Single-cell Manipulation Using Nanopipettes

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Abstract

Manipulation and analysis of individual cells is key in understanding processes that control single-cell behavior in a complex environment. Nanotechnology-based tools having high sensitivity and low invasiveness are holding great promises as new biomedical devices for single cell manipulation[1]. The fully electrical read-out as well as the ease and low cost of fabrication are unique features that give nanopipette technology enormous potential. Our group has used nanopipettes as a biosensor[2], and is now continuing to use nanopipettes for single-cell manipulation. We developed a single-cell manipulation platform based on quartz nanopipettes. The system uses scanning microscopy techniques to position the nanopipette with nanoscale precision. The nanopipette is fitted with electrodes to mediate voltage-dependent injection or aspiration from individual cells. Nanopipettes improve current injection methods due to its high controllability and high viability of cells post injection. Nanopipette tips cause less disruption to the cell membrane and allows injection into single-cells while in their normal plating conditions which improves viability. This technology also allows for multi-component injections. We have shown successful injections into mammalian cells, a technique that is a historically difficult task when using a micropipette. Another needle-based injection method which uses Atomic Force Microscopy (AFM) based techniques have also been demonstrated for injection into mammalian cells but they are limited in terms of throughput and control of injection volumes[3]. Using a similar feedback mechanism as our single-cell injections, our group has continued to use nanopipettes for single-cell manipulations for a project called Single Cell Biopsy, which allows for precise aspiration of contents from a single-cell. We present preliminary results showing the aspiration of minute amounts of cytoplasmic material from a single cell. To further optimize the single-cell manipulation, the devices are interfaced with a microfabricated fluidic chip to immobilize cells onto a 6x6 array, the Cell Sifter. Our technology forms the basis for a fully automated system for high-throughput immobilization and precise injection and aspiration of single cells.

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Introduction

Single cell biology is an emerging field in which a culture of cells is not only observed by its mass behavior but on an individual cellular level[4]. Single cell injections can allow direct manipulation on the individual cellular level. It is useful in regulating cell fate by introducing foreign material into a cell. Single cell injections have been a historically difficult task due to technological drawbacks. By semi-automating our technique, some of the difficulties of manipulating and analyzing single cells will withdraw.

There are various current methods for injecting cells, such as injections using a micropipette and atomic force microscopy, but each of these has their own disadvantages. One common method for injecting cells is using a glass micropipette. This technique is the current standard for *in vitro* fertilization, in which a single sperm is injected directly into an egg (oocyte) to form a zygote. Pillarisetti *et al.* has shown this technique successful using a zebrafish egg cell, a known large cell with the diameter of each egg being 600-700 μm[5]. However, if a cell is smaller than an oocyte, there will be great disruption to a cell during penetration due to the diameter of a micropipette tip being large. The technique described in this research was less invasive to the cell due to the large diameter of the cells relative to the size of the micropipette. In my project, the micropipette would be extremely invasive to mammalian cells due to their smaller diameter (20-30 μm). Also, micropipette injections require a skilled technician who has to position the cell using a holding pipette and then bring the micropipette into the cell for injections which creates a low-throughput and high error rate. The literature shows the successful reprogramming of mammalian cells to induced pluripotent stem cells. Therefore my

work has been primarily to use mammalian cells for cell injections to answer more biologically relevant questions.

This led to research being conducted to allow single cell injections into cells of a smaller diameter which includes most of the cells useful in manipulating for real-world application, for example stem cells. Atomic Force Microscopy (AFM) is a nanofabrication tool allowing cell manipulation on the nanoscale level[6]. This technique allows for less invasive penetration and injection into single cells, in this case mouse myoblast cells which are on a similar size scale as our work. Meister *et al.* was able to inject these myoblast cells using AFM but have disadvantages when it comes to controlling cell injection volume and high throughput. This led to our goal of integrating our single cell injection method with a cell capturing device to form the basis for a semi-automated single cell injection platform. Single cell immobilization has been created in the past but has been limited in ways of integration[7-8]. Ideally, immobilizing cells in a specific cross-section will allow for high throughput cell injections.

