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Integrated Electrowetting Nanoinjector/Aspirator for Single Cell Studies

DISSERTATION

submitted in partial satisfaction of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Engineering

by

Elaheh Shekaramiz

Dissertation Committee:

Professor H. Kumar Wickramasinghe, Ph.D., Chair

Associate Professor James P. Brody, Ph.D.

Assistant Professor Michelle Digman, Ph.D.

2017

DEDICATION

To

my family, teachers, and friends

in recognition of their worth

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CURRICULUM VITAE

Elaheh Shekaramiz

Education

2008 B.S. in Biomedical Engineering
University of California Irvine

2012 M.S. in Biomedical Engineering
University of California Irvine

2017 Ph.D. in Biomedical Engineering
University of California, Irvine

FIELD OF STUDY

Bioinstrumentation, Biosensor and Nanotechnology

Publications

- **Shekaramiz, E**. *et al*. Integrated Electrowetting Nanoinjector for Single Cell Transfection. *Sci. Rep.* **6**, 29051; doi: 10.1038/srep29051 (2016).
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ABSTRACT OF THE DISSERTATION

Integrated Electrowetting nanoinjector/aspirator in Single Cell Studies

By

Elaheh Shekaramiz

Doctor of Philosophy in Biomedical Engineering

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Professor H. Kumar Wickramasinghe, Chair

Single cell transfection and analysis techniques are crucial for comprehending the heterogeneity that exists between cells. Many techniques have been introduced and developed in the recent decades for single cell studies. However, there are many shortcomings to the current single cell transfection/analysis techniques. These techniques have limited transfection efficiency, dosage control, cell viability, and ease of fabrication. In addition, single cell analysis techniques are not powerful enough for spatiotemporal studies of single cells in their original microenvironment. In order to address these shortcomings, we have developed a technique called integrated electrowetting nanoinjector/aspirator that can modify the genetic expression of single

cells and aspirate femtoliter amounts of cytoplasmic content for protein quantification on a single cell level.

Chapter 1

Introduction

1.1 Significance of single cell studies

Cells are the units of life and together with their micro-environment present the basic integer of tissues and living organisms. In a population of cells, each cell is different from the neighboring cells. Many diseases may go unrecognized at early stages due to sampling of the average cell population not necessarily providing accurate representation of an individual diseased cell state.[1] The variations between cells can arise from the cell cycle, circadian oscillator, microenvironment, or epigenetic status of a genome.[2] The process of gene expression is known to differ from one cell to another.[3] Therefore, to better understand gene function of an individual cell, dependable single cell techniques are needed.[4]

There are two types of single cell studies, one is single cell analysis and the other one is single cell transfection.

1.2. Transfection

Transfection is the process of transferring nucleic acids into cells.[4] DNA by itself is a large hydrophilic negatively charged polymer, and thus cannot readily traverse into the cell.[5] As a result, many techniques have been developed both at the bulk level and single cell level to facilitate this transfer including physical, chemical and viral methods.[5] These techniques help scientists study gene function and regulation.[6] One of the main applications of transfection is corrective gene therapy in which genes are inserted into host cells for therapeutic effect by giving cells new functions or correcting a defective/missing gene.[7]

With single cell transfection, there is an increased assurance that the molecules inserted inside a cell are in fact the anticipated molecules.[8] Controlling the composition mixture of various reagents is very important in some applications such as fluorescent tRNA translation.[8]With bulk transfection, not all cells may be treated equally.[8]In comparison to bulk transfection, single cell transfection can be applied to any kind of cells.[4]Bulk cell transfections are hard to optimize and lack single cell resolution.[8] Methods of single cell transfections include microinjection, photoporation, micropipette electroporation, and Atomic Force Microscopy (AFM). (Figure 1.1)

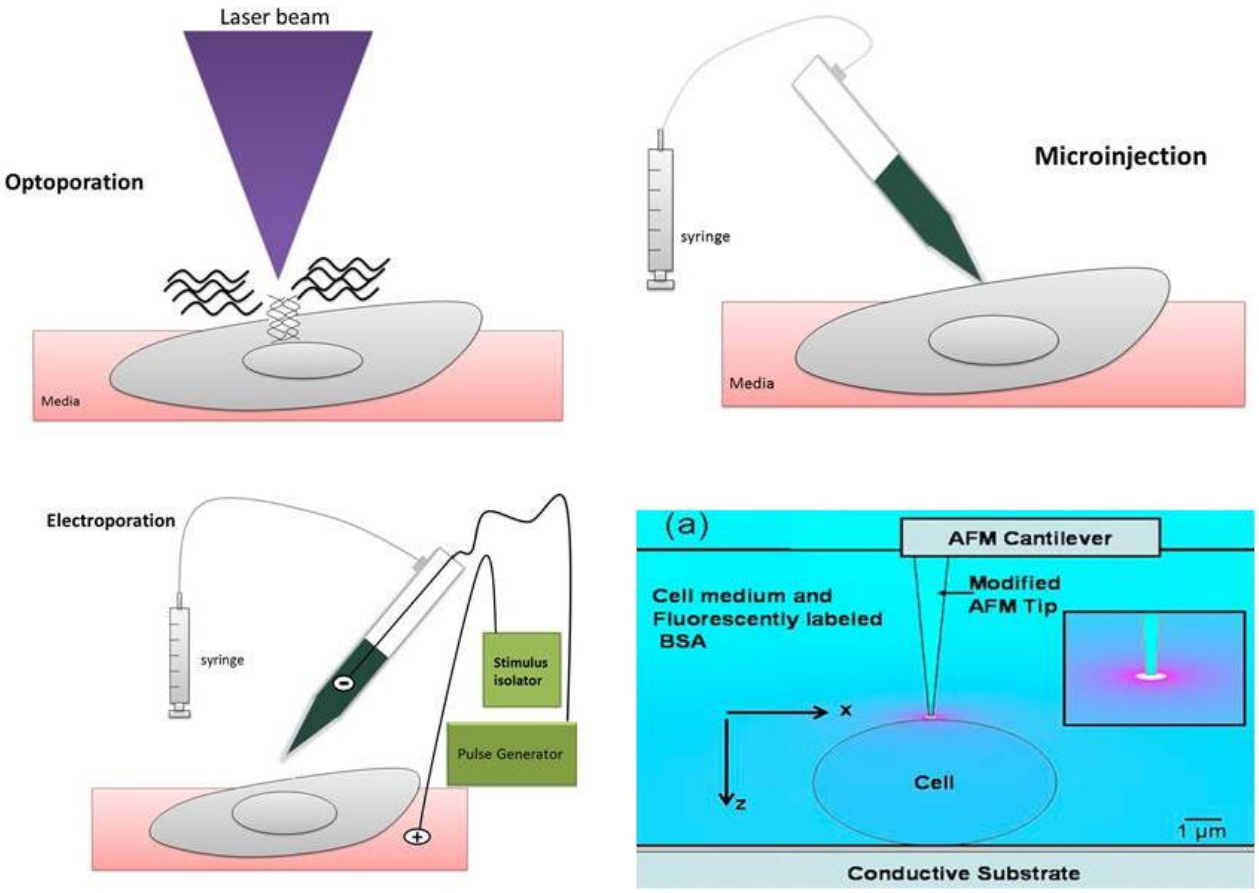

Figure 1.1. Single Cell Transfection Techniques including optoporation, electroporation, microinjection, and and AFM

1.2.1. Microinjection

Microinjection refers to delivery of molecules with pressure into cells through 0.5-5µm diameter glass pipettes (Figure 1.1). [10] In early 1970s, scientists applied microinjection to study gene expression of regulatory pathways in maturation of Xenopus oocytes. [11] By the 1980's, genes from membrane receptors, heat shock proteins, and secretory products isolated from oocytes were studied using microinjection.[11] Today, microinjection has a lot of applications including in vitro fertilization, reproductive cloning by somatic nuclear transfer, and production of transgenic animals by DNA injection into embryos.[12] The basic components of a microinjection system include an inverted microscope, micromanipulator, micropipette holder, micrometer syringe, gas pressure regulator, glass capillary tubes, and vibration isolation table. There are two types of microinjection systems including pulsed flow and constant flow systems. In the constant flow system, a constant flow drives the sample into cells and the amount of substance delivery depends on the duration that the micropipette is inside a cell. [10] This method is inexpensive, but not well controlled. There is more control in terms of injection parameters with pulsed flow system. The most common pulsed flow system is the Eppendorf femtojet injector and Eppendorf Injectman. The diagonal needle insertion is less damaging, faster and makes a direct piercing in the cells compared to the constant flow system.[13] The Eppendorf products are not user friendly and are expensive. When transfecting adherent single cells, the needle is held 30 to 45 degrees horizontal near the cell membrane, and it is lowered to pierce the membrane. Suspended cells however are held by a negative pressure and are injected by a positive pressure.[14]

1.2.2. Photoporation

Photoporation describes the delivery of genes into cells using laser(Figure 1.1). [15] Transfection with photoporation technique was demonstrated in 1984 by Tsukakoshi et al. using a Nd:YAG laser in rat kidney cells. In their work, a 355nm nano-second pulsed laser was focused on 0.5µm membrane of rat kidney cells surrounded by plasmid DNA.[15] They realized several days after the experiment that the cells were transfected. Initial transfection efficiencies of photoporation are reported to be 14%. In photoporation, a laser beam is focused through a high numerical aperture microscope objective lens on the cell membrane to create pores on the membrane and as a result, DNA in solution can enter the targeted cells. Photoporation is contactless and aseptic.[15] Both continuous and pulsed laser sources have been used for photoporation in single cells.[15] Continuous wave (CW) lasers are based on localized heating of the cell membrane.[15] Although both transient and stable transfection is possible with CW lasers and cells seem viable after photoporation, the highest efficiency reported with CW lasers is 30%.[15]With pulsed lasers, the pulse duration is an important factor in the reaction of a cell. Short pulses (femtoseconds) create low density plasma openings in the cell membrane. Long pulses (nanoseconds) on the other hand produce free electrons, as well as thermoelastic stresses and bubbles.[15] Nanosecond lasers have not been promising for single cell photoporation, but they have been useful for photoporating groups of cells. For these reasons, femtosecond pulsed lasers are the best choice for single cell transfection as of now.[15] The laser source

wavelength of 800nm is known to give higher injection rate (90%) compared to 1554nm (77%) with the same repetition rate (1MHz).[15]

1.2.3. Micropipette electroporation

Electroporation is using electrical field to induce pores in the cell membrane which can in turn permit exogenous DNA uptake by cells.(Figure1.1) [16] Based on the electroporation theory, applied electrical field creates large hydrophobic holes. [9] The hydrophobic holes become hydrophilic (change in the position of lipid bilayers) allowing molecules to pass through. For viability of cells, these pores need to reseal in the absence of voltage.[9]

Compared to bulk electroporation, single cell electroporation allows for the optimization of electric field on an individual cell with a certain shape, size, and status, and can locally electroporate cells with lower voltage applied.[17] Unlike bulk electroporation, the electrical field applied is not homogenous.[9] However, the principle behind the single cell electroporation is the same as bulk electroporation.[9] The first single cell electroporation was done in 1998 using carbon fiber microelectrodes to put vector pRAY1 into individual COS7 cells. Nowadays, single cell electroporation via microelectrodes, micropipettes, microfabricated devices, and electrolyte-filled capillaries is possible. Orwar's lab was the first to report single cell electroporation with solid microelectrodes.[18] Two electrodes, each the size of a cell or smaller are used on both sides of a cell and a voltage more than the transmembrane potential threshold is applied across the electrodes. The potential drop increases as the distance between the

two electrodes increases. Some of the potential is lost at the liquid/electrode interface because of double layer formation and electrode reactions. DNA is either in solution or can be delivered to the targeted cells.[9] Although the efficiency rate of microelectrodes is high, there is a concern with carbon electrodes or microelectrodes' cytotoxicity in cells because electrode reactions form reactive oxygen species.[17] The greater the distance of electrodes to cells and the shorter the pulse time, the less harmful it would be for the cells. Due to the cytotoxicity with microelectrodes, researchers started using glass pipettes for single cell electroporation. To apply a voltage, a wire is placed inside 0.5-3 μm diameter borosilicate glass pipettes and the other electrode is inside the solution.[9] The pipettes are connected to an external pressure to push materials inside cells, but the transferred material can also be in solution where the cell resides.[9] Because the electrodes are further away from the target area, cytotoxicity is not a problem in the case of micropipette electroporation.[19] The first single cell electroporation with 0.6-1*μ*m glass micropipettes was done by Hass et al. and the transfection efficiency reported was 30%.[19] With electrolyte filled capillaries (EFC) made of fused silica, capillaries are not in direct contact with cells and are placed 6.5μm or less away from the cell and voltage of 500v should be applied to electroporate cells.[9] With the EFC method, cells undergo lower strength electrical field because the pipette is further away, but the durations of field is seconds compared to milliseconds. Because of the high resistance of the orifice of electrolyte filled capillaries, most of the potential is lost and thus higher voltages should be applied.[19] With both micropipettes and filled capillaries, the volume of solute is around 1μL.[9, 19]

Huang and Rubinsky were the first to show single cell electroporation on a chip on which cells were placed between the two electrodes in 2001.[17] Lee et al. have used polydimethylsiloxane (PDMS) chips which are disposable and easy to use to immobilize single cells and electroporate them locally. With PDMS, fluorescent detection and monitoring is also possible. Furthermore, the format allows parallel electroporation as well as reversible electroporation. The cells are trapped with an applied negative pressure through a syringe as they are passing through. Once they are trapped, a voltage of less than 1v is applied for electroporation. Once voltage is applied, different behavior of DNA has been claimed including formation of endosome like vesicles, insertion of DNA into bilayers, electrophoretic forces, and DNA complexion with lipid bilayers.[19]

1.2.4. Atomic Force Microscopy (AFM)

The concept of atomic force microscopy was introduced by Binning and Quate in 1986, but the first demonstration of vibrating cantilever happened in 1987 by Wickramasinghe.[20] More than any other scientists, biologists were very interested in using AFM for imaging small resolution biological samples, but the problem was that the AFM was only functioning in air at the time. The potential of AFM for biologists became a reality when AFM was shown to be functioning under liquid in 1989.[21] The first biological sample that was imaged with AFM was amino acids and polymers. Later, topographies of protein membranes with molecular resolution, images of native protein in photosynthesis ,and high resolution of DNA images were achieved.[21] The hallmark of AFM was when the scientists started using AFM not only as a microscope, but rather

as a tool.[22] In 1990s, the interaction forces between biomolecules as well as cell-cell, cell-substrate interactions were investigated. Hoe and coworkers used AFM as a dissecting device to dissect the junctions between cells. AFM tips were also applied to extract DNA and amplify the genetic material with PCR.[22] Thus, AFM has been very crucial for many scientists to unravel various scientific questions.

AFM is one of the scanning probe microscopies that can scan over an area to map out the surface topographies. AFM is not limited by the wavelength of radiation unlike classical microscopes because AFM does not acquire images based on light.[23] AFM is made of a cantilever probe with a sharp tip mounted on a piezoelectric actuator which moves the tip in a 3D pattern, a detector that detects the laser beam deflected off of tip, and a feedback loop that monitors the interaction between the tip and the surface of a sample. As the tip interacts with the sample surface, the cantilever deflections are monitored and are used for topography of the sample. AFM probes are made of silicon or silicon nitride. The size and shape of the tip and the spring constant of the cantilever varies and should be chosen based on the application.[23]

AFM has also the capability of being used as a tool for single cell transfection (Figure 1.1). Scientists have delivered molecules with AFM probes in three ways. The first use of AFM is via attaching DNA plasmids to the AFM tips and allowing DNA to be diffused inside cells. The second method is creating channels inside AFM cantilever for delivery of DNA molecules. Lastly, our group has applied AFM tips for electroporating and transfecting cells.

Grandbois et al. delivered plasmid DNA attached to commercial pyramidal AFM tips.[24] They reported minimal damage as well as 30% efficiency in Human embryonic kidney 293 (HEK 293) cells. Jun Miyake investigated AFM probes for DNA delivery further.[25] They first started delivering DNA with regular pyramidal AFM tips into mesenchymal stem cells and they realized that the efficiency of these probes were as low as 30% due to the insertion rate of pyramidal tips.[25] Moreover, the cell viability was affected by the shape of the tips.[25] Thus, later they fabricated cylindrical 200nm diameter silicon nanoneedle AFM probes for injection into mesenchymal stem cells which are flat and small. They attached plasmid DNAs electrostatically to poly-L-lysine coated AFM tips. In their studies, they compared AFM technique in transfection with microinjection and lipofection. The efficiency of gene expression with AFM tips, microinjection, and lipofection were 74%, 8%, and 42% respectively. The low efficiency rate of microinjection is because of the insertion rate as discussed before. Furthermore, the lipofection technique is an irregular in terms of delivering DNA molecules in each cell.[25] With the fabricated needles, the efficiency rate is higher due to higher insertion rates (90%) and there is no damage to cells because the shape of the tip is cylindrical.[25] From this study, they also have shown that AFM can be used for both small and flat cells as well as large cells.[26]

Zambelli et al. have fabricated AFM cantilevers to have a hollow opening for dispensing of liquids by hydrostatic pressure inside single cells.[26] In their FluidFM technique in which they combined atomic force microscopy with nanofluidics, they reported that injection of materials is possible through “gentle contact” [26] or penetrating the cell

membrane. Using FluidFM, they have successfully delivered dyes into cells. The advantage of FluidFM compared to functionalized AFM tip is the limitless amount of material for injection.[26]Furthermore, the “gentle contact” allows for studying the behavior of viruses.[26] Any material can be injected with FluidFM and multiple injections should be possible although not studied yet.[26] One of the main advantages of AFM and microinjection is passing through the membrane and direct delivery into cytoplasm or nucleus.[25, 27] With AFM, there is a robust feedback signaling and possibility to access subcellular regions in the cell.[26] There is no study on the throughput of AFM, but since each/ cell has to be injected individually, the throughput is low.

Lastly, AFM has been applied to electroporate single cells and transfect them.[28] AFM tips were fabricated by growing SiO2 layer and then exposing bare silicon layer by machining to obtain a 0.5um diameter tip. The cells were grown on top of conductive indium tin oxide (ITO) substrate. The AFM tip then approached the cell and contacted the membrane. The point of contact could be detected by deflection of cantilever. Once in contact, the AFM tip was retracted 200nm and a voltage of 1v, 0.5Hz was applied between the AFM tip and the ITO substrate for creating membranes inside cells. Based on the simulations, the electric field was locally applied in the region where tip was located and thus the damage to cells was none to minimum. Cy5 labeled BSA molecules were visualized to be diffused inside the cell in less than 10s.[28]

1.3. Single cell analysis

Proteomics is studying of protein abundance, variations, and modification inside cells.[29] Proteins are vital to cells because they regulate cell functions, cell adhesion and growth, regulate signal transduction and transport molecules across cell membranes. As discussed before, due to differences between cells, studies need to be focused on individual cells rather than a whole population of them (Figure 1.2). [30] Understanding proteins at the single cell level helps us apprehend the molecular mechanisms governing disease progression. [31]

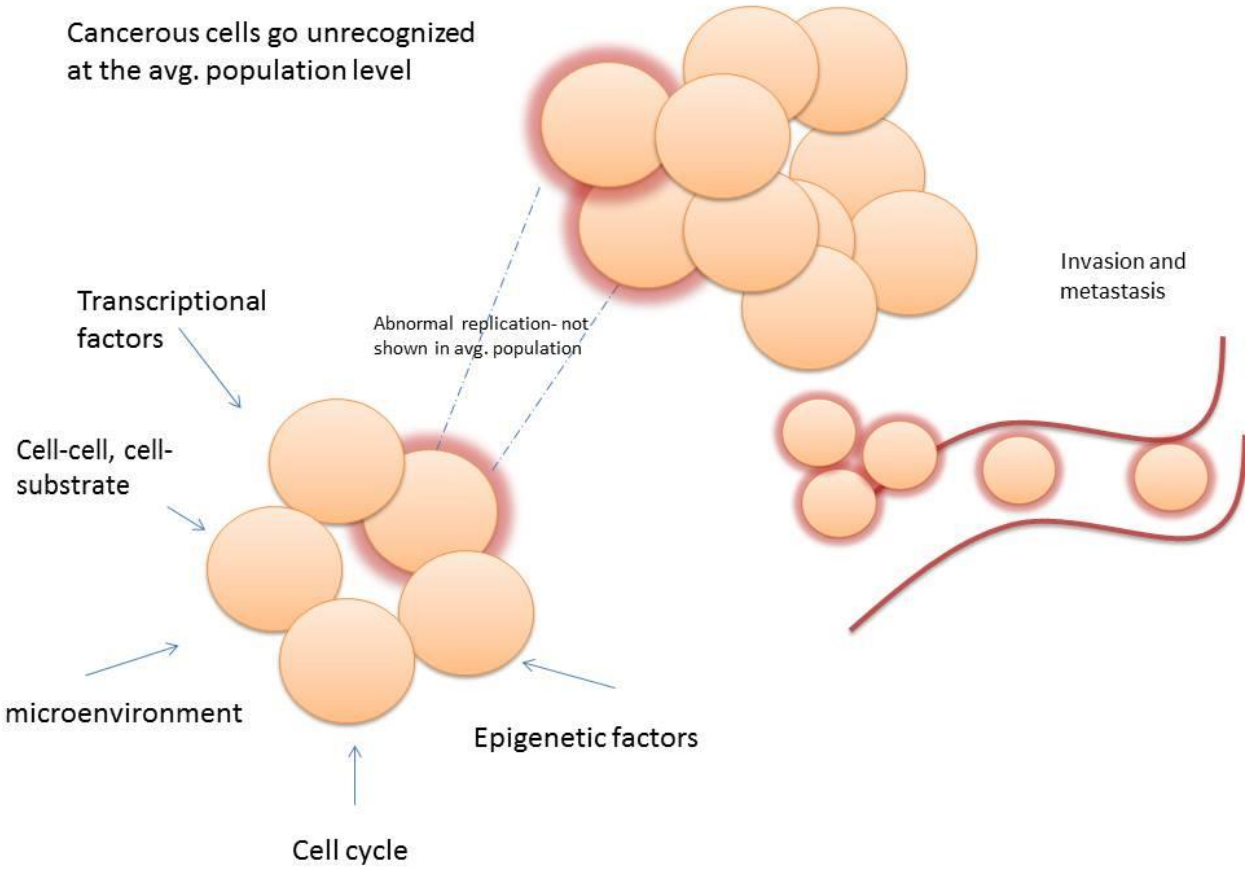

Figure 1.2. Response of a population of cells to a stimulus: an actual response versus an averaged response

Many methods have emerged for single cell protein analysis such as Fluorescence activating cell sorting (FACS), mass spectrometry and microfluidic platforms[32].

