iterations involving new semiconductor fabrication. A new mask should be created to incorporate many more holes. Theoretically you can include several hundred-thousand captures sites on the device so they will fit within the field of view under 4X magnification on an optical microscope. More holes in the membrane will allow you to capture more cells, which will increase the likelihood that you will capture a rare cell of interest. More holes in the membrane will also increase fluid flow through the device, which will decrease the hydrodynamic resistance of the device, allowing you to increase the applied negative pressure and in turn allowing you to capture cells faster and more efficiently. Increased fluid flow will make the devices easier to clean and may allow the device to be usable with cell media that contains serum, the portion of the media that contains the large proteins that clog the holes in the current device.

The final limitation I will discuss, although there are others, is the pressure control issue. The current setup uses an air-compressor with a precision regulator, a digital pressure gauge, and plastic tubing and valves that allows the user to apply a negative pressure between approximately 0 and 32 kPa. Changing from one pressure to another involves turning the regulator by hand and this takes up valuable time. This lack of response and control is an issue when trying to capture larger cells out of solution, applying a capture pressure and holding pressure, and when releasing cells. A electronic computer-controlled pressure controller would provide the response and control necessary for capturing cells and holding them down. Pressure controllers are not cheap, but not unreasonably priced either. This equipment would greatly improve the usability of the device.

5. Future Work.

Single-cell biology will greatly benefit from the further development and characterization of the single-cell nanomanipulation system and the associated cell-capture device. Future work should address the challenges associated with this form of single-cell immobilization, such as membrane integrity,

pressure control, and cleaning for reusability. Several design modifications should be included in future iterations of the device, such as a thicker membrane and an increased number of through-holes. These two modifications would greatly improve the device's capabilities. Other design-work can include the optimization of the cell-capture device to include individually addressable through-holes that will enable greater control and targeting of captured cells. These modifications would help support a fully-automated single-cell nanomanipulation system.

A completely automated system can make this technology accessible to a broader community. Major steps must be taken to make the system completely automated. Computer vision technology can be used to make the system sample-in, data-out, capable. Incorporating structures with arrays of nanopipettes, or "nanopipette-like" structures such as nanopillars, would allow users to perform many nanomanipulations in parallel. You can envision an assembly-line like process where cells are shuttled to different compartments in which they are captured and interrogated by nanopipettes. A system where a sample is introduced and the entire capture and nanomanipulation procedure is computer controlled would make this technology accessible to users with minimal training. This kind of system could be used to produce engineered cells. An exciting application is in the cellular therapeutics field. Imagine a system that fits on your desktop and produces tubes of engineered cells.

Exciting developments in imaging technology point to a future where single-cell capture and nanomanipulation will be even more powerful. One can imagine combining the cell capture device and nanomanipulation system with adaptive optics and super-resolution microscopy. Theoretically, this could afford nanomanipulation system users an unprecedented level of precision. Imagine being able to remove not only a population of mitochondria, but one single identified mitochondrion.

The cell-capture device is a step in the right direction for providing a means of holding down non-adherent cells so that they can be investigated with the nanomanipulation system. This technology holds great promise and will help biologists better understand biological phenomena.

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