Viability for the cells post-injection has had limited investigation. Many researchers have been able to successfully deliver molecules inside of a cell but a viability test has not been performed. Cell viability is important post-injection because depending on what molecule was injected, it may take time to integrate into the genome of the cell and the cell staying viable is necessary for analysis. Also, in some cases more than one molecule needs to be injected into the same cell for a certain reaction to occur. Rodolfa *et al.* show that using double-barreled pipettes eliminates the issue of having to re-inject the same cell with two different pipettes. This also aids with cell viability. An electric potential is applied between the two barrels and the molecules are deposited in the single cells by applied voltage[8]. However, this group did not use

the double-barrel pipette to inject single cells, they only deposited molecules on top of a surface.

In my study, we have created and tested a technology for precise injection into single cells using a double-barreled nanopipette. We have shown successful viability post-injection, control of volume, and control of injection from each barrel respectively. The injection technology is integrated with an immobilization microfabricated fluidic chip to allow for high-throughput injections. We have also shown the ability to perform a single cell biopsy using a similar mechanism to our injection system. The biopsy allows for precise aspiration of minute amount of cell contents for further analyzes.

Methods and Results

My work was primarily to apply the technology that R. Adam Seger developed. His work created a system where single cells could be injected using an applied voltage and a feedback system. The feedback system measures the current and determines where the cell is in relation to the tip of the nanopipette (Figure 1). The system knows how far from the cell membrane the nanopipette is and will inject the cell after the nanopipette penetrates the cell of choice.
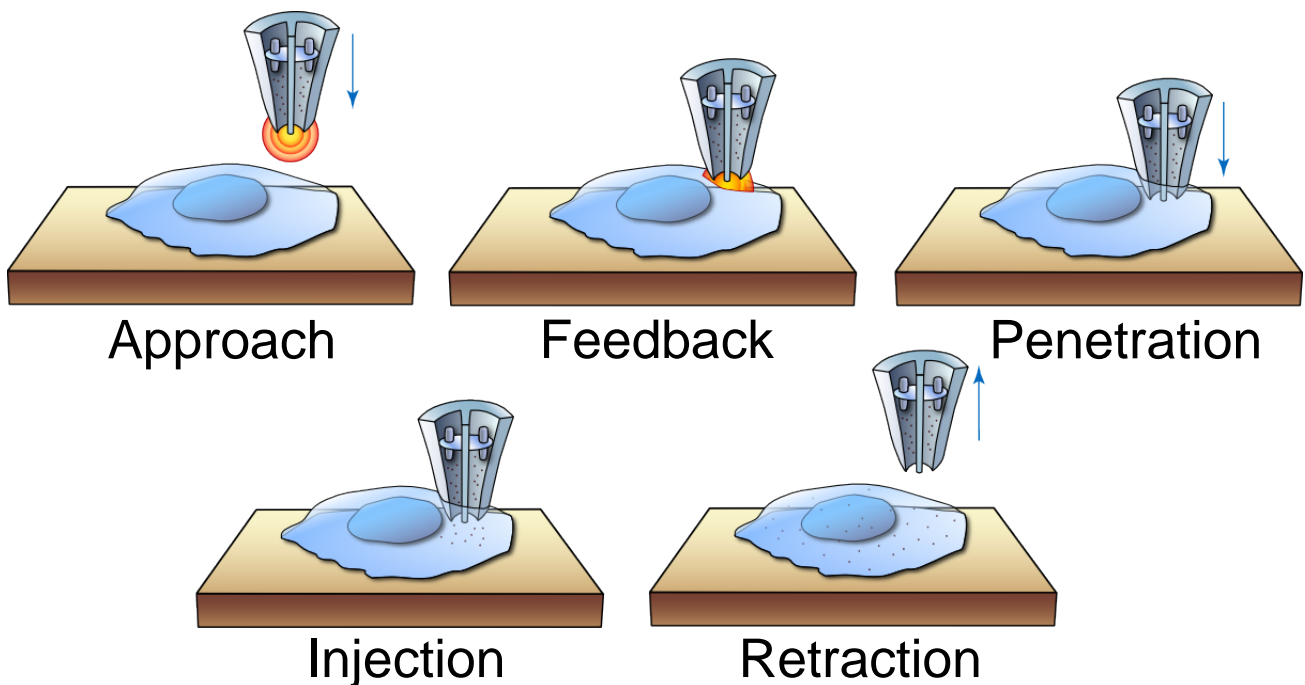

Figure 1: Nanoinjection process: Here is a step by step illustration of the nanoinjection system. The nanopipette is fitted with two electrodes that measure a current as the nanopipette is lowered into the cell where it is automatically penetrated and then injects the cell.