1.3.1. Microfluidic Platforms

Microfluidic techniques increase the performance of protein analysis by decreasing volume and result time with high throughput. [33] Microfluidic techniques also require lower number of cells (100-1000) for protein analysis. [34] Single cells are either intact or they are lysed in microfluidic platforms. The advantage of keeping cells intact is that the proteins are at their intracellular concentration which is much higher than their concentration upon lysis. [30] The dimension of the microfluidic chips are usually from 10µm to 100 µm. Klug and Willison reported a label free microfluidic antibody capture (MAC) chip to quantify the number of proteins in a multiplexed single assay format.[35] After each cell is optically trapped and lysed, the content of that cell is released inside 4.6nl chambers. The protein of interest is then bound to the antibody captured on the glass slide on the floor of the MAC chamber. The protein is tagged with a second antibody and is counted with TIRF illumination and CCD camera recording.[32] Microfluidic techniques have been combined with flow cytometry to overcome some of the flow cytometry limitations in single cell analysis. [31]

1.3.2. Flow cytometry

Flow cytometry is a laser based technique used for protein engineering, cell sorting and cell counting. Fluorescent activated cell sorting (FACS) is one of the special forms of flow cytometry. FACS machine is able to separate the cells of interest from a mixture of cells. The cells are tagged with fluorescent-labeled antibodies. The antibodies bind to proteins, which are expressed by the cells of interest. Once the cell passes through the nozzle, the laser light excites the dye. The light is captured by the photomultiplier tube. Some light is also scattered which can indicate the size of the cell. Before generating droplets at the end of the nozzle, computer determines how to separate cells. Once the droplets are formed, an electrical field is applied which makes positively or negatively charged droplets. Droplets with no charge are thrown away. At the end of the process, the number of cells as well as the amount of fluorescence is determined. Quantitative flow cytometry is accomplished by comparing fluorescent intensity versus calibration standard. FACS is powerful because it has a high throughput and high level of multiplexing.[36] However, FACS is expensive due to the huge amount of antibodies that needs to be used. The instrument itself is costly and requires a skilled trainee to operate it. [29] Cells are still alive after FACS, but they are not in their original environment. [37] FACS is also limited in the number of fluorescent markers that it can simultaneously measure.[37]. Because FACS needs a lot of cells (more than 10,000), rare cells such as biopsy cells or primary tissue cells can not be analyzed. [30] Moreover, spatial studies on captured single cells are not possible with FACS. [37]

1.3.3. Mass Spectrometry

Mass spectrometry (MS) is widely used in proteomics nowadays due to the electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) in 1980. ESI and MALDI are two of the techniques used for ionizing proteins. Before Mass spectrometry, scientists used chemical or enzymatic degradation such as Edman degradation to sequence proteins. In MS, sample is ionized, accelerated by an electrical field, deflected by electrical or magnetic field and then separated based on mass to charge ratio. There are different types of MS such as hybrid quadrupole time-of-flight (Q-Q-ToF), tandem time-of-flight (ToF-ToF), linear ion trap and many others. Each of these mass spectrometries is capable of each task with different performances and standards.

The common mass spectrometry for analyzing proteins in a single cell are electro-spray MS, laser desorption ionization (LDI-MS) and secondary ion MS (SIMS). [32] Sweedler et al. have successfully quantified neuro-peptides with MALDI MS. [38] The major limitation in using protein mass spectrometry is the low amount of proteins in a single cell due to loss of sample during the process.[39] In other words, the sensitivity of mass spectrometry is a huge limitation of it. Recently however, groups of scientists have developed Single Cell Proteomics by Mass Spectrometry (SCoPE-MS) to address the issue of sample loss in mass spectrometry at the single cell level and quantify thousands of proteins in mouse embryonic stem cells. [39]

Currently, 10-18 different proteins can be measured simultaneously in single cells by FACS.[40] The number of measurable proteins can go up to 20-30 proteins by

conjugating antibodies to metal isotopes of different masses in mass spectrometry. [40] However, secreted proteins can not be measured effectively and that's where recent microfluidic platforms have played a role. [40] Recently, Abbaspourrad et al. demonstrated a label free technique for quantification of proteins at the single cell level using microfluidics. [40]

However, with all these conventional techniques, cells are lifted with trypsin causing environmental disruptions to cells. Moreover, protein analysis only occurs after cell lysis or cell fixing and perming. Thus, a necessity arises for a technique that disturbs the cell minimally during protein analysis. Compared to other protein analysis methods, the Integrated Electrowetting nanoprobe technique developed in this study is capable of spatiotemporal analysis of single cells.

1.4. Organization of the Document

Chapter 2 provides detail description of the Integrated Electrowetting nanoinjector/Aspirator technology.

In chapter 3, the cell viability of cells using the technique is explored.

In chapter 4, the technique is used for injecting quantified amount of plasmid DNA into single cells.

In chapter 5, the technique is used for extraction of cytoplasmic or nuclear content and for protein analysis.

Chapter 6 concludes the dissertation with some direction for future work.

Chapter 2

Technique

2.1. Current Techniques in Single Cell Transfection

The current techniques for single cell transfection have limited transfection efficiency, dosage control, cell viability, and ease of fabrication.[41] For instance, microinjection, which is the most common transfection technique, damages cells due to the large microinjector needle diameters of 0.5–5 μm and the needles tend to clog before injection.[27] Other single cell transfection techniques such as optoporation lack controlled dosage delivery and need optimization of parameters for each cell type.[42, 43] Single cell electroporation techniques have low cell viability given the high electric fields applied to each cell. [44] Grandbois *et al.* transfected cells by inserting plasmid-decorated Atomic Force Microscopy (AFM) probes and incubating them in cells for one minute. The number of molecules injected was not quantified and the efficiency was 30%.[24] Recently, Vorholt *et al.* injected plasmids into single cells using specialized AFM tips fabricated with integrated fluidics. [45] However, the fabrication of such AFM tips is cumbersome and they possess a reported efficiency of 40%. [45] Most importantly, injection volumes could only be determined after injection with the help of a co-injected fluorescent marker. [45]

Pourmand's group used double-barrel nanopipettes to inject fluorescent dyes inside human BJ fibroblast cells. [41] Mirkin *et al.* also developed a method where one electrode was placed inside culture media and another inside the micropipette and used it to deliver positively and negatively charged fluorescent dyes into cells. [46] However, neither of techniques was used to change the genetic expression of living cells. [41], [46]

In the current study, we present a new simple method using an integrated electrowetting nanoinjector (INENI) with controlled dosage delivery and high transfection efficiency. Furthermore, with thistechnique, we demonstrate both transfection and single cell analysis. (Figure 2.1)

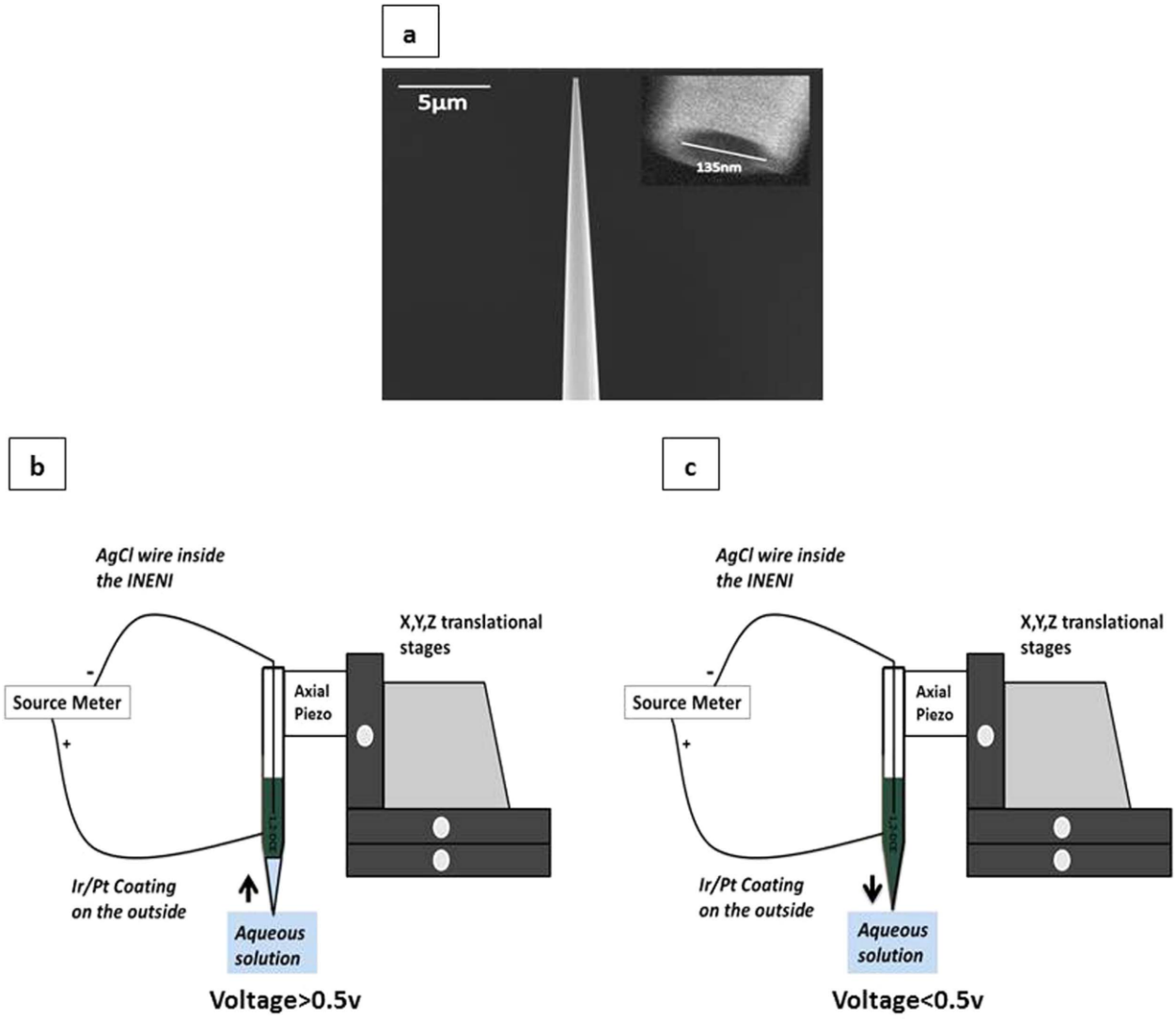

Figure 2.1. (a) Scanning Electron Microscopy (SEM) image of INENI. (b) Schematics of INENI device for pick up, where voltages higher than 0.5v produces solution uptake. (c) Schematics of INENI device for ejection., where voltages lower than 0.5v gives rise to solution ejection.

2.2. Fabrication of Nanoprobes

2.2.1. Size of Integrated Electrowetting Nanoprobes

INENIs were fabricated from borosilicate glass capillaries (Sutter Instrument, Novato, CA) using a P-97 puller (Sutter Instrument, Novato, CA). The parameters of pulling including velocity of pull, heat, and strength of pull were optimized so that the nanopipettes are rigid enough to enter into cells and small enough to not damage the cells. After the pulling, the nanopipettes were coated with 10nm iridium (Ir) and were imaged under scanning electron microscopy (SEM). There was a variation in the pulling and the mean diameter was determined to be 139 ± 3.5 nm. The cone angle was measured to be 4.3 degrees (Figure 2.2).

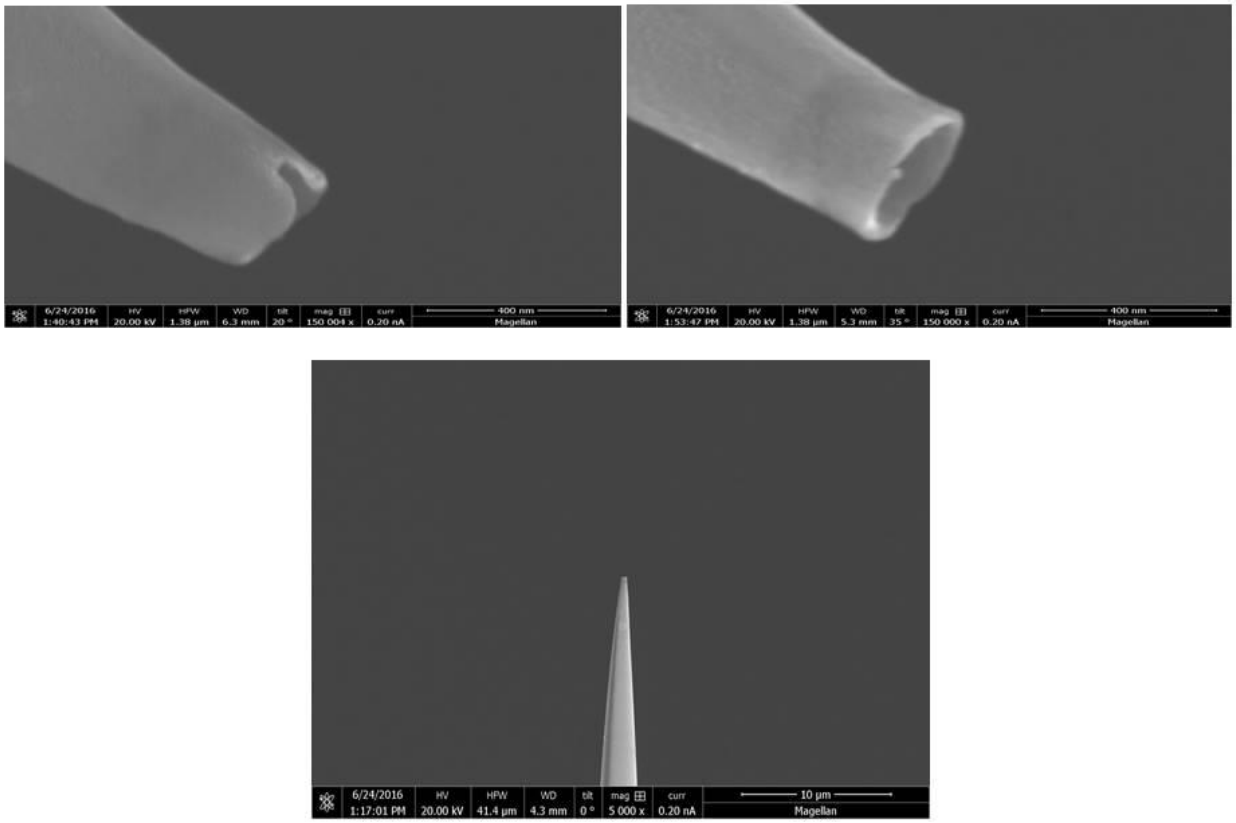

Figure 2.2. SEM images of nanoinjector/aspirator. The figures show the size of nanoinjector/aspirators at different magnifications

2.2.2. Coating of Integrated Electrowetting Nanoprobes

There were two approaches in coating nanoinjectors. The first approach was coating capillaries and then pulling them and the second approach was pulling capillaries and coating them with the appropriate target electrode. (Figure 2.3)

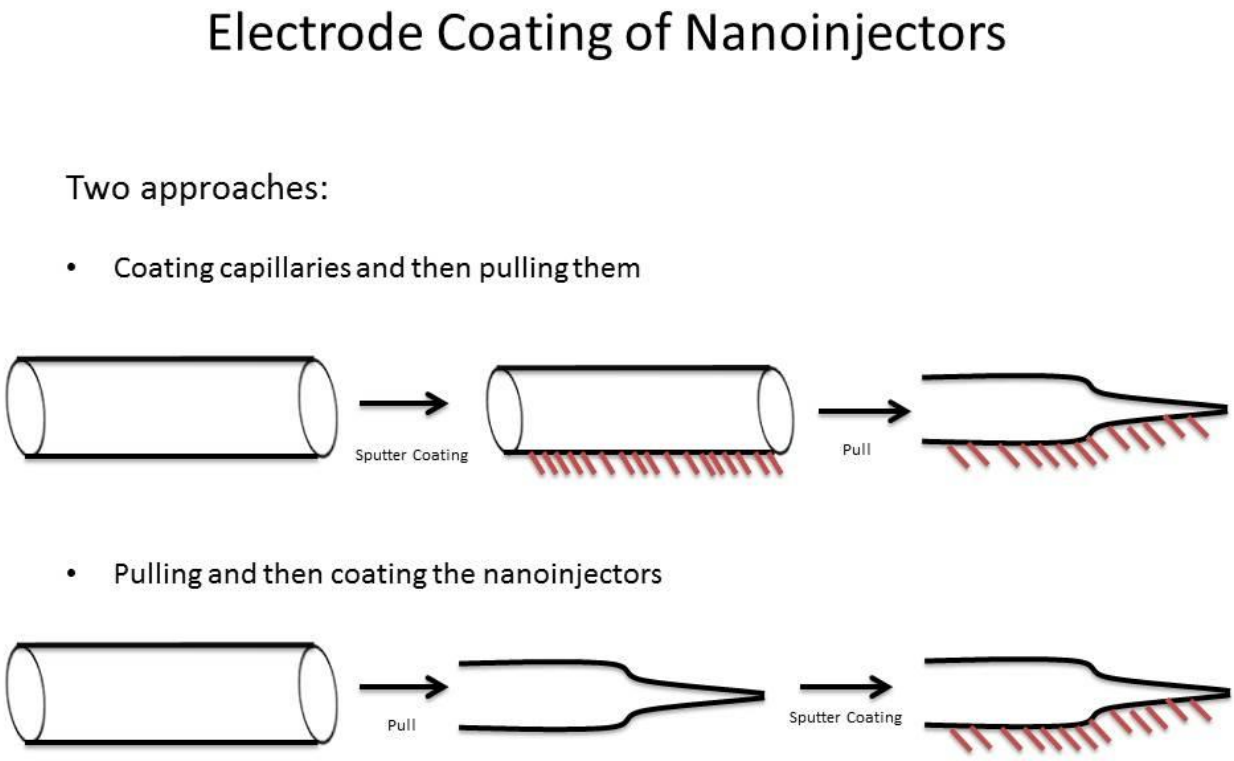

Figure 2.3. Schematics of the two approaches for coating an outer electrode. Upper panel shows coating capillaries and then pulling them. Lower Panel shows pulling capillaries and then coating them.

2.2.2.1. Material and thickness of coating

With the first approach, two coating materials were selected including gold and titanium. We wanted a material such that when we pull the glass, it would melt with the glass. Thus, the melting temperature of the material chosen were in close proximity of the melting temperature of glass. Gold (Au) and Titanium (Ti) has a melting temperature of 1064degrees and titanium's melting temperature is 1668degrees (Table 2.1). In order for gold to attach to glass, it needs chromium (Cr) adhesion layer. The melting

temperature of chrome is 1907 degrees which is 300degrees more than the melting temperature of glass, but since the for the adhesion layer we only needed 3nm, the argument was that the puller filament can still heat it up and pull it.

Table 2.1. Melting temperatures of different target materials

Target Material	Melting temperature(°C)
Gold	1064
Titanium	1668
Platinum	1768
Chromium	1907
Iridium	2447
Glass	1400-1600

Nanopipettes were coated only on one side of glass with 3nm of Cr and 90nm of gold and then pulled. However, after pulling patches of gold were observed on glass. (Figure 2.4) In other words, not a continuous film of gold was covered on top of glass nanoprobes.

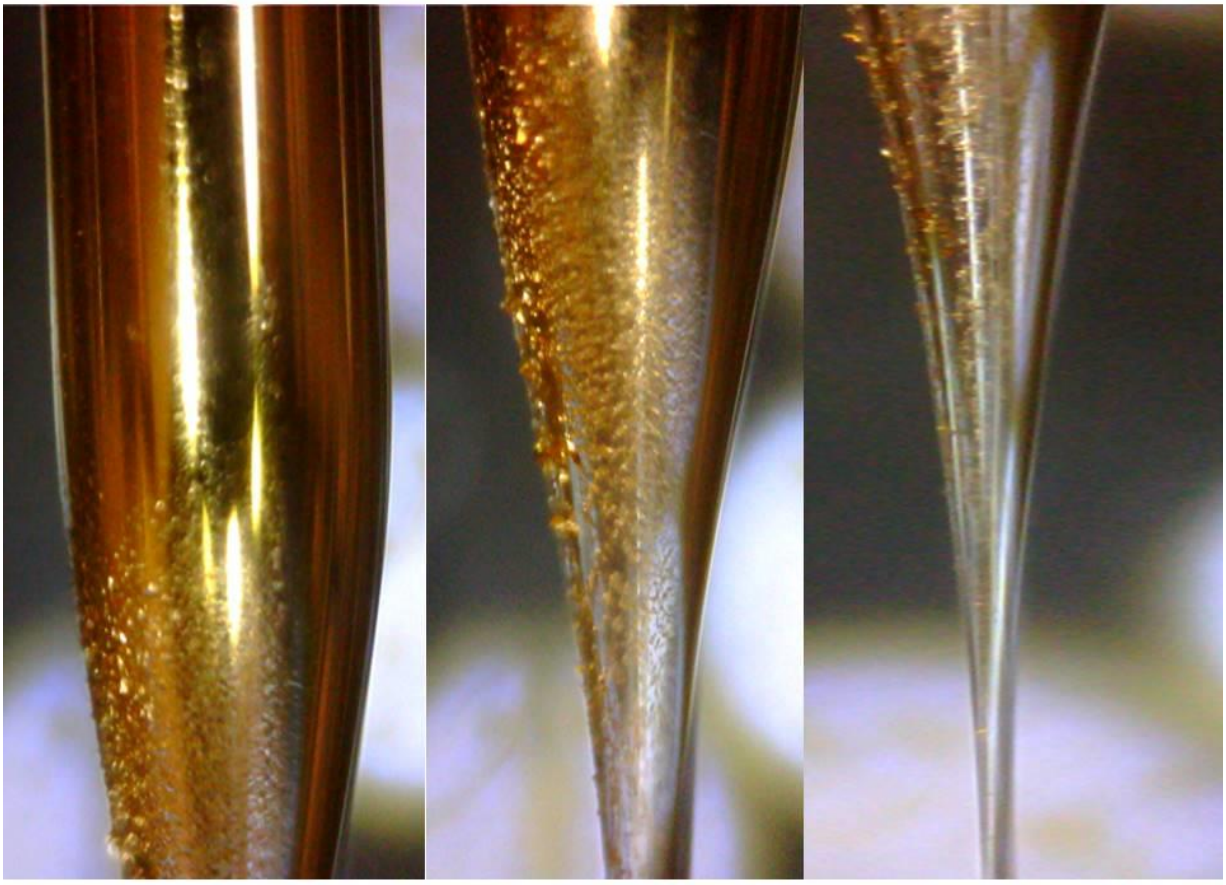

Figure 2.4. Coating of nanoprobes with gold and pulling them after. Lef-t panel shows upper part of nanoprobes. Middle part shows the middle part of nanopipette. Right panel. The very end coating of gold on top of nanoprobes

Since gold coating did not work out for our application, we switched to titanium coating. After coating glass capillaries with a thick film of titanium (100nm), the capillaries were pulled. However, coating with Titanium was also not successful because there was not a continuous layer pf the coating material. (Figure 2.5)

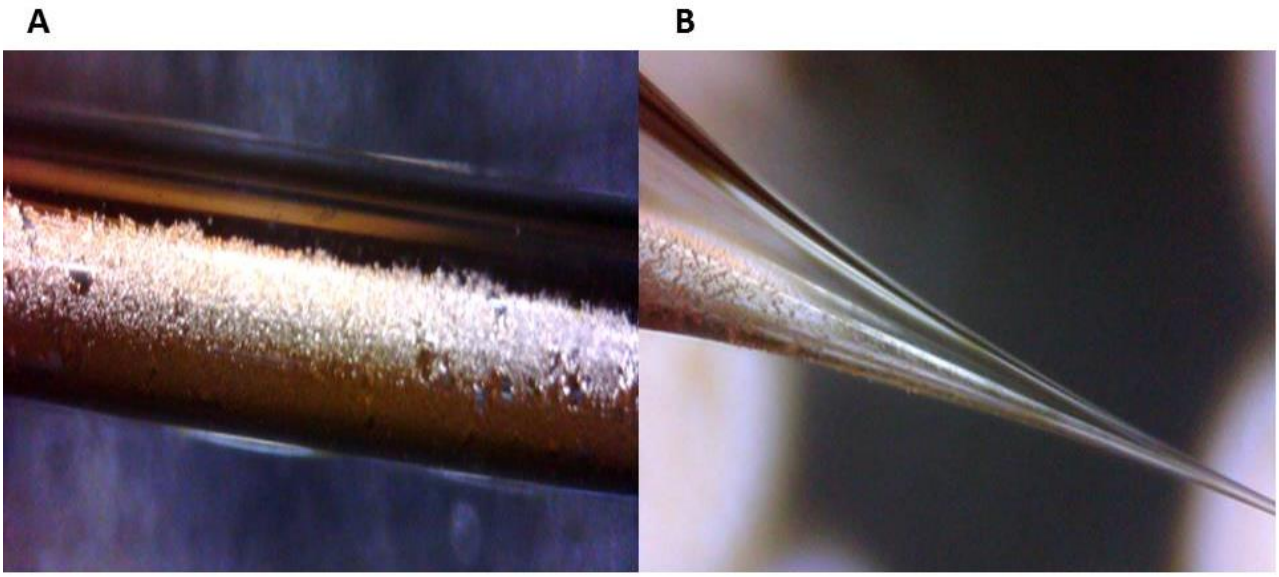

Figure 2.5. Coating of nanoprobes with Ti and pulling them after. A. Upper part of the nanopipette. B. lower end of nanopipette

Since gold and Titanium coating both did not provide satisfactory outcomes, a second approach was undertaken. While going through the SEM pictures of nanopipettes, one picture was noticed where the coating of iridium covered the entire length of the nanoprobe. (Figure 2.6)

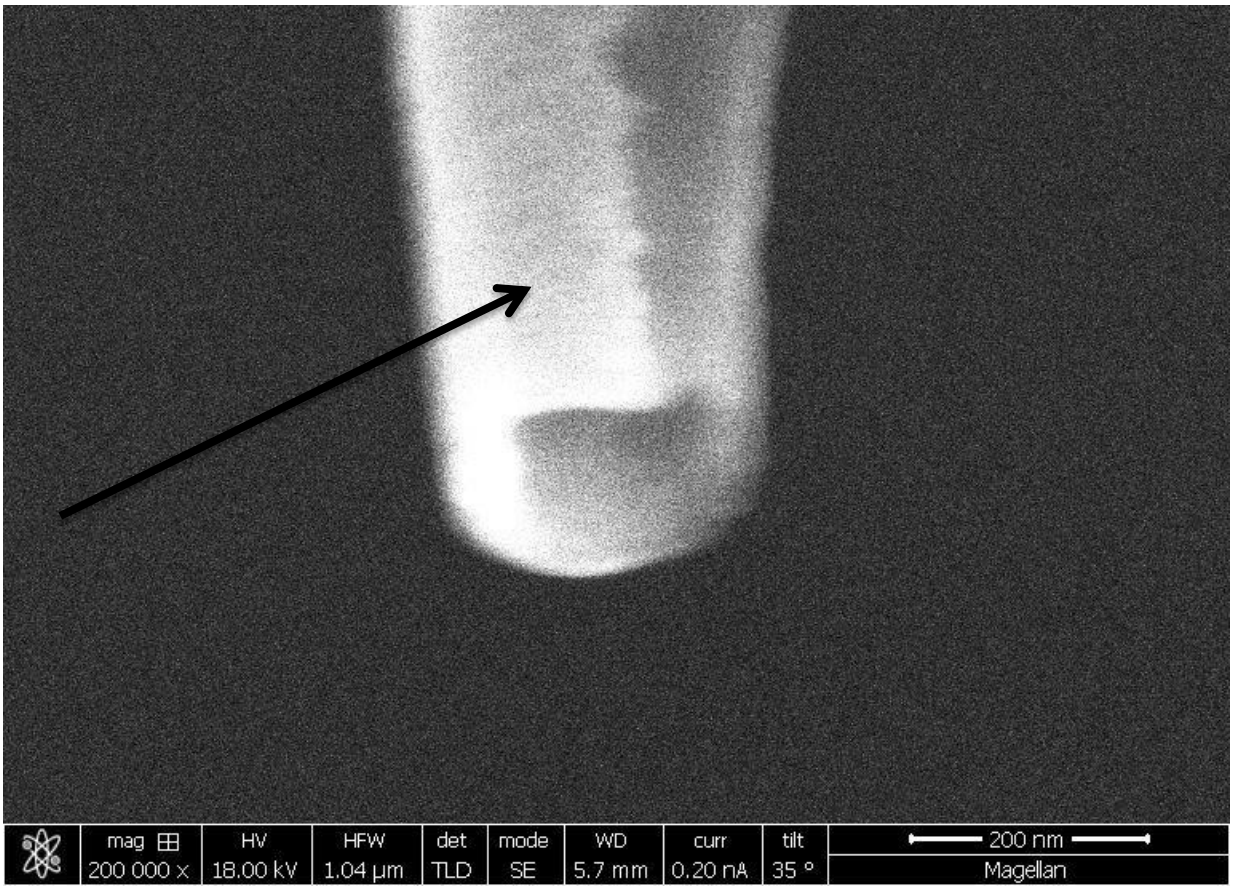

Figure 2.6. Iridium coating of nanoprobes. 10nm of Iridium coating visible on the very end of nanoinjector

It was hypothesized pulling the nanoprobes and then coating them with Ir would make a good outer electrode. Based on the literature search, Pt and Ir/Pt are used for biomedical devices and do not provoke any cytotoxicity.[48]

Coating with 60nm of Pt on one side of nanoprobes after pulling created stress on the coated side causing the nanoprobes to bend (Figure 2.7).

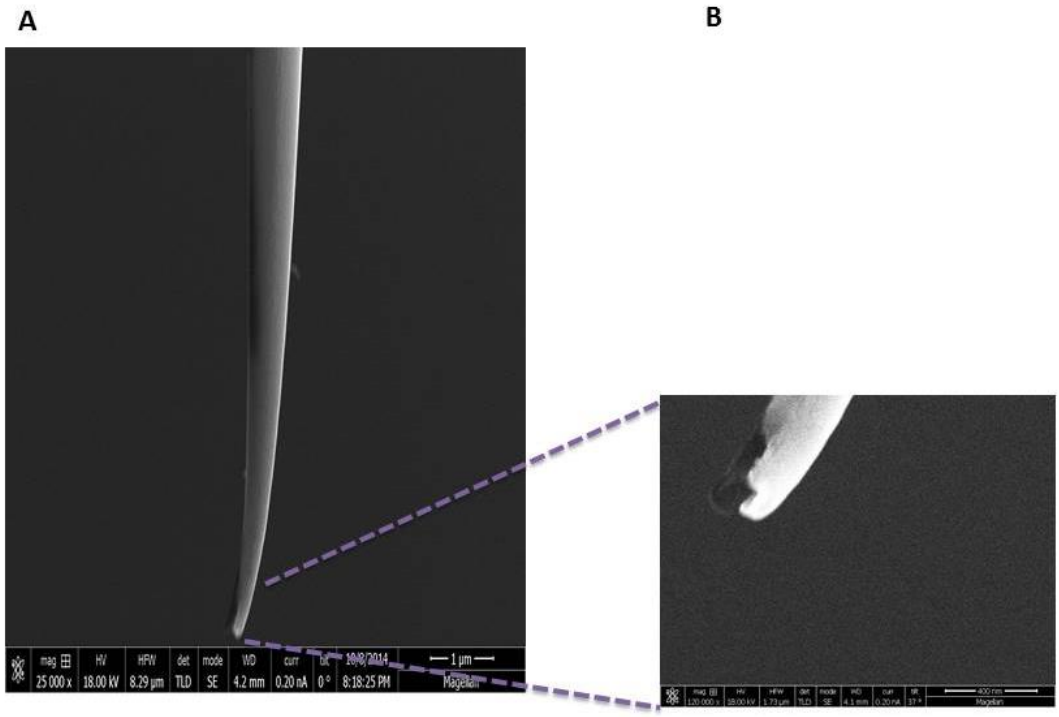

Figure 2.7. Ir/Pt Coating of nanoprobes. A. Coating 60nm of pt causing a bend at the end of nanoprobes B. Zoom in image of A.

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The amount of material deposited was reduced to half and 10nm of Ir and 20nm of Pt yielded the best parameters for our application (Figure 2.8). The nanoprobes were then oxygen-plasma treated for 5-10 minutes for sterilization purposes at the end.

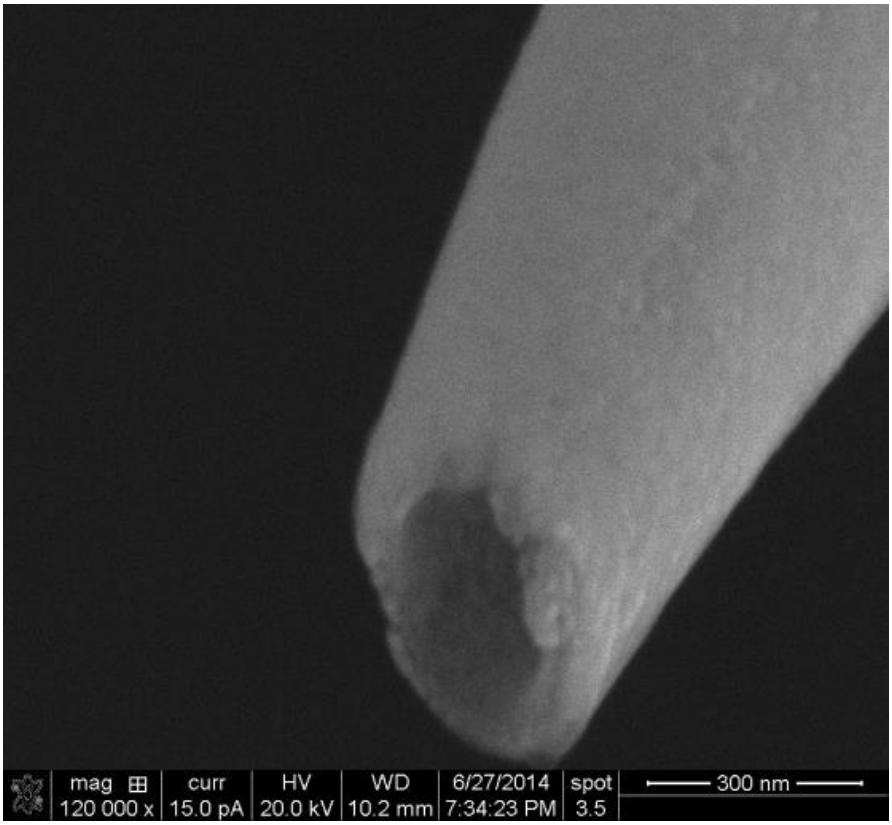

Figure 2.8. Fabricated nanoprobes. Ir/Pt coating and treatment of nanoinjectors with oxygen plasma

2.2.3. Characterization of Nanoprobes

2.2.3.1. Experimental Results

After fabrication of nanoinjectors, they were tested to ensure liquid can be picked up and ejected. Upon applying 2.5v positive voltage with respect to the outer electrode, liquid was picked up and with 0.1v, PBS was ejected (Figure 2.9).

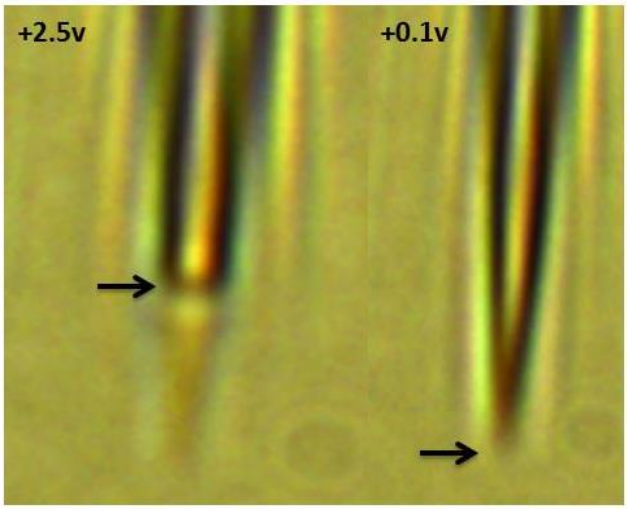

Figure 2.9. Pick-up and ejection of PBS with different voltages. left panel. With higher voltages, liquid is picked up in PBS solution. Right panel. With lower voltages, PBS is ejected. Arrows indicate the height of liquid.

To quantify volumes of pick up, different voltages were applied and the height was measured using a known distance as a reference with Image J software (Figure 2.10). Radius corresponding to the height of liquid was calculated based on the angle of cone. Our cone angle from SEM images was calculated to be 4.3 degrees and average radius was 70.2 nm.