Fabrication and analysis of nanopipettes used for Injection

We use double-barrel nanopipettes to inject Human BJ Fibroblast cells. The nanopipette is fabricated from a theta quartz capillary with filament. The capillary is placed in a laser puller and is heated and pulled into two nanopipettes. The ideal nanopipettes for injections have a tip diameter around 50-100nm. The size of the nanopipette tip can be controlled by altering the pulling parameters while pulling the nanopipette. The nanopipette is fitted with two electrodes to mediate voltage. To test each tip to determine if it is a good pipette for injection, I checked for linearity between the applied voltage and current prior to injection. This also determines if the tip is broken or if the tip is too blunt for injections. If the tip is too blunt then the injections will be less controllable and a large volume of injection dye will cause the cell not to survive. If the current is too low or not linear, the system has internal noise and will slow down the typical time for injections often causing the feedback system to stop working entirely.

Injection Parameters

My work with injections has included injection of fluorescent dyes. The injection is confirmed by fluorescence microscopy. There are two filters used in my work, Fluorescein isothiocyanate (FITC) and isothiocyanate derivative (TRITC) filters. These filters will fluoresce the cell injected with each filters respectively. To prepare for injections, it is important to fill the nanopipette with the fluorescent dye of choice and bring dye to the absolute tip by centrifugation. A short circuit may occur if the two dyes from each barrel are connecting in anyway, causing a slowdown to the system or other malfunctions. To prevent this, it is necessary to silanize the back of the nanopipette. The silane creates a hydrophobic region, and as a result the two dyes filled in each barrel will not connect. The three fluorescent dyes I have

injected cells with are Carboxyfluorescein succinimidyl ester (CFSE), Carboxyfluorescein, and Rhodamine 101. CFSE is known to be a highly cell permeable dye that covalently couples to intracellular molecules. At certain concentrations, we have found that when injected with CFSE, cells are able to remain viable for many hours. Cell morphology also stays intact post-injection with CFSE. Figure 2 illustrates six human fibroblast cells injected with 250uM CFSE at injection and 1 hour post injection. Besides one hour, cells were also determined to remain viable and fluorescent over 24 hours post-injection even after a cell division event occurs. Post-injection viability was tracked by plating cells on a gridded plate.
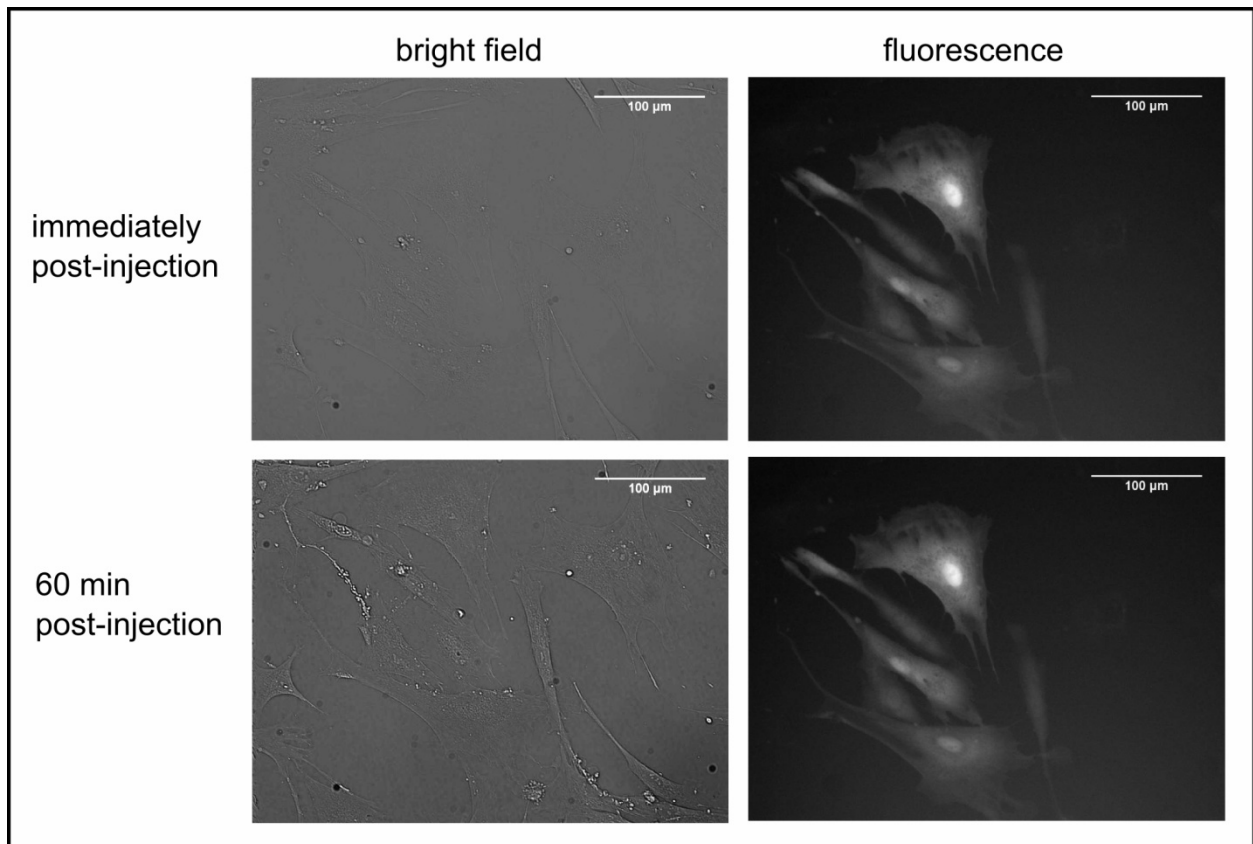