$V = \frac{1}{3} h\pi r^2$

Radius corresponding to height of liquid = radius of cone from SEM + h$tan\theta$, where $\theta = 4.3°$

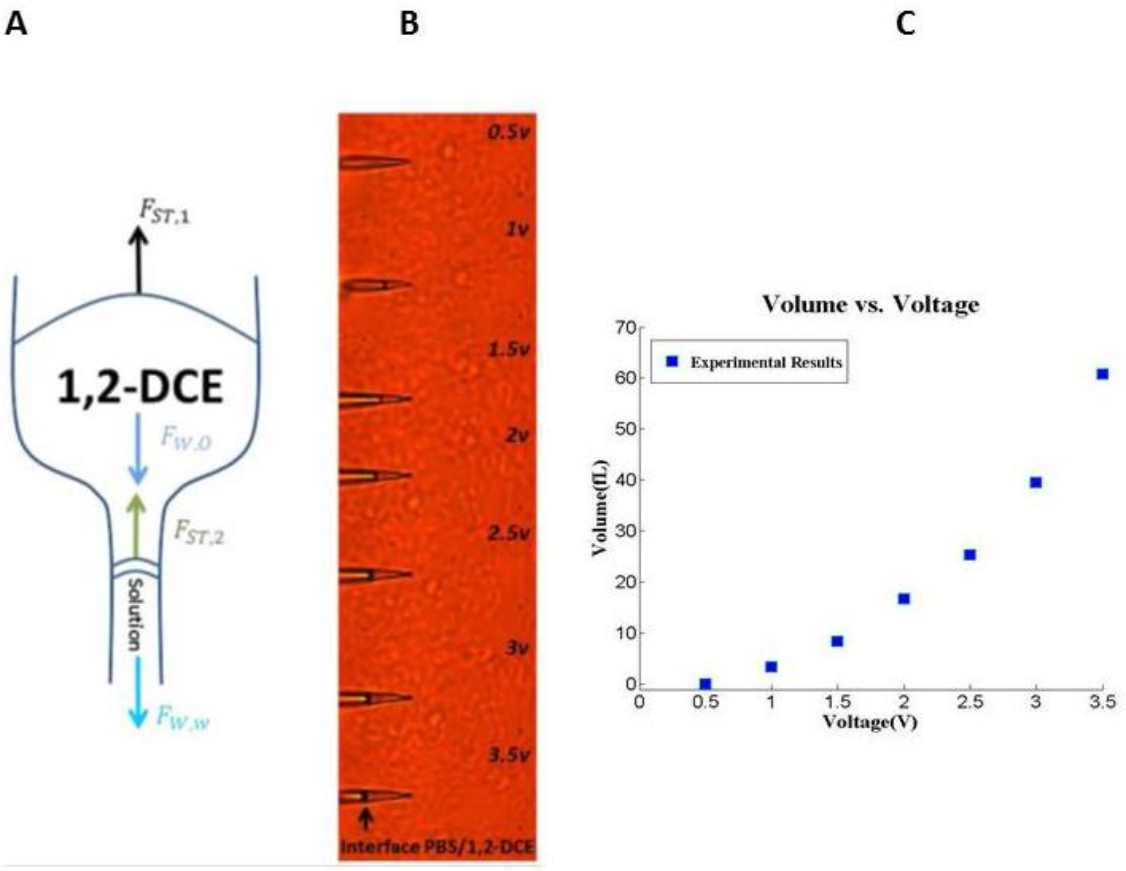

Figure 2.10. Study of Injected Volume versus Applied Voltage. A. Schematic of governing forces in an INENI. B. Image of PBS/1,2-DCE meniscus for different applied voltages. C. Experimental data of ingress volume.

2.2.3.2. Proposed Theory of the Technique

The proposed theory of this technique is as follows. An equation to balance the forces acting on the liquid column inside INENIs is given below:

$$F_{ST,1} + F_{ST,2} = F_{W,O} + F_{W,w} \quad (1)$$

where,

$F_{ST,1} = 2\pi r\, \gamma_{L,V}$ Cosφ, force due to surface tension between liquid- vapor in a capillary[49]

$F_{ST,2}$=2πr $\gamma_{L,L}$ Cosθ, force due to surface tension between liquid- liquid in a capillary[49]

$F_{W,O}$= ρ_Ogv_O , weight of organic phase

$F_{W,w}$= ρ_wgv_w, weight of solution

Based on Lippmann's equation [50],

Cosθ= Cosθ$_0$+ $\frac{1}{2\gamma_{L,L}} C(V - V_0)^2$ (2)

And,

$\gamma_{L,V}$ is the surface tension between 1,2-DCE/vapor(38.7mN/m),

$\gamma_{L,L}$ is the Surface tension between water/1,2-DCE(28.2mN/m)[51],

φ $_0$ is the wetting angle between organic phase and glass(135.9°C)[52],

θ is the variable angle with applied voltage between water and glass,

θ$_0$ is the angle between water and glass without applied voltage(10°C),

v_O is the volume of organic phase(49fL),

$v_w$is volume of water,

ρ_O is density of organic phase(1.25$\frac{g}{cm^3}$)[53],

$\rho_w$is density of water(1$\frac{g}{cm^3}$),

r is the radius of INENI cylinder ,

V_0 is potential at the point of zero charge(0.37v)[51],

V is the applied voltage, C is the capacitor of double layer per unit area.[54] Assuming constant capacitor, the capacitance of double layer at the point of zero charge is $\frac{\mathrm{k1}\,\varepsilon1\varepsilon2\mathrm{k2}}{4\pi(\mathrm{k1}\varepsilon1+\varepsilon2\mathrm{k2})}$, where

k_1^{-1}(0.7nm)[55] and k_2^{-1}(calculated to be 30nm) are the Debye length of the two immiscible solutions, and ε_1 and ε_2 are the permittivities of water ($7.1*10^{-10}$) and 1,2-DCE($8.9*10^{-11}$) respectively. [55],[56]

Combining equation 1 and 2, we get,

$$v_w = \frac{F_{ST,1} - F_{W,O} + 2\pi r\, \gamma_{L,L}(\mathrm{Cos}\theta_0 + \frac{1}{2\gamma_{L,L}} C(V - V_0)^2)}{\rho_w g} \qquad (3)$$

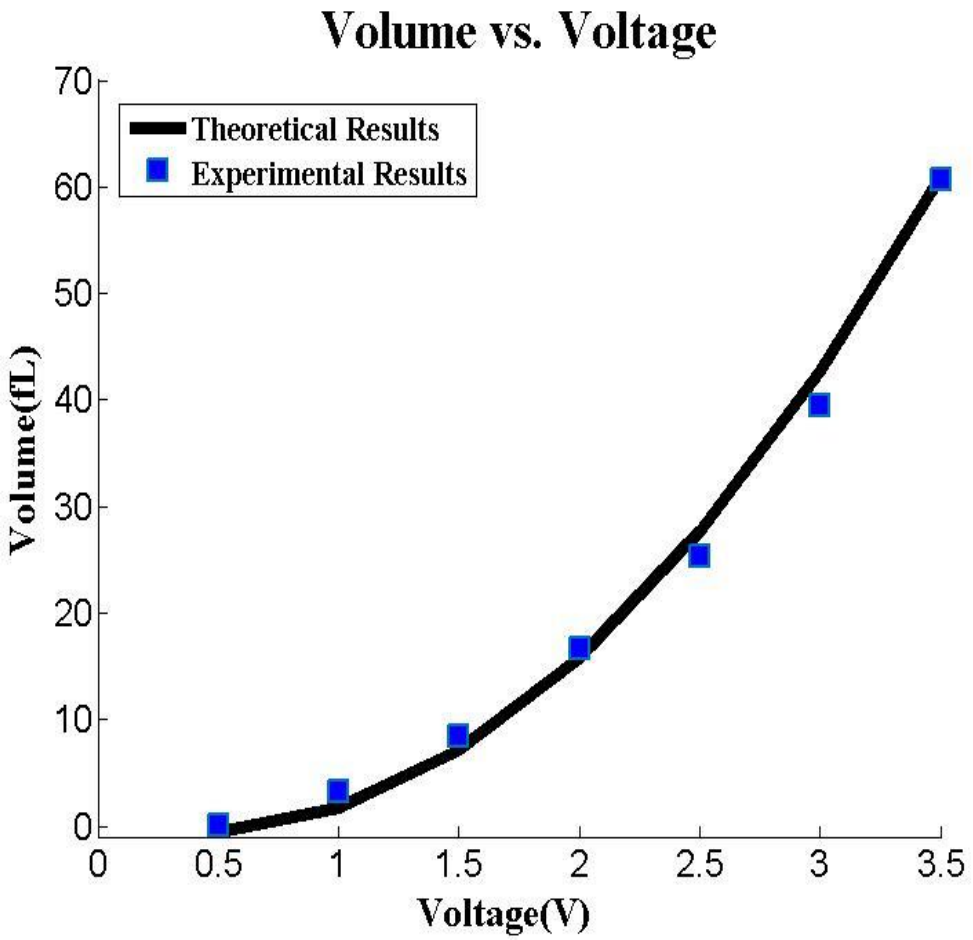

Figure2.11. Graph of theory and experimental results. The proposed theory matches with experimental results.

The theory (solid line in Fig. 2.11) matches closely with the experimental data (markers in Fig. 2.11) where voltages exceeding 0.5 V result in increased solution uptake in the

INENI and voltages less than 0.5 V result in solution being expelled from the INENI. One advantages of the INENI is the ability to accurately control the dosage delivery via applied voltages.

2.3. Single Cell Injection

Since the technique was working as expected, the next aim was to determine whether injection into cells is possible. Early on however with manual manipulators, cells were not surviving the process of manipulation. Often, the pipettes were breaking by hitting the bottom of petri dishes because there was no fine movement.

2.3.1. Set up for Injection

X, Y, and Z stages were purchased from Thorlabs. The three stages were connected to one another by 3D printing a 90 degrees bracket. The whole set-up was attached to the microscope with a 3D printing piece. In order to have a successful injection, the angle of injection should be between 30-45°; this piece was also 3D printed. The last step was adding a fine adjustment which was done by attaching a piezo actuator to the angle 3D printed piece (Figure 2.12).

Figure 2.12. Platform Set up. The x, y, and z translational stages were attached to the microscope with a 3D printed piece and piezo was attached to the translational stages for fine movements.

To have estimation as to how far integrate nanoprobe moves, the piezo movement was calibrated by placing a TEM grid and applying different voltage to the piezo (Table 2.2).

Table 2.2. Calibration of Piezo movement

Voltage Applied (v)	***Distance traveled by Pipette***
2	*2µm*
1.5	*1.5 µm*
1v	*1µm*
0.5	*0.5µm*

From the calibration of piezo, applying 0.3v to piezo was sufficient enough for entry into cells because the membrane of cell is around 7.5-10nm thick.

By setting up current measurement using labview, I was able to deteremine when entry into cytoplasm has happened (Figure 2.13). Besides current measurement, upon entry into cells, a white spot will appear on the image under the phase contrast microscope.

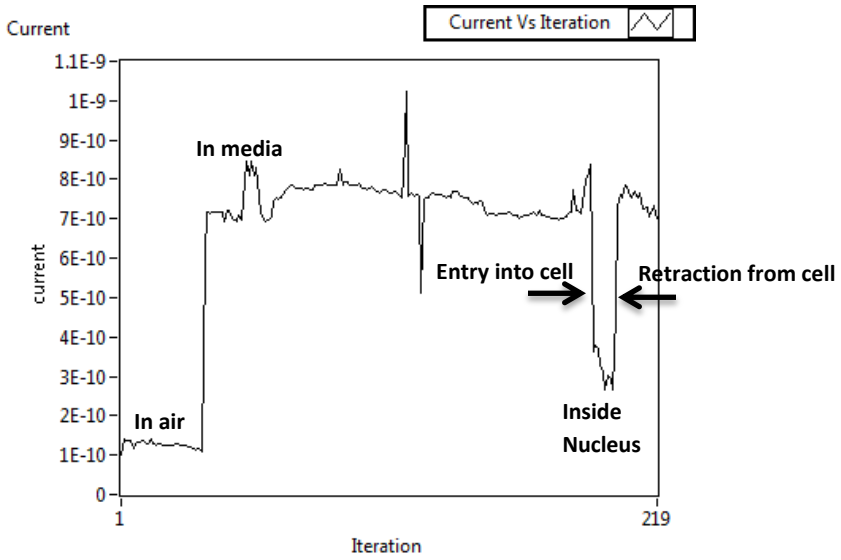

Figure 2.13. Current measurement for entry into cells.

2.3.2. Injection of Dye into cytoplasm and nucleus

2.3.2.1. Methods

Injection Set up. The injection set-up comprised X, Y, and Z translational micrometer stages for coarse movement (Newport, M-433 for Z movement and M-TSX-1D for X and Y movements) and a nanocube piezo actuator (Physik Instrument, P-280) for finer axial motions of INENI. These stages were placed on an IX71 Olympus microscope via adaptors that were 3D printed in our lab. For current measurement and voltage application a 2116 Keithly Sourcemeter was employed. For pulsing purposes, an Agilent

function generator was used (Model number 33220A), and fluorescent image acquisition employed a SBIG camera (Model number ST-7xMEI). The system was operated using custom coded software written in Labview (National Instruments).

Volume of liquid entry was measured via the height of solution using ImageJ software and a transmission electron microscopy (TEM) reference grid.

Dye Preparation. Tetramethylrhodamine isothiocyanate–dextran was purchased from Sigma Aldrich. The final concentration of the dye injected was 8–10 mg/mL.

DNA tagged fluorescein was purchased from IDT and was diluted to 10 μM in Schneider's drosophila medium (Thermofisher scientific). After the injection, DNA tagged FAM was removed and the cells were washed 3 times with PBS and were imaged with the fluorescent camera.

2.3.2.2. Results

The nanoinjectors were used to inject 50-70fL of 10mg/ml Tetramethylrhodamine isothiocyanate–Dextran (average molecular weight 65,000-85,000Da) into cytoplasm as well as nucleus of mouse NIH 3t3 cells (Figure 2.14). The large molecular weight of dextran allowed dye molecules to stay localized and not escape the membrane of cell or nucleus.

Figure 2.14. Injection into cells. Left fig. liquid was picked up, inserted into cell. Middle fig. liquid injected inside cell. Right fig. nanoinjector outside of cell.

After injection, cells were imaged under Fluorescent microscope and they were observed to fluoresce under TRITC filter (Figure 2.15). As can be seen in figure 2.15, we have the ability to spatially control where the injection happens whether in nucleus or cytoplasm of a cell.

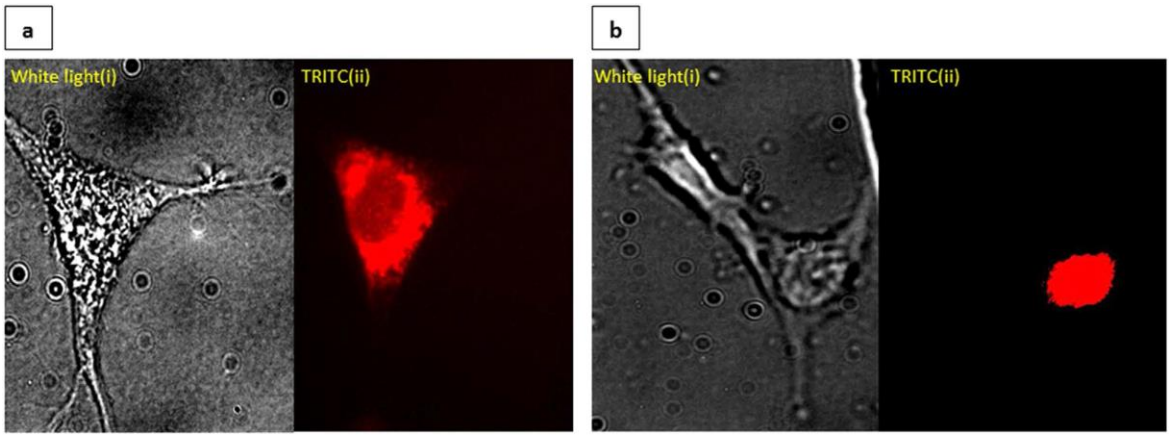

Figure 2.15. Injection of TRITC into cytoplasm and nucleus (a). Upper Left Panel. Injection of tetramethylrhodamine isothiocyanate–dextran into cytoplasm. (i) Left. Optical image of cell. (ii) Right.Fluorescent image of the cell. (b) Upper Right Panel. Injection of tetramethylrhodamine isothiocyanate–dextran into nucleus. (i)Left. Optical image of cell. (ii)Right. Fluorescent image of the same cell.

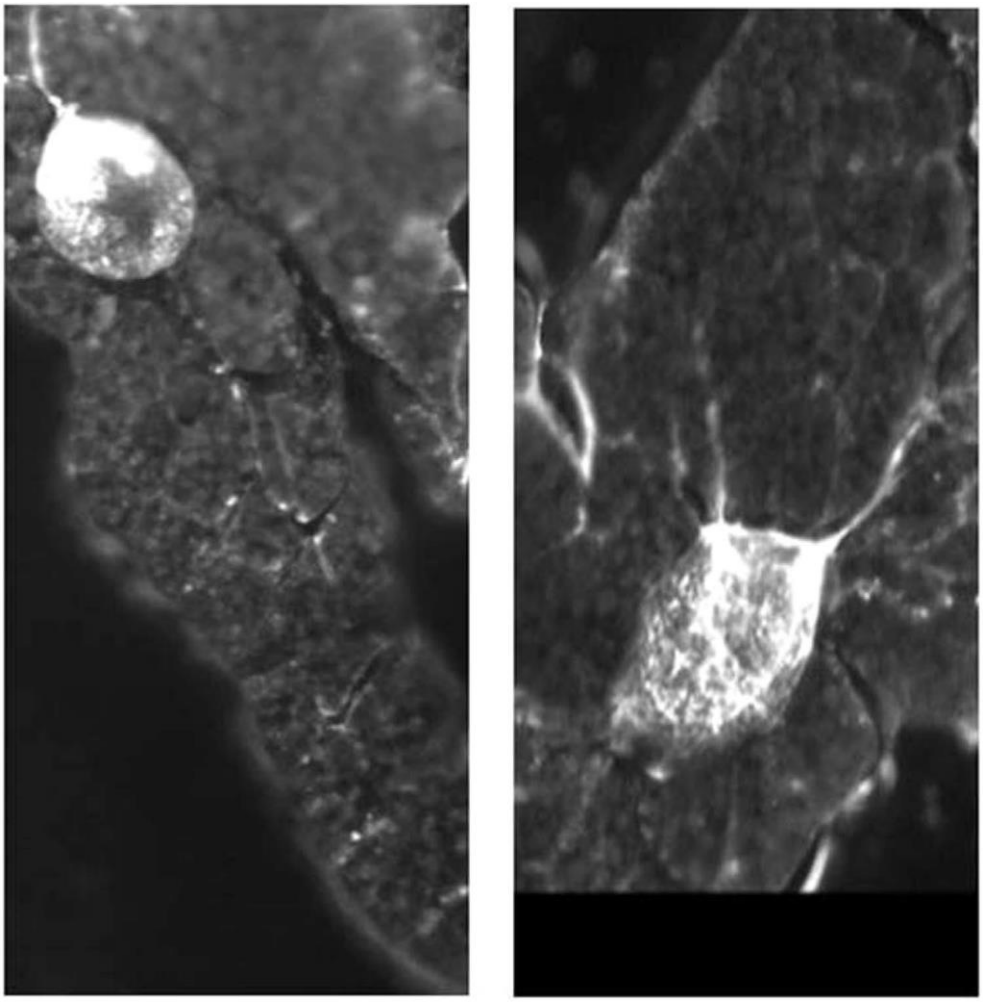

Figure 2.16. Application of INENI in tissues. Injection of DNA tagged FAM into two representative fat body cells.

Cell viability experiments were not performed after injection of this dye. The injection of the dye into nucleus or cytoplasm was solely performed to prove that this technique can be used to inject and localize molecules in different compartments of a cell. It is not known whether the dextran conjugated dye at the concentration used had long term toxic effects on cells.

Additionally, the technique was used to inject 10 μ M DNA tagged Fluorescein amidite (FAM) into several fat body cells, cell number 1 and cell number 2 are shown in Fig.

2.16. Since DNA is negatively charged, with no injection,DNA tagged FAM did not get into cells.

2.4. Discussion

We have established a new technique for single cell studies. The fabrication of the probes is done by coating the glass nanopipettes on one side using iridium and platinum metals. These small size (around 150nm in diameter) nanopipettes are utilized to both inject into single cells and extract from individual cells. In this chapter, we demonstrated the capability of the technique by injecting different dyes into the nucleus or cytoplasm of cells. The next step for this study was to determine cell viability using this technique.

Chapter 3

Viability of Cells with Integrated Electrowetting Nanoinjector/Aspirators

3.1. Cell Viability of cells with Injection of biomolecules

In order to study gene expression, injected cells had to be viable for up to two days. For this reason, many experiments were performed to make sure that the cells are viable after probing.

3.1.1. Cell Viability without Probing Cells

3.1.1.1. Methods

Cell Culture. NIH3T3 mouse Fibroblasts were purchased from the ATCC and cultured with high glucose DMEM media supplemented with 10% fetal bovine serum (ThermoFisher Scientific). All cells were cultured in 5% CO2 at 37 °C and washed with 1x phosphate buffered saline (PBS). Polydimethylsiloxane (PDMS) were made
by 10:1 w/w base prepolymer, curing agent formulation. PDMS was cut into small pieces and square holes of 1 mm by 1 mm by 1 mm were created in each PDMS chamber. PDMS chambers were cleaned with 70% ethanol, dried and were placed on top of sterile Petri dishes (Sigma Aldrich). Gridded Petri dish (polymer coated, Ibidi, Germany) was used for tracking cells. Cells at 60–70% confluency were used in these set of experiments.