Figure 2: Post Injection Viability: Images of Human Fibroblast cells in brightfield and under fluorescence at injection time and after 1 hour. This figure illustrates no obvious morphological change and cells seem to remain alive and unharmed while still fluorescent.
Parameters: 30V 100ms 250uM CFSE

There are many electrical parameters also tested for injection to obtain maximum success in cell injection and viability. The maximum voltage able to be applied is 100V, however all of the injections were performed with a range of 1V-30V. Injections higher than this voltage cause high cell disruption. This is seen by vesicles forming around the edges of the cell post injection due to the cell lifting from the petri dish and not surviving. The duration of the injections is between 100ms-10s, typically. Typical procedures are to use a low voltage if using a long injection duration time and vice-versa. Cells were penetrated at 1µm depth for injection.

Injection volume is controlled by changing the voltage and injection duration. Figure 3 demonstrates two human fibroblast cells of similar sizes injected both at 10V and at 100ms and 1000ms, respectively. It is noticeable that the cell injected for a longer time constant has higher fluorescent intensity relative to the cell injected for lower time duration.
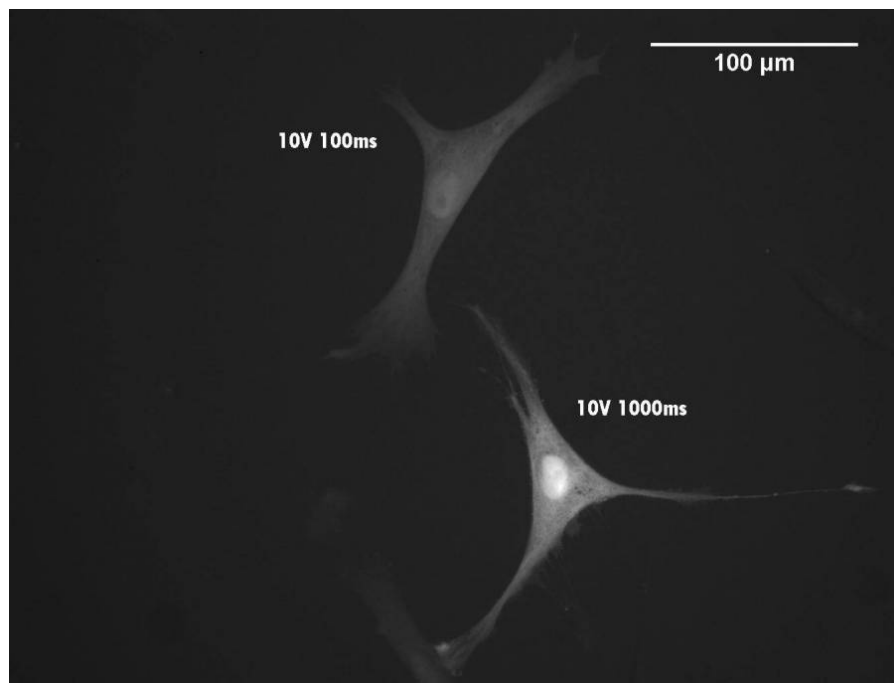