Wild type Fruit flies were purchased from Bloomington Stock Center and were dissected to isolate fat body cells. Adipocyte tissues were attached to coverslips that were coated overnight with Poly-L-lysine (Sigma-Aldrich).Cells were used immediately after dissection.

Media condition. Three media conditions were tested to comprehend which environment cells like best during the one hour period in which they reside outside the incubator. The

first media was DMEM and 10mM HEPES. HEPES is a buffer which keeps the PH the same in physiological condition. The second media was DMEM and the third condition was complete media.

3.1.1.2. Results

Cells had great viability rates in all the three media conditions. The viability rate inside 10mM HEPES, DMEM, and complete media was 99%, 100%, and 98% respectively (Table 3.1).

Table 3.1. Cell viability of NIH 3t3 cells in different media conditions

Media	***Number of cells, Time = 0***	***Number of cells, Time = 30min***	***Number of cells, Time = 1 hour***
10mM HEPES inside incubator	***101***	***97, 96%***	***97, 96%***
10mM HEPES outside incubator	***78***	***72, 92%***	***72, 92%***
DMEM inside incubator	***60***	***60, 100%***	***59, 98%***
DMEM outside incubator	***50***	***50,100%***	***50, 100%***
FBS+DMEM inside incubator	***57***	***57, 100%***	***53, 93%***
FBS+DMEM outside incubator	***55***	***55, 100%***	***54, 98%***

Based on this table, DMEM was the best choice for cell viability. This media was used for later experiments of cell viability.

Cells were monitored 1 and 2 days after being outside of the incubator for an hour. The results indicated that cells were replicating even after one and two days (Table 3.2).

Table 3.2. Cell Viability in DMEM 2 days after being outside of incubator for one hour

Time	***0***	***30min***	***1hr***	***1 day***	***2 days***
# of cells, repeat 1	***23***	***23***	***22***	***36***	***>64***

3.1.2. Cell Viability Experiments with Probing

3.1.2.1. Methods

In the next set of experiments, cell viability experiments were performed with glass and Ir/pt coated nanoinjectors to figure out whether probing or Ir/pt coating causes dramatic cell death.

3.1.2.2. Results

With glass nanopipettes as well as Ir/pt coated nanoinjectors, cells survived for 2 days and they also replicated (Table 3.3 and 3.4).

Table 3.3. Cell viability after probing cells with glass nanopipettes

	Before Probing	***After Probing***	***Day 1***	***Day 2***
Number of cells- repeat 1	***12***	***11***	***18***	***>28***
Number of cells- repeat 2	***15***	***15***	***24***	***>40***

Table 3.4. Cell Viabiltiy of Ir/pt coated nanoinjectors

	Before Probing	***After Probing***	***Day 1***	***Day 2***
Number of cells- repeat 1	***12***	***12***	***22***	***43***
Number of cells- repeat 2	***10***	***10***	***20***	***>32***

3.2. Cell viability of Cells after Sampling Cytoplasmic and Nucleus Content

In the next set of experiments, we sampled around 20-50fL of nucleus and cytoplasm of HeLa cells and tested their viability with trypan blue dye. HeLa cells were cultured on top of gridded petri dish to easily locate the sampled cells. The cells were probed with the same nanoejector and they were outside of the incubator for no more than 30-45minutes.

As can be seen in figure 3.1, after sampling the nucleus of the cell between B&C, the cell is not visualized as blue and therefore it is alive. Also in figure 3.2, the cytoplasm of indicated cell (the cell next to number 4) was extracted and based on trypan blue results, it is alive after sampling.

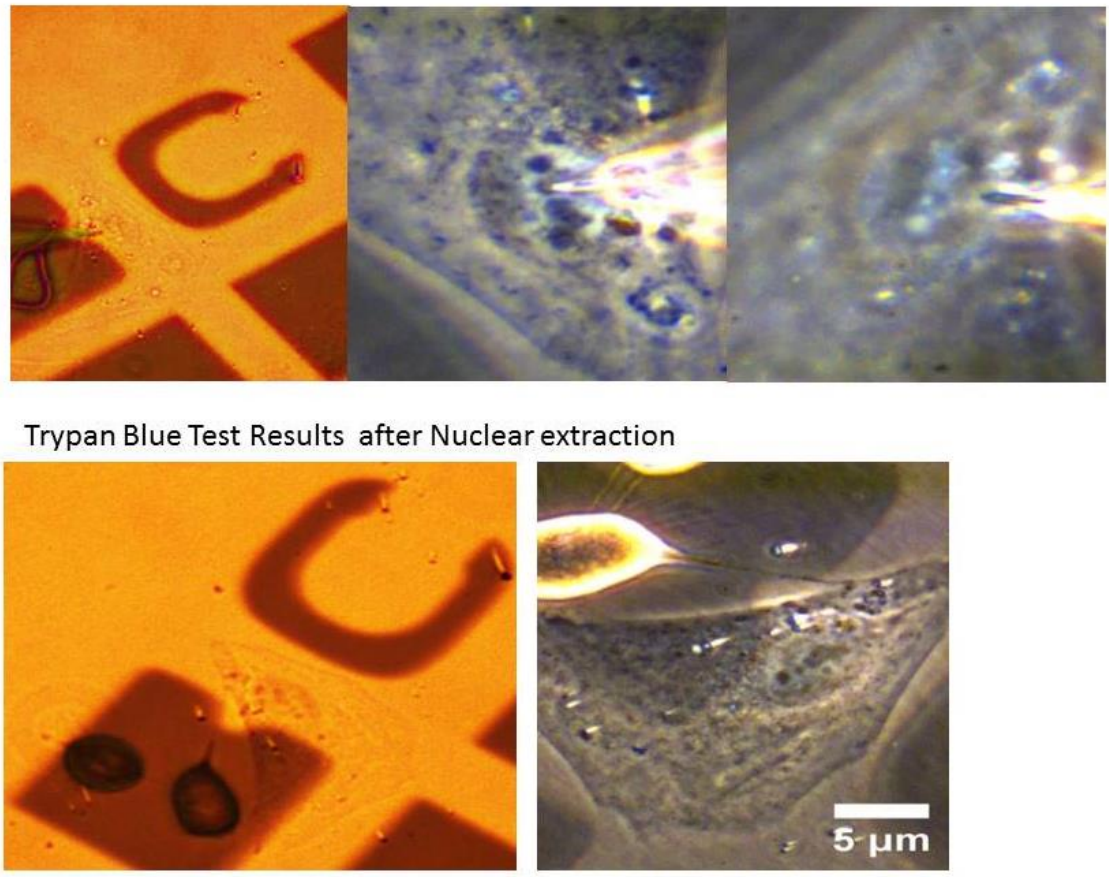

Figure 3.1. Cell viability after nuclear extraction. Upper panel shows in which cell the extraction took place. Lower panel shows the trypan blue experiment where the probed cell is alive after extraction. The left lower panel. Cell is under 10 x magnifications. Lower right panel. The same cell under 40x magnification.

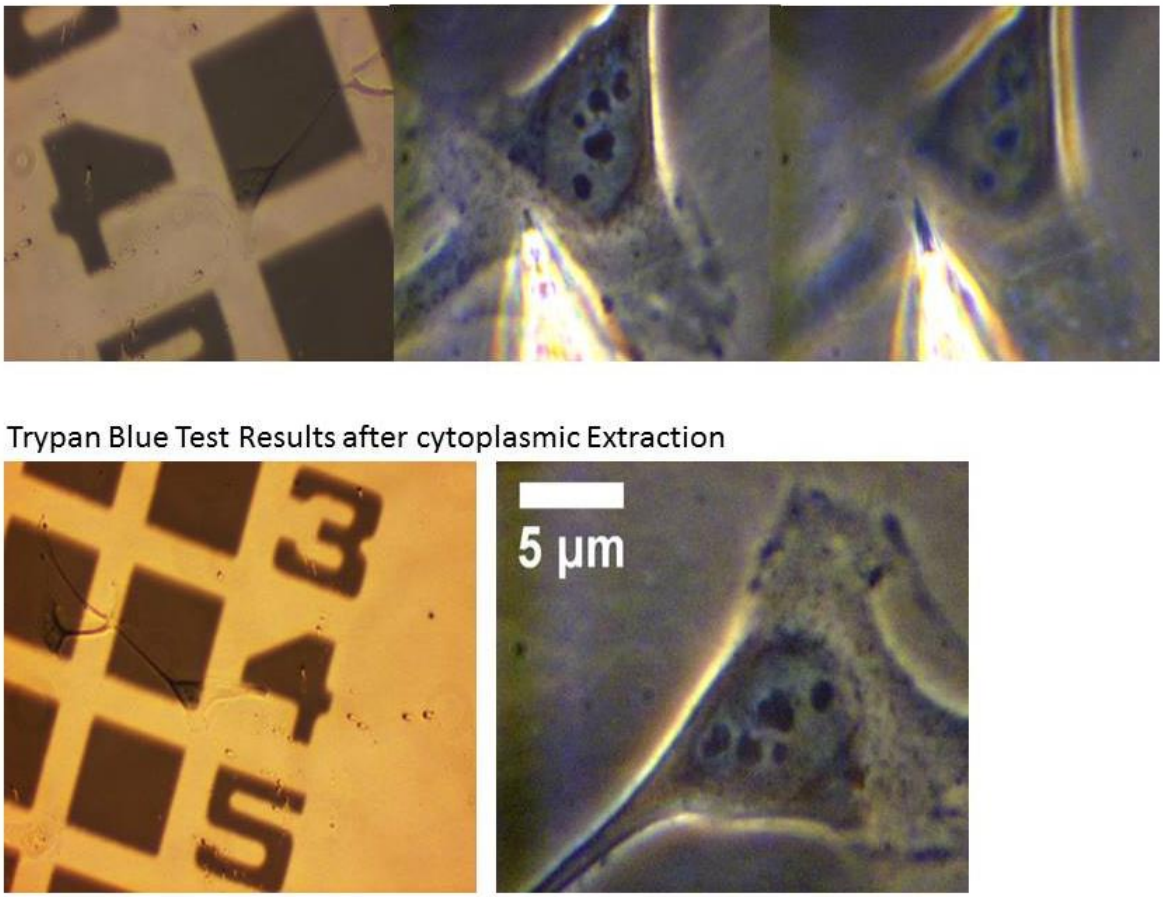

Figure 3.2. Cell viability after cytoplasmic extraction. Upper panel shows in which cell the extraction took place. Lower panel shows the trypan blue experiment where the probed cell is alive after extraction. The left lower panel. Cell is under 10 x magnifications. Lower right panel. The same cell under 40x magnification.

The cell viability percentage after nucleus extraction was determined to be 85% and the cytoplasmic extraction was up to 100%.

3.3. Discussion

The cell viability experiments were detrimental to perform in order to assess the possible applications of the technique. Cell viability for injections of biomolecules into single cells was up to 100%. Without cells being viable, gene expression studies could

not be applicable because after injection of plasmid DNA, the injected cells can take up to two days to express the corresponding proteins. Moreover, the technique becomes more valuable because many various applications can be performed. The cell viability experiments were also completed for small volume extraction of cytoplasm and nucleus. The nucleus extraction viability was up to 85% and the cytoplasmic extraction viability was up to 100%. This result makes sense as many crucial DNAs and also nucleoli are located inside the nucleus of a cell.

Chapter 4

Application: Plasmid injection

One of the applications that came into mind using this technique was changing the genetic expression of cells by injecting plasmid DNAs. In this chapter, the focus is on injection of plasmids into single cells.

4.1. Validation of plasmids with electroporation at the bulk cell level

4.1.1. Methods

Since cells survived after probing with the nanoinjectors, the next experiment was injecting plasmid molecules inside cells. First, plasmid which was bought from Lonza was validated with electroporation technique.

4.1.2. Results

In the presence of plasmid, GFP was expressed and could be observed 17 hours after the electroporation experiment. With negative controls, where there was no plasmid added, to test cell viability after electroporation, no GFP was seen. Also, with another negative control where the experiment was performed in the absence of electroporation program to test the toxicity of plasmid DNA to cells, GFP was not expressed (Figure 4.1).

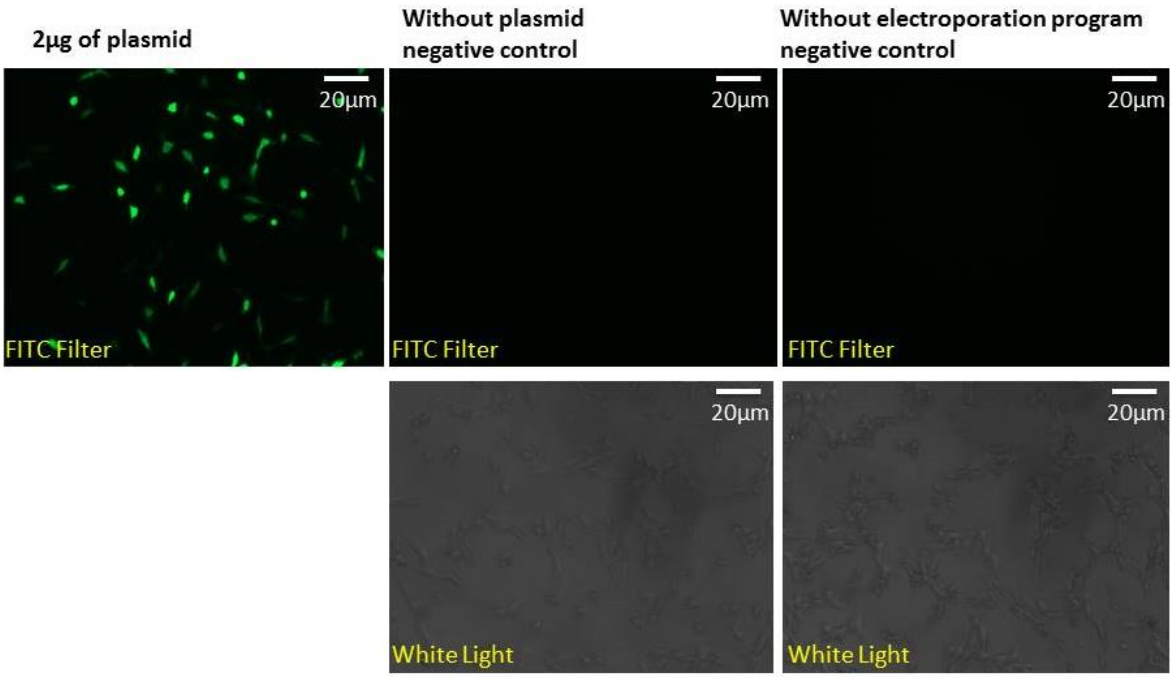

Figure 4.1. Left fig. 2ug of plasmid with electroporation. Middle fig. negative control, no plasmid added. Right fig. negative control without electroporation program

4.2. Plasmid injection using INENI

Finally, 1,800 molecules of plasmid were injected with nanoinjectors inside 3T3 cells and they were imaged with fluorescent microscope one day after the experiment. The cells expressed GFPs one day after injection (Figure 4.2).

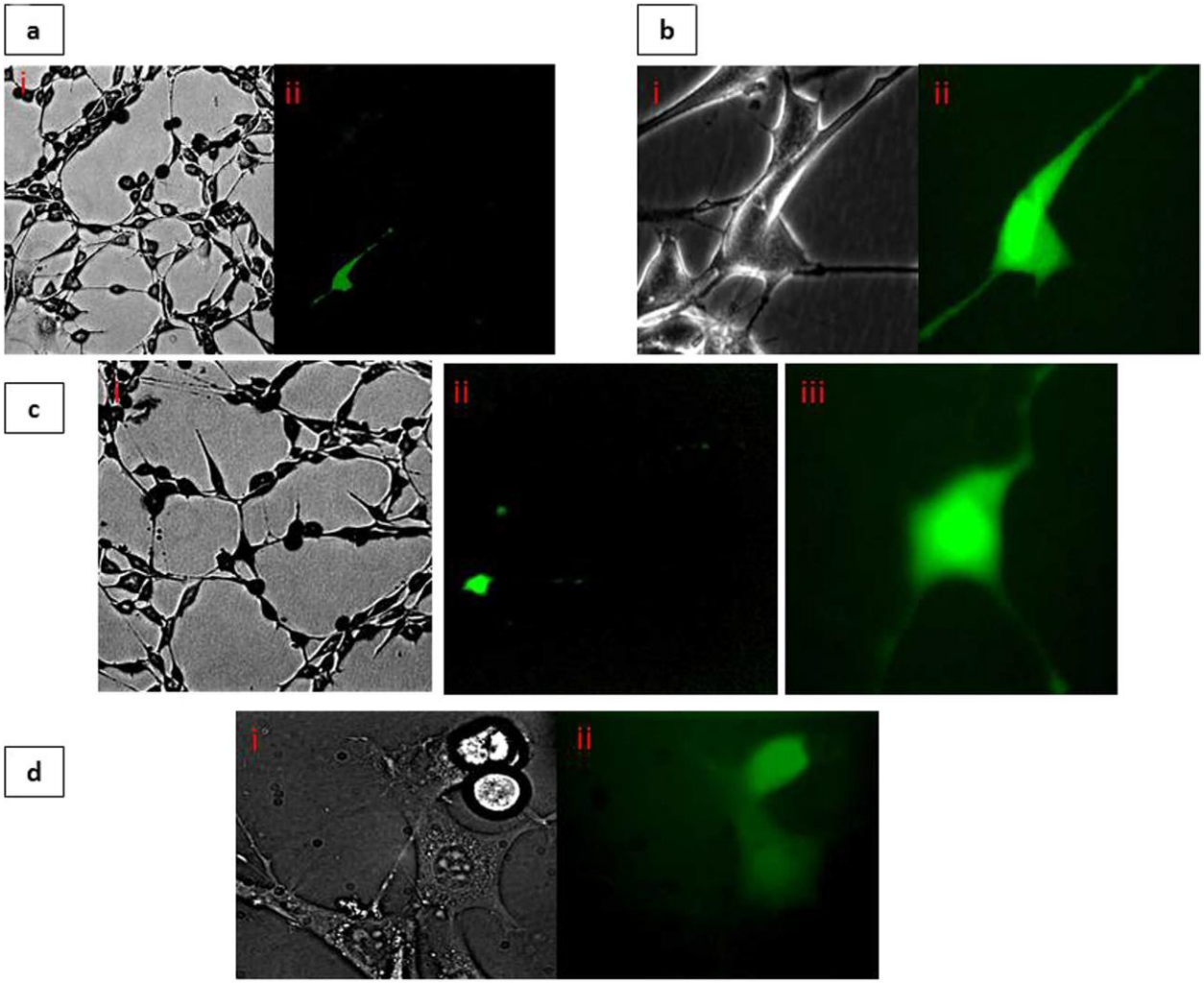

Figure 4.2. GFP expressing cells 48 hours after plasmid injection using INENIs. (a) (i) Bright field image of cell at lower magnification (10x). (ii) Cell fluorescence image using Fluorescein Isothiocyanate (FITC) filter. (b)(i) Bright field image of the same cell shown in A under 40x. (ii) Cell fluorescence image using FITC filter under 40x. (c) (i) Bright field image of cell at 10x. (ii) The same cell using FITC filter at 10x. (iii) Same cell using FITC filter 40x. (d) (i) bright field image of daughter cell expressing GFP 48 hours after transfection. (ii) The same cell using FITC filter.

Published works on microinjection indicate that 1,000 molecules of injected plasmid resulted in a faint fluorescent signal, whereas 100,000 molecules resulted in bright fluorescence.[57] Below 1,000 molecules, cells were rarely transfected using microinjection.[57] The same INENI could be used for injection of up to six cells. To

have a high throughput within the hour, collection and ejection of plasmid happened inside the chamber where cells resided. Plasmid was combined with DMEM solutions inside a small PDMS chamber and INENIs were not withdrawn from solution each time for pick up. PDMS chambers were designed to minimize the amount of plasmid needed for injection. To maintain cell viability, cells were kept outside of the incubator for no more than one hour.

4.3. Transfection Efficiency

The transfection efficiency of the technique was evaluated to be close to 100%. In a series of two experiments, twelve cells were injected with plasmid, and after 48 hours, 13 cells were fluorescing indicating GFP expression not only in the injected cells, but also in daughter cells (Fig. 4.3& Fig. 4.4& Table 4.1).

$$Efficiency = \frac{number\ of\ cells\ expressing\ GFP}{number\ of\ cells\ injected} * 100\%$$

Table 4.1. Transfection efficiency of two sets of experiments performed on NIH3t3 cells

	Number of Cells Injected	Number of Cells Expressing GFP	Transfection Efficiency
Experiment 1	12	13	100%
Experiment 2	8	8	100%

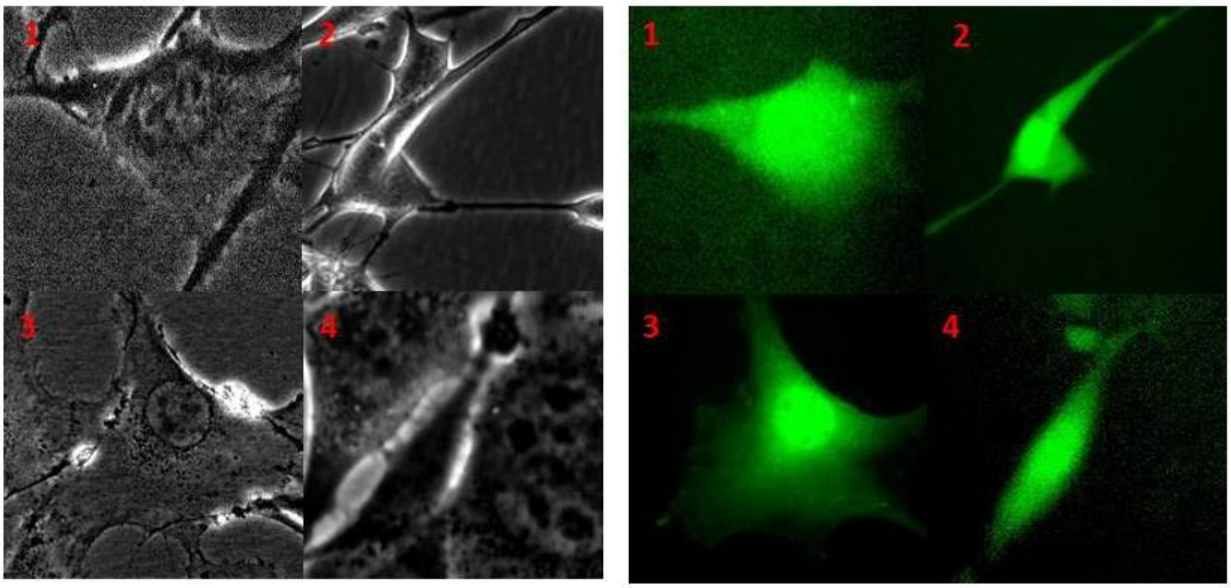

Figure 4.3.Transfected NIH 3t3 cells. Left panel. Bright field images of transfected NIH 3t3 cells. Right Panel. Fluorescent images of the same transfected cells.

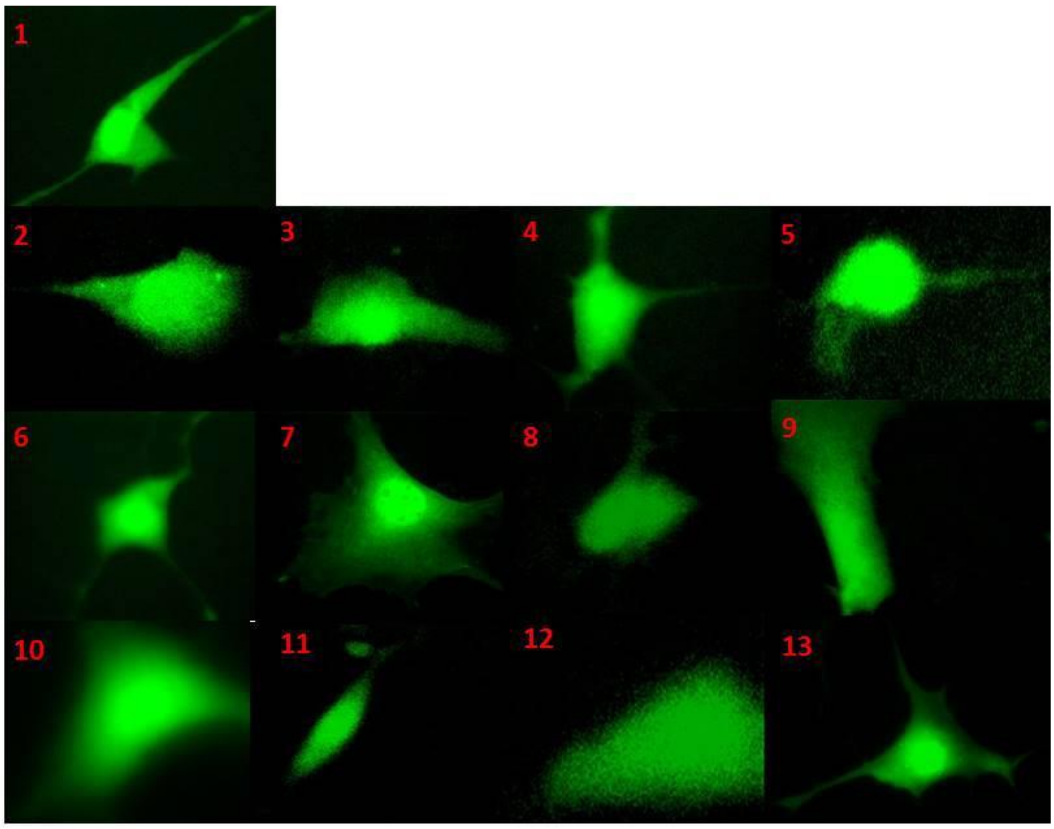

Figure 4.4.Fluorescent images of one out of two set of transfected cells that expressed green fluorescent proteins.

Within an hour, up to15 cells could be injected. A negative control was performed where cells residing in the chamber were exposed for one hour to the same concentration of plasmid without any injection. No GFP expression was observed 48 hours after the experiment. Higher throughput can be achieved by automating the system. By combining the INENI with vision system and registering the INENI and cells with respect to a reference coordinate in the image plane, the movement time of the injector from cell to cell can be reduced to one or two seconds. The transfection time from cell to

cell is primarily limited by fluid ejection time (10 seconds) rather than movement of INENI from cell to cell.

4.4. Discussion

In brief, using this technique, we have successfully transfected NIH 3t3 cells with 100% efficiency. The volume of delivery can be controlled via voltage application. With higher voltages, more liquid enters the INENI and with lower voltages liquid is expelled. This technique can be used to deliver plasmid DNA directly into the nuclei of cells. The INENI requires only the use of a single probe since both electrodes are integrated into the same nanopipette. Hence more space is available, and ergo the INENI offers a simplistic means for direct injection of metered amounts of exogenous material into the confines of a cell cytoplasm and/or nucleus while retaining full cell viability.

The number of plasmid molecules used in this study was 1,800 copies. Since this is well below the 100,000copies usually used, our technique requires nearly 50 times less plasmid for successful transfection.[57] We conjecture that the transfection efficiency of this technique reaches 100% due to the small diameter of the nanoinjector compared to microinjectors and direct plasmid insertion into the nucleus rather than to the cytoplasm with little disruption to the nuclear membrane.

Chapter 5

Application: Protein analysis

In order to figure out whether our technique could be applied for single cell protein analysis, we tried picking up and ejecting some commercial proteins. We then moved into in vivo studies by extracting GFP from NIH 3t3 cells. Later, the probes were utilized for extracting actin proteins from single cells. The amount of proteins quantified matched the amount of proteins reported in the literature. Cell viability was tested with trypan blue for cell viability after nuclear and/or cytoplasmic extraction. To further examine the potential of this technique, the P53 expression levels were examined after treatment of HeLa cells with actinomycin D drug.

5.1. In vitro studies using various proteins

The Integrated electrowetting nanopipettes were used to show that proteins can be picked up and ejected. The proteins that were tested were Bovine albumin serum (BSA), and streptavidin. As can be seen in Figure 5.1 and figure 5.2, both of these proteins could easily be picked up and ejected.

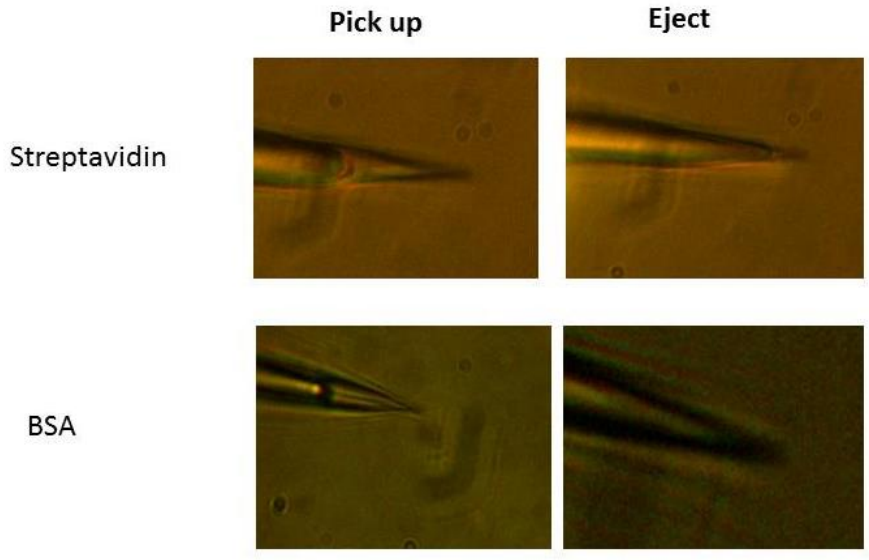

Figure 5.1. Capability of the technique for pick up and ejection of proteins

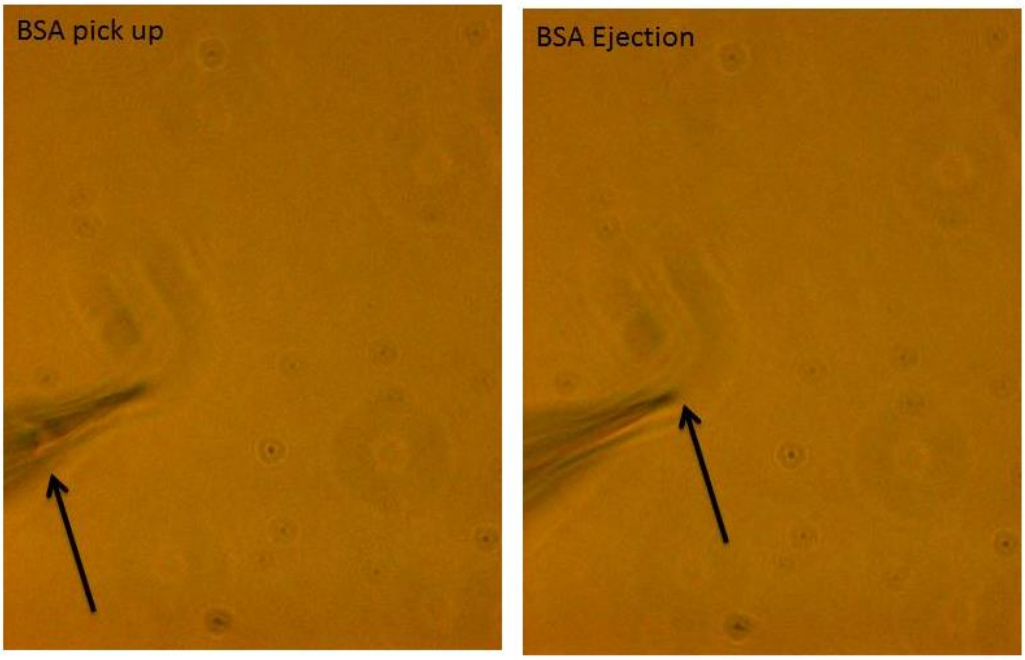

Figure 5.2.Pick up and Ejection of BSA protein using nanopipettes

BSA fluorescein conjugate were purchased from Thermofisher scientific and were used to show that proteins can be picked up and ejected in vitro on top of glass coverslips (Figure 5.3 & 5.4).

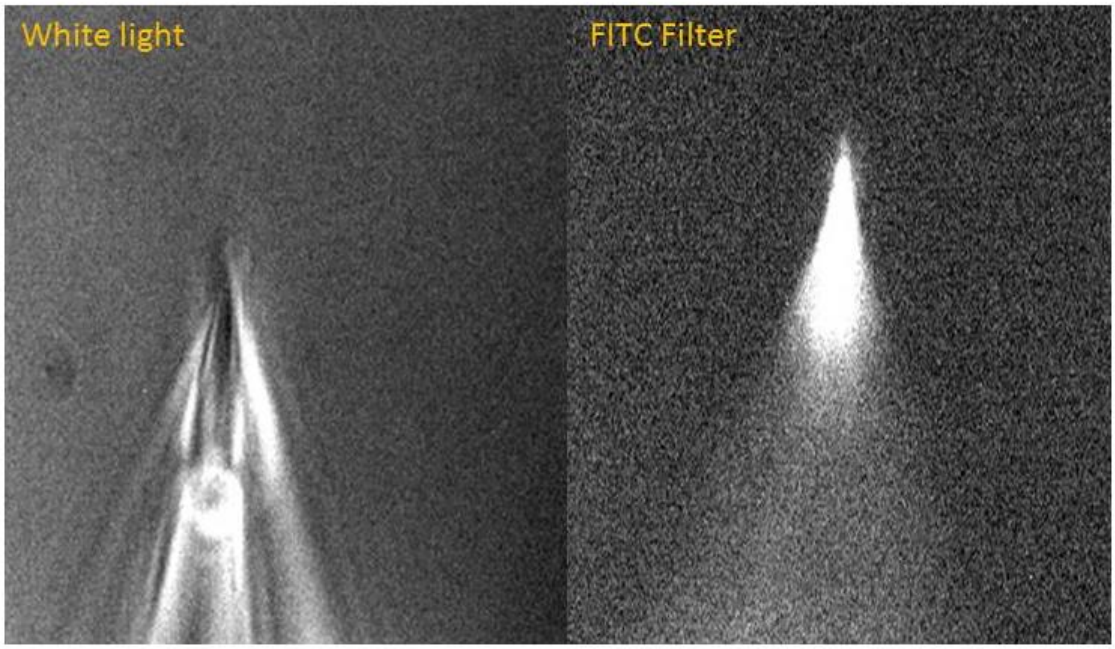

Figure 5.3. BSA tagged FITC picked up by the Integrated Electrowetting nanoprobes

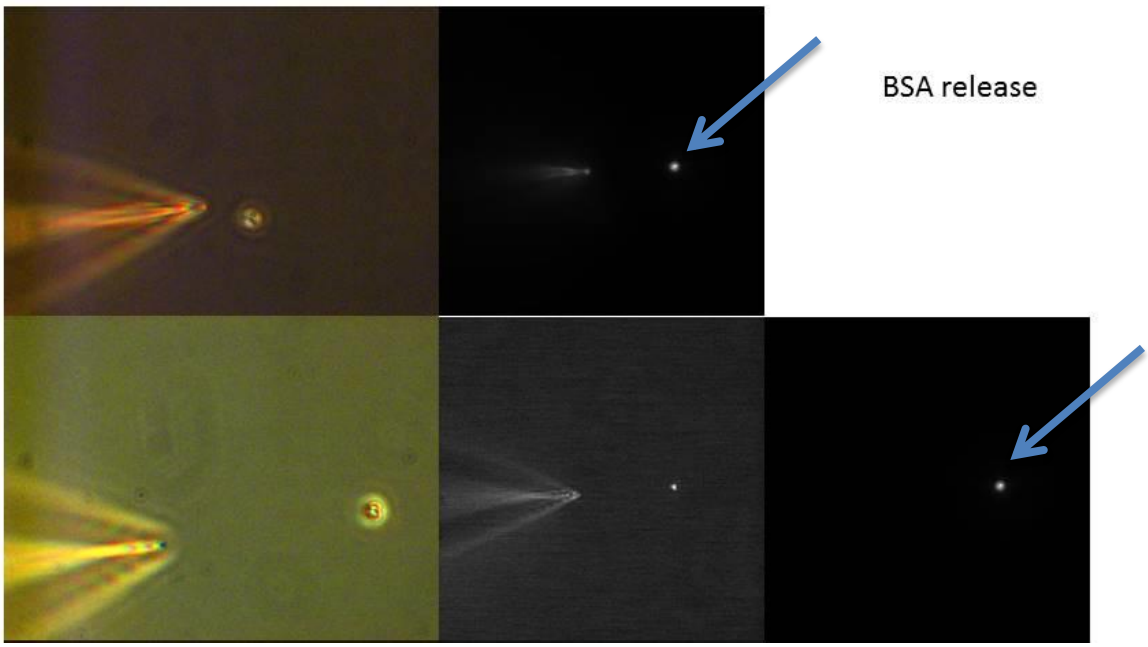

Figure 5.4.BSA tagged FITC released on top of coverslips. The figures show two different nanoprobes and their release on top of coverslips.

5.2. GFP Extraction and release from cells

5.2.1. GFP Extraction

NIH 3t3 cells were electroporated to uptake plasmid GFP. After 24 hours, cells were observed to express GFP. The cultured cells were used to sample GFPs at the single cell level. As can be seen in figure 5.5, extraction of green fluorescent proteins using our technique was possible.

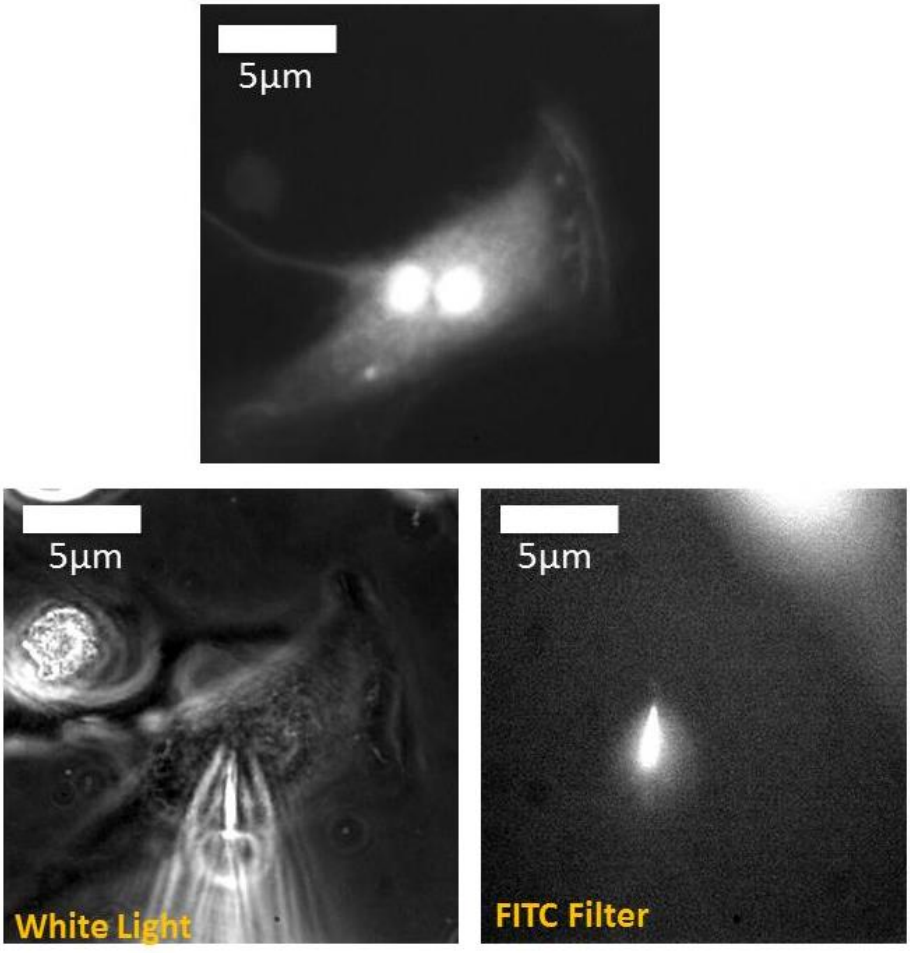

Figure 5.5. GFP pick up after plasmid electroporation. Upper Panel. Graph of GFP expressing cell. Lower left panel. The white light image of the nanoprobe that went inside a NIH 3t3 cell to pick up GFP. The lower right panel. The same image only under FITC filter

5.2.2. GFP Release

Once GFP was extracted, the nanoejector was removed from the cell culture dish and extracted on top of an uncoated coverslip and imaged under FITC filter. (Figure 5.6)

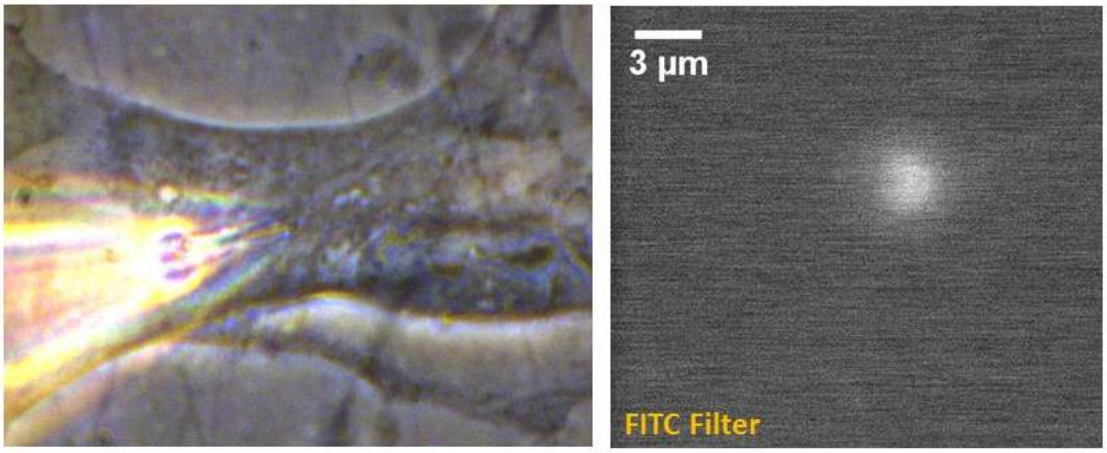

Figure 5.6. GFP extraction using nanoprobe(left panel) and ejection onto a coverslip under FITC filter (right panel)

5.3. Actin quantification at the single cell level

Actin protein is one of the most abundant housekeeping proteins in cells.[58] Actin and myosin form the basis of muscle contraction. To further prove the potential of the technique, we decided to extract proteins from the cytoplasm of the cell and stain for actin specifically and quantify the number of actin proteins using our technique.