Figure 3: Volume Controllability: Human Fibroblast cells, of similar sizes, injected with CFSE at both 10V for 100ms and 1000ms, respectively. The cell injected at a longer duration displays higher fluorescent intensity showing controllable injections.

Multicomponent Injections

Our injection system is unique in the fact that we can perform multicomponent injections using the same nanopipette. The double-barrel nanopipettes allow for bicomponent controllable injection where each barrel of the nanopipette can be filled with a different molecule and independently injected into a cell by changing the polarity of the applied high voltage (Figure 4). To show this, I filled a nanopipette with one barrel Carboxyfluorscein (green) and the other barrel with Rhodamine (red). I then injected six cells, changing polarity after 3 cells. Figure 5A shows cells injected with each barrel respectively at 20V for 500ms and the nanopipette used for injection.
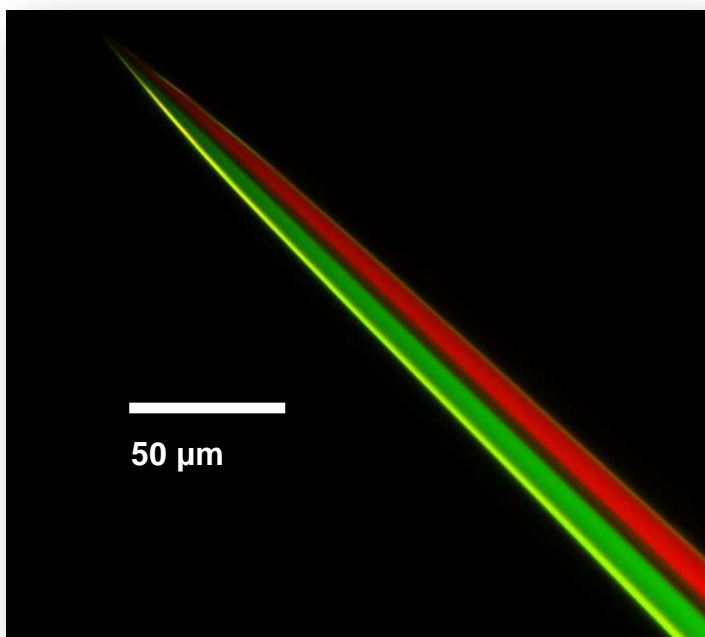

Figure 4: Nanopipette filled with two separate dyes: The double-barrel nanopipette allows for the unique ability for injection using two different molecules without any noticeable cross-talk. This false color image of the nanopipette is filled with both Rhodamine and Carboxyfluorescein. This nanopipette was used for both injections in figure 5A and 5B.

On the same note, I also showed that one cell can be injected by both barrels controllable. Figure 5B shows two cells injected with both red and green dyes at opposite times. The cells show fluorescence with both colors with the higher injection duration dye being more prevalent.
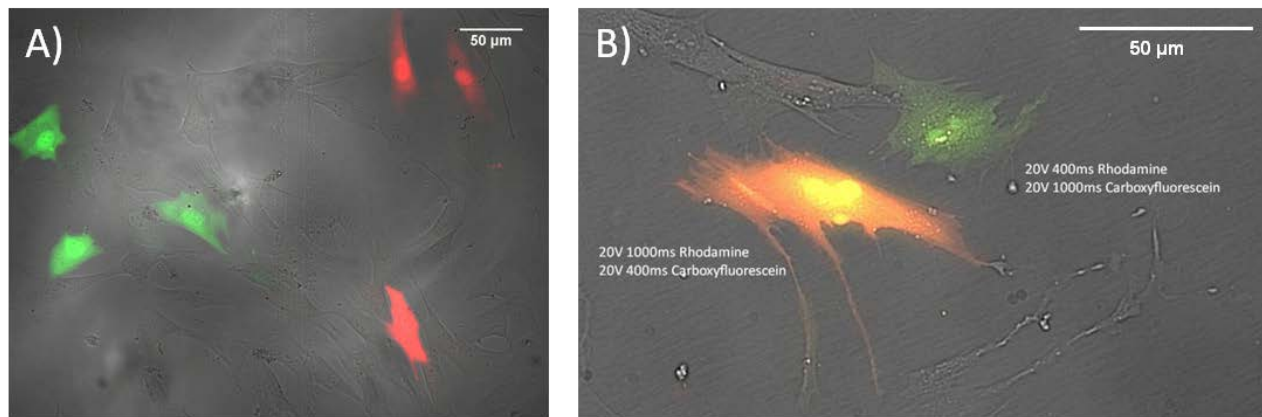

Figure 5: Bicomponent injection **A)** False color image of human fibroblast cells injected with carboxyfluorescein (green) and sulforhodamine (red) independently at 20V for 500 ms. **B)** False color image of cells injected with both dyes showing the ability of the technology to achieve intermediate compositions by controlling the duration and polarity of the applied voltage.

Single Cell Biopsy

Using a similar feedback mechanism, we have continued to use nanopipettes for analyzing the contents within a cell. The single-barrel nanopipette is used to aspirate a minute amount of intracellular mass (Figure 6). These contents are then analyzed by performing a cDNA synthesis from the RNA extracted followed by amplification of the DNA and then a gel electrophoresis is run. In theory, the contents can be redistributed to another cell using our injection system for cell to cell transfection.

We have tested both quartz nanopipettes and borosilicate nanopipettes. The advantage of borosilicate is the melting temperature is lower than quartz, therefore creating a larger pore during the laser pulling of the nanopipette. Using a larger pore allows for greater intracellular volume aspirations and can be a cause for easier and more accurate post-aspiration analysis.
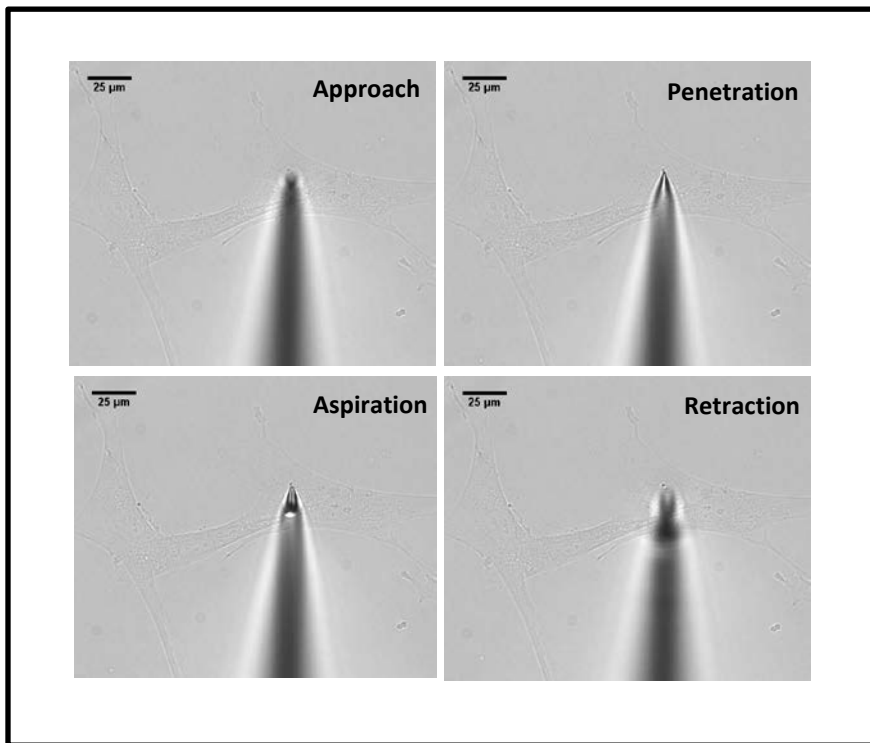

Figure 6: Single Cell Biopsy: Still images from a short video during single cell biopsy. The nanopipette is lowered within 100µm of the cell, and nanopipette penetrates the cell, aspirates cell content, and the nanopipette is retracted. During the aspiration stage, subcellular mass from the cytoplasm enters the nanopipette.