5.3.1. Methods

5.3.1.1. Cell culture.

Cells were cultured with 90%DMEM and 10%FBS. They were incubated at 37°C and 5%CO2. NIH3t3 cells were used at 70% confluency.

5.3.1.2. Extraction on top of coverslips

In order to locate where the proteins were released, grids were created on top of coverslips. TEM grids were purchased from SPI and were placed on top of coverslip. 20nm of Iridium was sputter coated on top of it. The coverslips were coated with 2% (3-aminopropyl) triethoxysilane (APTES) in ethanol overnight. They were washed with ethanol and DI water after the coating and were used for protein extraction.

The experiments with the anti-actin antibody tagged FITC were performed by simply picking up and releasing the different volumes of antibody on top of coverslip and measuring the raw integrated density signal using Image J software. However, the standard curve for the actin antigen was performed by picking up various volumes of actin molecules and performing the staining procedure on top of APTES coated coverslips.

5.3.1.3. Staining

Primary antibody staining was implemented in this study. Anti-Actin antibody tagged with fluorescein (FITC) was purchased from abcam and Anti-P53 antibody tagged Allophycocyanin (APC) was purchased from R&D systems. After extraction of proteins from cytoplasm, they were released on top of APTES coated coverslips. 1%BSA in PBS was used for one hour for blocking the regions were no proteins were spotted on. The coverslip were then washed with 1x PBS three times. The proteins would then be incubated for one hour with their corresponding antibodies.

5.3.2. Anti Actin Antibody Standard Curve

The first set of experiments was designed to show that using the technique quantification of proteins is possible. The anti-actin antibodies tagged with FITC were picked up using the nanoejector and were released on top of coverslips. (Figure 5.7)

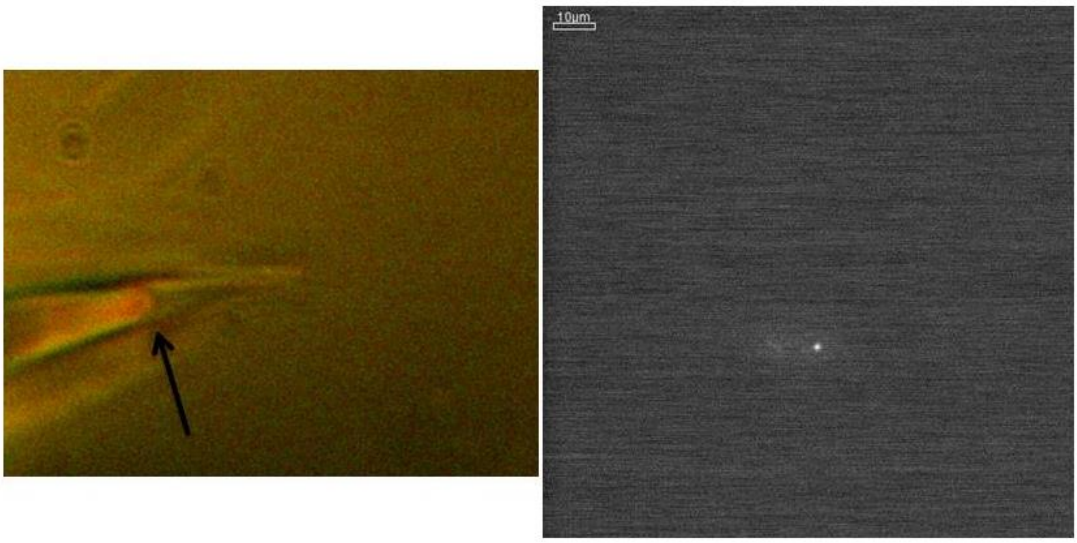

Figure 5.7. Anti actin antibody pick up and release on top of coverslip. Left panel shows the anti actin antibody solution inside the nanopipette and right panel is the fluorescent image of the solution released on top of coverslip

The volumes of each release was calculated and plotted versus fluorescent intensity of the signal. (Figure 5.8)

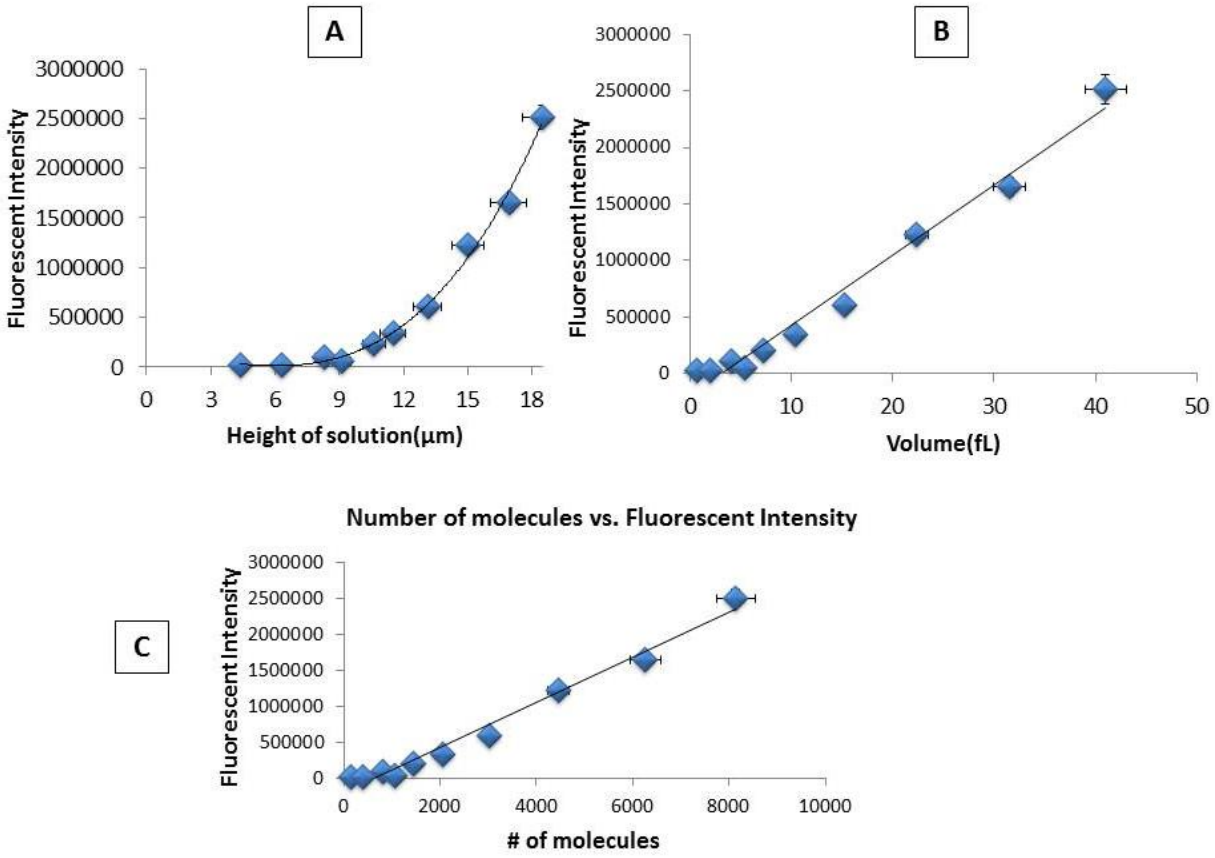

Figure 5.8. Standard curve for quantification of anti actin antibody . A. Height of Anti actin antibody solution in the nanopipette versus fluorescent intensity. B. Calculated volume based on the height of solution in the nanopipette vs. fluorescent Intensity calculated. C. number of molecules picked up versus fluorescent Intensity

5.3.3. Actin Antigen Standard Curve

The experiments with anti-actin antibodies were repeated with actin antigen to determine how many actin molecules exist in a single cell. (Figure 5.9)

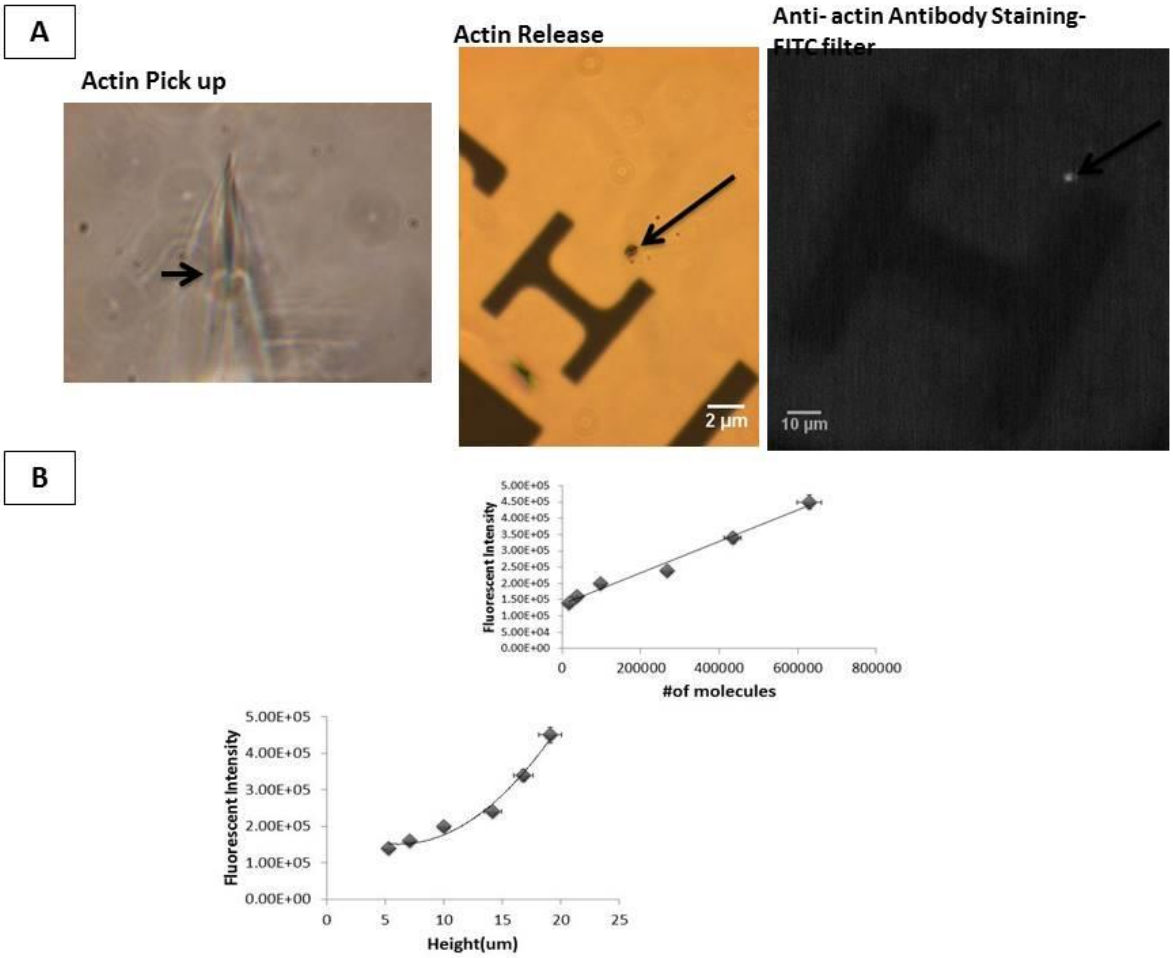

Figure 5.9. Actin antigen standard curve. A. left. Integrated Electrowetting Nanoprobe actin antigen pick up. Middle. Release of actin on an APTES coated grid. Right. Staining of actin antigen with anti actin antibody tagged FITC. B. The upper panel presents the number of molecules versus fluorescent Intensity. The lower panel presents the height of actin antign solution inside the nanopipette versus fluorescent Intensity of anti actin antibody bound to actin antigen.

5.3.4. Actin protein extraction from one cell

Around 50fL of cytoplasm of NIH 3t3 cells were extracted from each cell and were stained with anti actin antibody. The fluorescent intensity was then measured with image J and was correlated on the standard curve to quantify the number of actin proteins inside NIH 3t3 cells. (Figure 5.10)

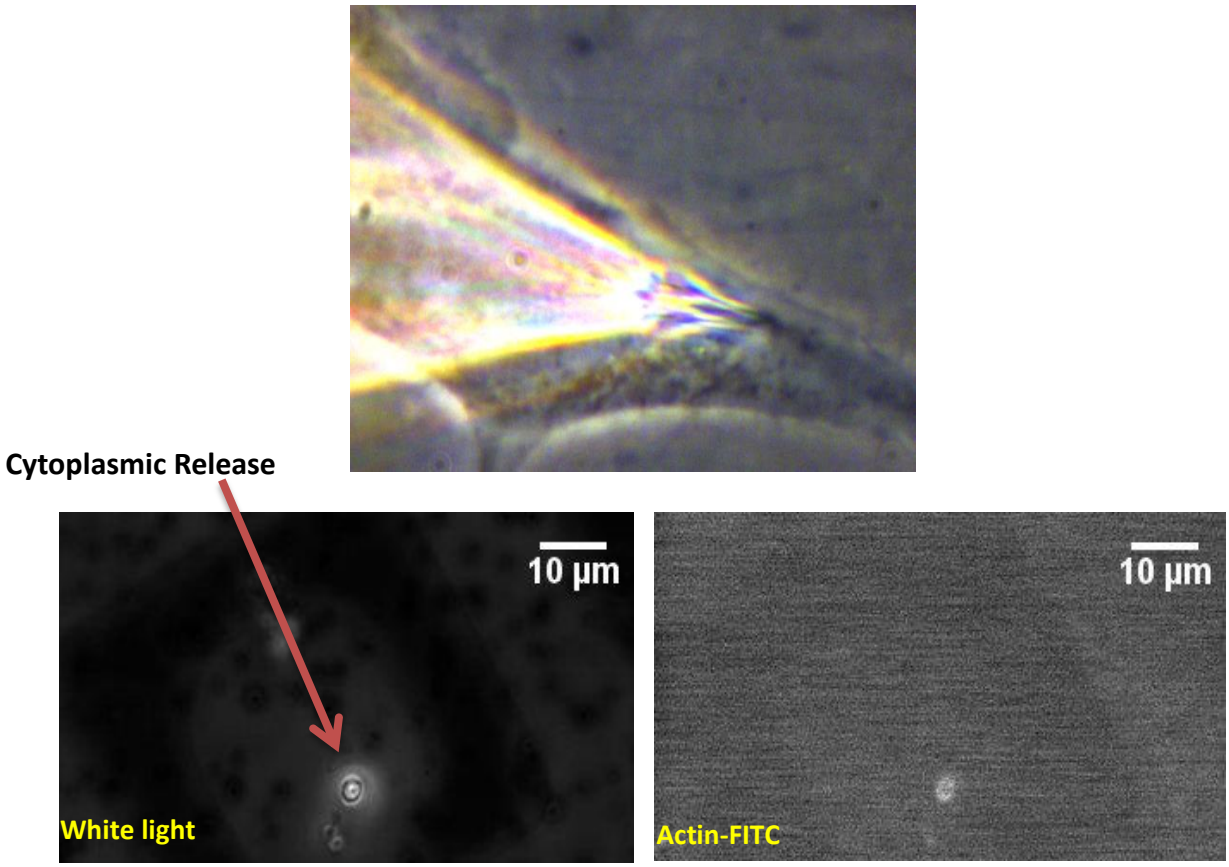

Figure 5.10. Extraction of cytoplasmic content and staining for actin with anti actin antibody Upper panel. Cytoplasmic extraction. Lower panel left. White light image of the cytoplasmic release. Lower right panel. The cytoplasmic extraction after actin staining under FITC filter

5.4. p53 Experiments with HeLa cells

p53 or T53 is a gene that codes for the protein that controls tumor suppression in cells. The p53 protein is mainly a transcription factor that regulates many biological factors such as apoptosis in response to stress.[59] When cells are unstressed, the p53 proteins are expressed at low levels. However, with the treatment of an anti-cancer drug such as actinomycin D used in this study, the p53 accumulates inside the nucleus. [60] The actinomycin D has been shown to inhibit transcription by interfering with RNA polymerase. [60] In this work, we intended to demonstrate the accumulation of p53 proteins inside nucleus after treatment with actinomycin D using our technique.

5.4.1. Methods

HeLa Cells were treated with 50nM of actinomycin D drug one day after their passage and they were probed 24 hours after treatment. The cells were also probed before treating the cells with actinomycin D.