We have also decided to use a different cell line for the aspirations than the fibroblasts during these preliminary stages. We use HeLa cell line that has green fluorescent protein. The fluorescence is confirmed by using optical fluorescent microscopy. We have been able to aspirate from the HeLa cells but are still optimizing the parameters. Post-aspirate analysis is still to be determined.

Discussion

In this study, I have shown multiple experiments showing the feasibility of our single cell injection system using a double-barrel nanopipette. The easy fabrication of this nanopore is an ideal noninvasive way to inject single cells while in their normal plating conditions. Other current techniques require the cells to be in situations that cause cell stress and our system prevents this.

Our injection system is a reliable fairly rapid system. Figure 7 shows a high-throughput injection in which eight cells were successfully injected within five minutes. The short length of time to inject over a handful of cells shows feasibility and efficiency. This is important because even though cells remain in their normal plating conditions, still being in their medium, they are out of the incubator which causes cell harm if not at 37°C for over thirty minutes..
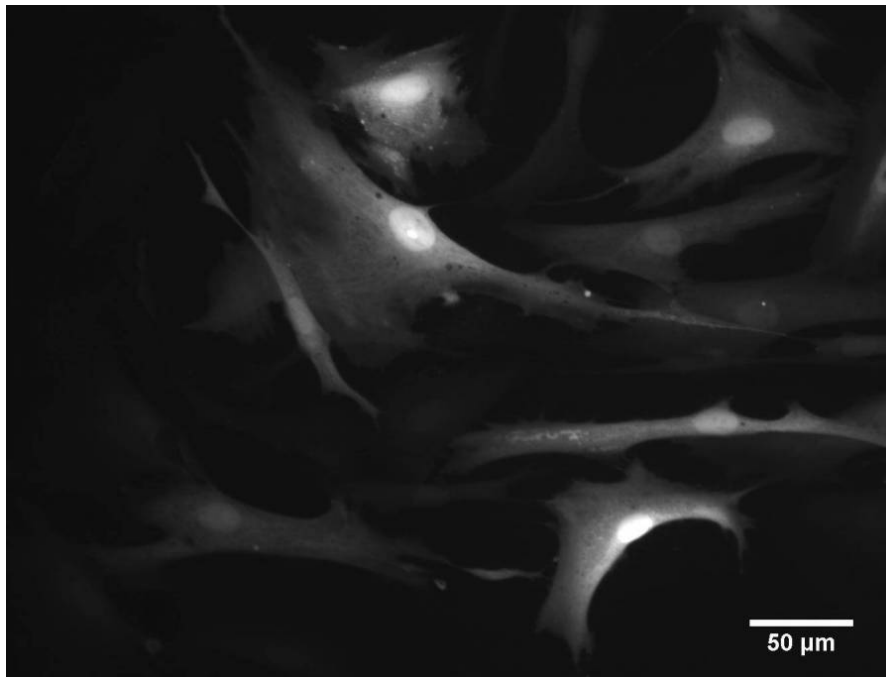

Figure 7: High-throughput Injections: Fluorescent image of human fibroblast cells injected with carboxyfluorescein at 30V for 100ms. This whole injection was accomplished within 5 minutes showing feasibility and efficiency.

Due to the experiments showing controllable, successful bicomponent injections, we can say that the flow of molecules from the nanopipette into the cell is induced by electroosmosis. The injections are therefore not controlled by the charge of the molecule but rather by applied voltage. This is an advantageous element allowing us to explore injection of any biologically relevant molecule without concerns of charge.

Conclusion

The single cell injections have been successfully shown to controllably inject fluorescent dyes into human cells with high post-injection viability. Multicomponent injections using a nanopore have not been shown in the literature and our double-barrel nanopipette is unique in this relation. Removing the need for an external electrode due to using double-barrel pipettes allows for less damaging cell injections in which repeated cell injections may remain viable. We hope that in integrating the cell sifter with injections will allow for the direct reprogramming of Human BJ Fibroblast cells into Induced Pluripotent Stem cells as described by Yamanaka et al. 2006[9]. The single cell biopsy will confirm RNA expression levels at different stages during the induction which takes several weeks. This technology has the potential to become a fully-automated single cell manipulation and analysis platform for many studies.