5.4.2. Results

Untreated Hela cells were probed both in cytoplasm as well as nucleus and stained for both p53 and actin. In the untreated cells, p53 were found to be inside the cytoplasm and not inside the nucleus. (Figure 5.11) However, in treated cells, p53 was found only in the nucleus and not inside cytoplasm. (Figure 5.12) We were also able to simultaneously measure actin with the same extract. (Figure 5.12)

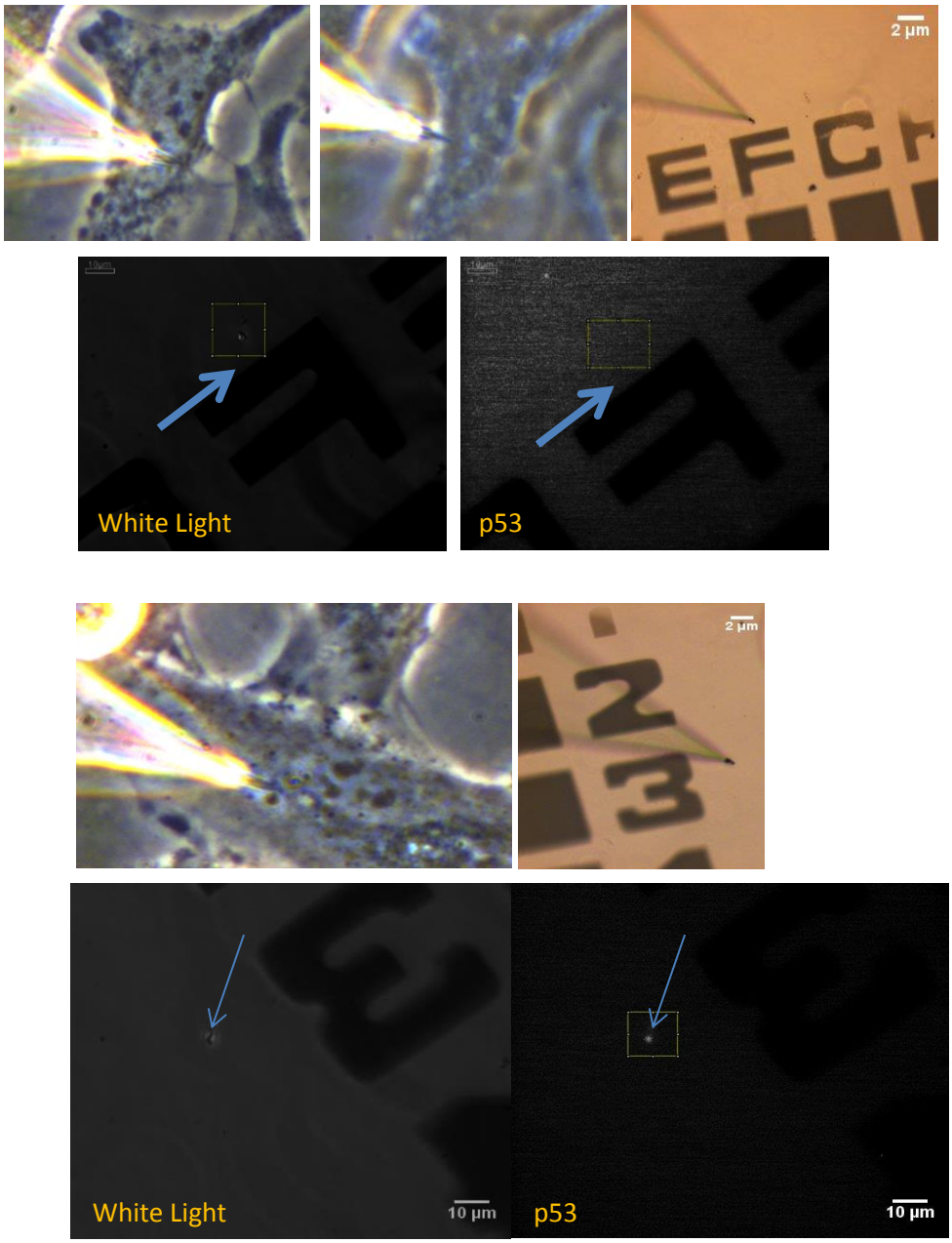

Figure 5.11. Untreated cytoplasmic and nucleus Extraction of HeLa cells. Upper Panel. Shows the extraction of nucleus and staining for p53. Lower Panel. Demonstrates cytoplasmic extraction and staining for p53 proteins.

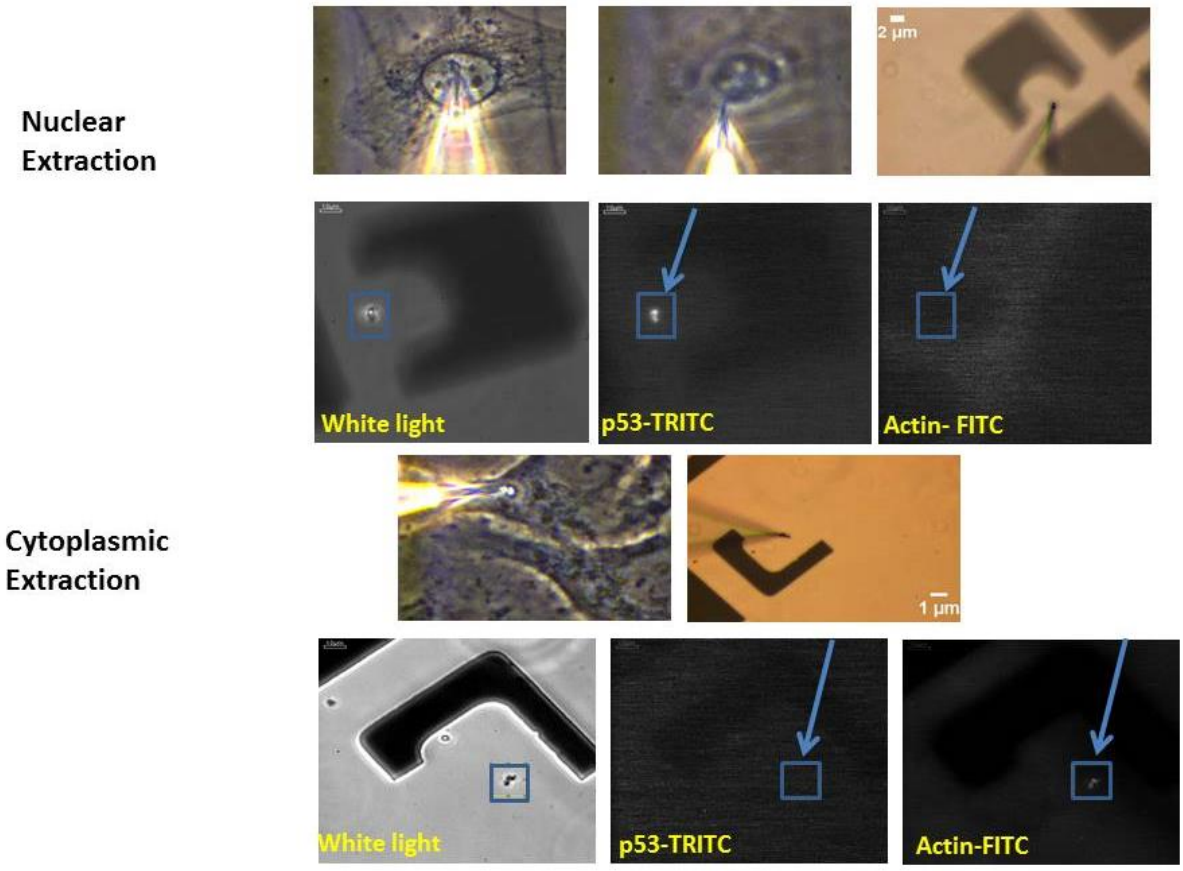

Figure 5.12. Sampling of HeLa cells 24 hours after drug treatment . Upper panel shows nuclear extraction and staining for both p53 and actin. Lower Panel. Shows cytoplasmic extraction and staining for both p53 and actin.

Finally, the nanopipettes were also applied to bigger cells such as salivary gland cells to show the wide application of the technique. (Figure 5.13)

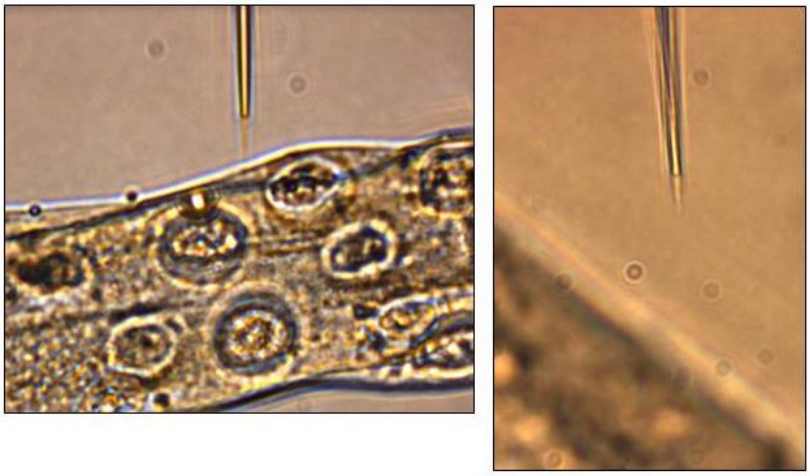

Figure 5.13. Probing of salivary gland cells. The picture shows the extraction from salivary gland cells.

5.5. Discussion

In this chapter, the goal was to prove single cell proteomic analysis and quantification using the integrated electrowetting nanoinjector/aspirator. Some in vitro assays were first performed to show that with this technique that proteins can be readily picked up or ejected. The GFP proteins were then aspirated from NIH 3t3 cells and were released on top of coverslip proving that proteins can be aspirated at the single cell level with the technique developed. We were not only interested in protein analysis, but also we desired to quantify proteins with our method. With the nanoprobes, we sampled actin proteins and quantified the number of actin molecules inside mouse fibroblasts using our calibration standard curve. The number of proteins inside mouse fibroblasts was reported to be between 10^2-10^8.[61] The number of actin molecules sampled was estimated to be around 4 to 9 x10^5. This number matches with the number of reported

actin molecules in the literature. [62] Schwanha¨usser et al. have reported 10^8 molecules of actin inside mouse fibroblasts. [62] Since with our technique we sample 1% of the volume of the cell (20-50fl of the 2000fl corresponding to the volume of the cell), that would leave us with 10^6 actin molecules which correlates with the number that we have estimated.

We were further interested to test the limits of the technique by analyzing p53 proteins after treatment with actinomycin D drug. In the control experiment where there was no treatment of cells with the actinomycin D drug, p53 was found in the cytoplasm of cells and not in the nucleus. The p53 upregulation however was observed in the nucleus after Actinomycin treatment and no p53 proteins were found in the cytoplasm 24 hours after the treatment of HeLa cells with actinomycin D drug. These results were as expected because actinomycin inhibits transcription and causes accumulation of p53 proteins inside nucleus.[63] [60] Additionally, from the same extract, the number of actin proteins was quantified to be in the same range before treatment and 24 hours after the drug treatment. This result was also as anticipated because actin is one of the housekeeping genes which means actin proteins are expressed relatively constant from one cell to another. [64]

Chapter 6
Future Works and Conclusion

The technique developed in this study is a unique method in a way that both injection and extraction can be executed.

6.1. Future Works

6.1.1. Mitochondrial Aspiration

One of the other applications of this technique that is very promising is extracting mitochondria from cells and analyzing the mitochondrial genome at the single cell level. Mitochondrial genome mutations are responsible for many hereditary diseases.[65]

As can be seen in figure 6.1, mitochondria can be stained with mitotracker dye and can be sampled for later analysis.

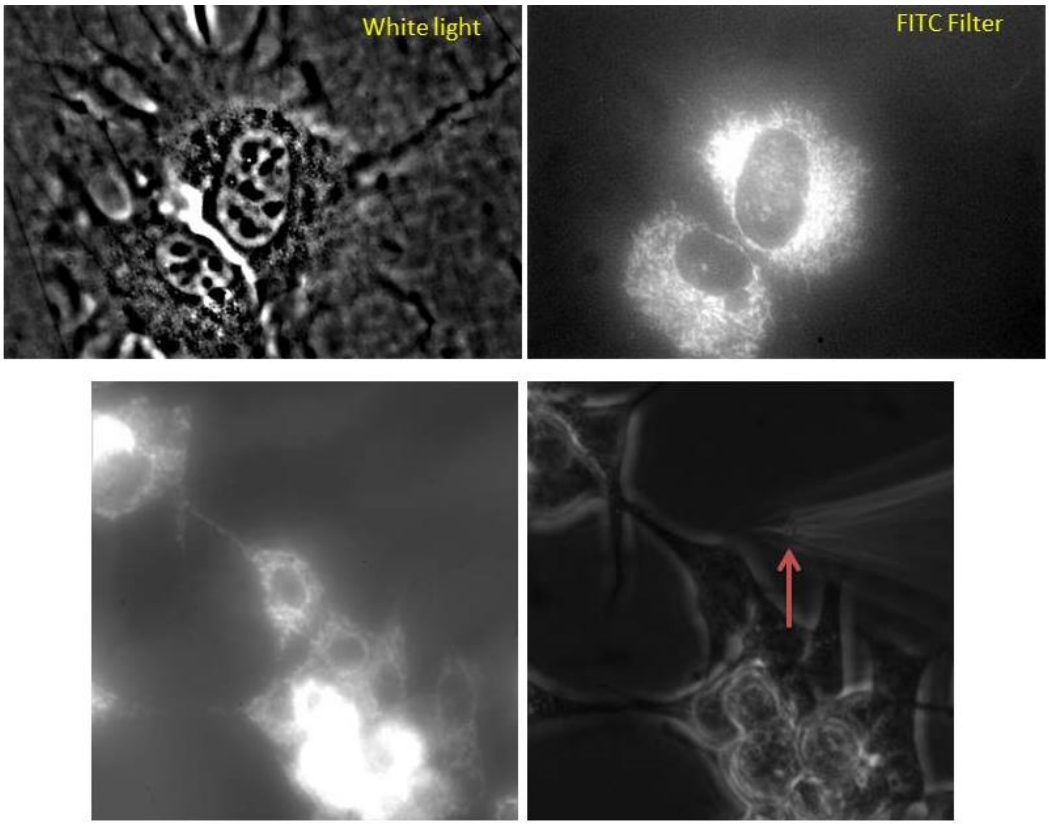

Figure 6.1. Mitochondrial Aspiration. Upper Panel. Staining mitochondria with mitotracker. Lower Panel. Aspiration of Mitochondria from cells

6.1.2. Combining Integrated Electrowetting Nanoinjector/Aspirator Technique with AFM

One of the many challenges in single studies is the correlation of mRNA copy numbers with protein copy numbers. The genome copy numbers is constant but the mRNA and protein numbers vary with time and from one cell to another. [61] Due to the lack of techniques for protein analysis, scientists use mRNA copy numbers to calculate the number of proteins.[66] However, recently, studies on this matter have suggested that the number of proteins is more likely dependent on the translational control than

transcriptional control. [61] For this reason, we propose combining the AFM technique that was developed in our lab for measuring mRNA at the single cell level and correlating the protein results measured with the Nanoaspirator. (Figure 6.2)[67]

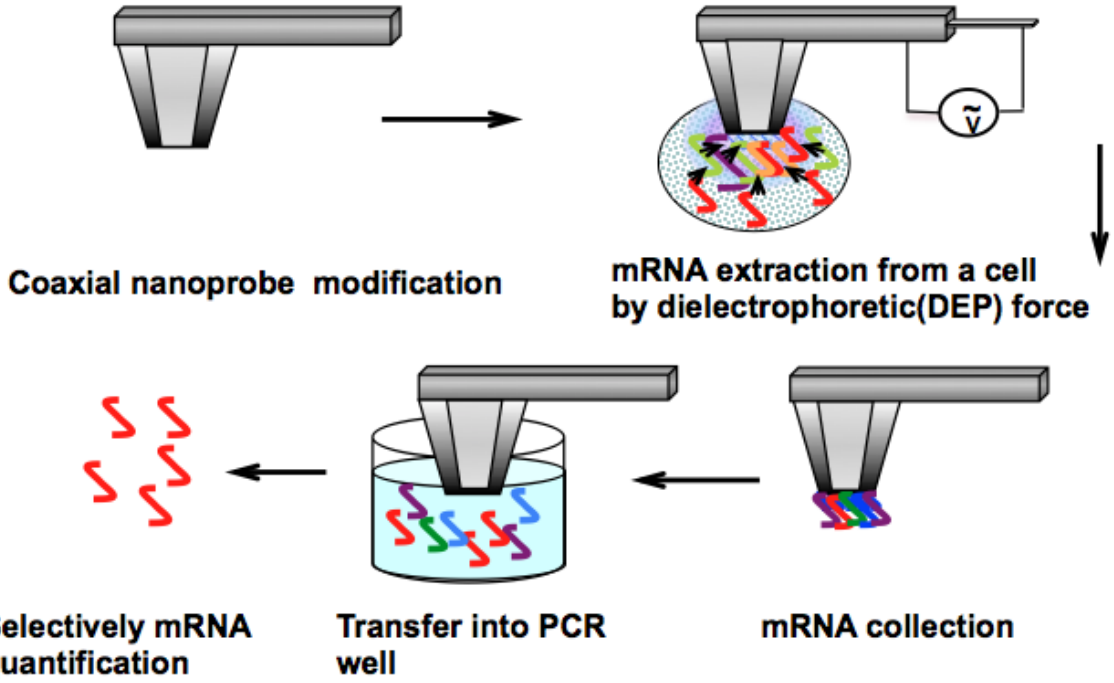

Figure 6.2. AFM technique in measuring mRNA at the single cell level. Applying DEP force inside cells to pick up mRNA and releasing mRNA inside qPCR wells for quantification

Basically, the cells will be placed on top of gridded petri dish where they can be tracked. The cells will then be probed with both the AFM coaxial probe and with the nanoejector. The number of mRNA and proteins for the indicated gene would then be quantified and would be correlated.

6.2. Conclusion

Due to the heterogeneity that exists between cells, single cell studies have gained more popularity. The single cell techniques have been slowly growing but scientists have had their challenges with single cell transfection and protein analysis. The technique described in this work can unravel the unknowns of cell dynamics and cell heterogeneity. With this powerful technique, the genetic expression of the cell can be modified and studied by extracting the cell content.

In this study, we proposed a new and simple technique called Integrated Electrowetting Nanoinjector/aspirator for injecting macromolecules into cells that are typically impermeable as well as aspirating cytoplasmic content for single cell studies.

The theory of this technique relating voltage to fluid volume flux inside the nanoprobe was derived and verified experimentally.

Our technique has several advantages over other similar transfection techniques reported in the literature. Currently, with microinjection, scientists need to use PCR or fluorescent molecules to quantify femtogram of DNA injected into cells. Furthermore, these micropipettes clog because of drying and aggregation of samples at the end of the tip before injection. High pressures are required for sample delivery dictated by the Hagen-Poiseuille equation where pressure is proportional to $\frac{1}{r^4}$; the smaller the radius, the higher the pressure needed for ejection. This is the reason why needles have been in the micrometer ranges for pressure based injection leading to lower cell viability from entry into cells.

Another delivery technique that has been discussed is the use of AFM probes for administering DNA into cells. AFM probes are subjected to the same issues as micropipettes when it comes to quantifying the number of molecules injected. Fabrication of AFM probes and low transfection efficiencies are two additional disadvantages of using AFM probes.

Single cell electroporation techniques also lack quantification of DNA delivery. Small molecules have been shown to enter cells immediately after pulsation, but large plasmids seem to accumulate near the pore for at least 10min. During electroporation, the pore size has been claimed to be around 1nm and these factors may be the reason why larger molecules do not enter cells as readily as small molecules. During electroporation, 500 volts is required for perforating the cell membrane. Such high voltages create heating and further impair cell viability.

In comparison to microinjection, AFM probes, and single cell electroporation techniques, the INENI technique gives us the ability to control the amount of delivery *in situ*. The INENI technique is an integrated device where one electrode is inside the capillary containing organic phase solution and the coating layer is another electrode. Since the organic phase solution (1, 2-Dicholoroethane) and the aqueous phase do not mix, it is very easy to gauge the height of solution once it enters the capillary. Quantification of molecules is critical when determining drug tolerance and point of efficacy at the single cell level. Furthermore, the DNA delivery to cell death ratio suggests a high efficiency of this technique. The fabrication of INENIs is simple and the diameters of INENIS are very small (roughly 140nm). In this study, INENIs have been successfully demonstrated for

biological applications. Other applications of this technique as discussed are single cell analyses of living cells where femtoliter volumes of cell cytoplasm can be extracted for further analysis.

One of the many challenges at the single cell level proteomics is quantification of proteins and correlating that number to the mRNA level. Protein quantification and analysis at the single cell level have been performed with other methods such as FACS, mass spectrometry, and microfluidic platforms. [40] While one technique can be less costly or more efficient, all of these techniques lack the urge to study single cells in their microenvironments. All of the existing techniques applied in single cell proteomics require lifting off cells from their environment for further analysis. In addition, most of the times, the cells are not viable after single cell analysis. Most importantly, spatiotemporal studies are not conceivable with the current single cell analysis techniques. Thus, the technique proposed here can untangle the unknown problems that could not be addressed before because of the lack of appropriate single cell analysis techniques.

With this technique, we modified the gene expression of living cells by inserting known number of plasmid vectors inside NIH 3t3 cells. We also proved protein quantification at the single cell level and we demonstrated the change in P53 expression after drug administration to HeLa cells while monitoring the level of one of the housekeeping proteins, actin.

The technique can be utilized in extraction of mitochondrial genome and quantification of that genome using qPCR. If combined with the AFM technique, mRNA and protein

expressions inside one cell can be monitored in time. This will address one of the challenges in the single cell analysis studies.

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Appendix A: APTES Coating

Cleaning:

1. Glass coverslips were rinsed with Acetone
2. They were then rinsed with Isopropanol Alcohol(IPA)
3. Next, they were cleaned with DI water

Oxygen Plasma treatment:

1. The coverslips were oxygen plasma treated for 10 minutes.

APTES Coating:

1. 2% APTES was made with pure ethanol.
2. The coverslips were coated with 2% APTES overnight.
3. The coverslips were rinsed with ethanol the day after
4. The coverslips were rinsed with DI water and dried