Future Work

Integration with Cell Sifter

The Cell Sifter (Figure 8A) is used for capturing single cells in a 6x6 array. The cell sifter is fabricated on a silicon wafer and the holes were defined using photolithography. There is a channel that connects the vacuum barb to the well. The goal is to integrate the cell sifter with

the injection system to create a fully automated high-throughput injection system. Each opening in the cell sifter will capture one cell, and the nanopipette will be able to identify the location of each hole and inject accordingly. The cells can remain in media and placed in the incubator for daily re-injections or viability tests. The cell sifter has shown to successfully capture fluorescent polystyrene beads seen in figure 8B. The cell sifter has been yet to be tested using human fibroblast cells.
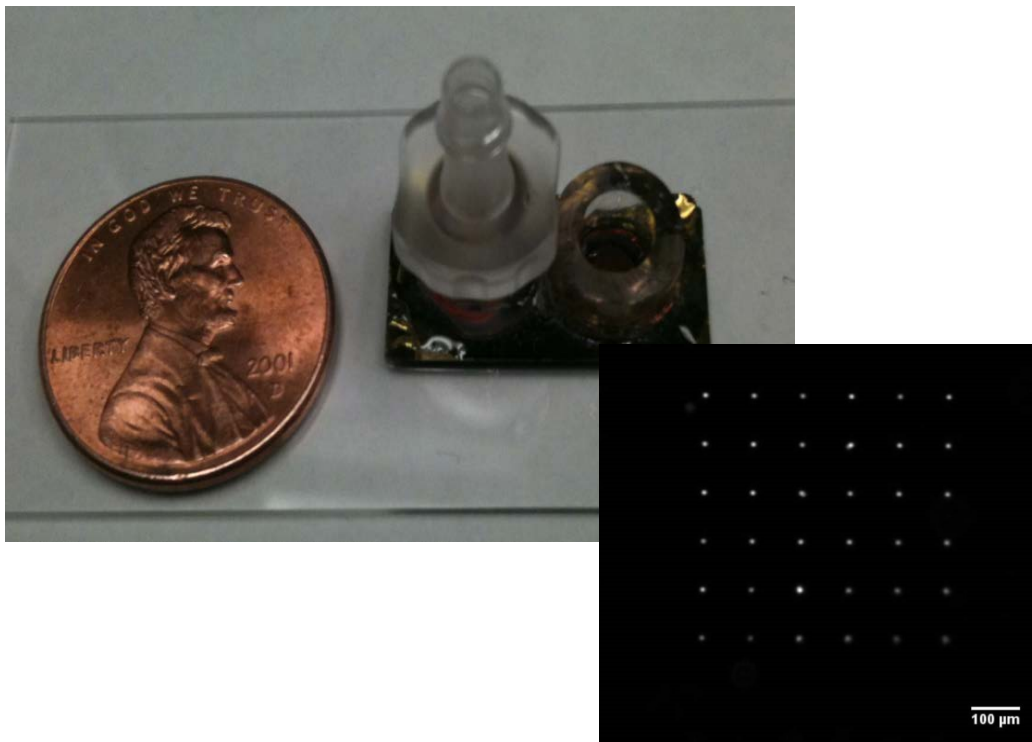

Figure 8: Cell Sifter: **A)** The cell sifter is a microfluidic chip that captures single cells. There is a well and a vacuum barb on the chip. The well contains a 6x6 array of holes that are able to immobilize one cell per hole. The chip is comparable in size to a penny. **B)** A close up image of the 6x6 array inside the well with fluorescent polystyrene beads captured. Each hole is 1.9m.

Appendix

Cell Culture Information: Cells were cultured in 5% CO2 at 37°C. Human BJ fibroblasts were a gift from Dr. S. Salama (UCSC) and cultured in FibroGRO™-LS Complete Media Kit (Millipore), supplemented with Pen/Strep L-Glutamine mix (BioWhittaker) on untreated ibidiGRID u-Dish 35mm plates coated with 1% gelatin.

Double-barrel nanopipette fabrication: Double-barrel nanopipettes were fabricated from theta quartz capillaries with an outer diameter of 1.2 mm and an inner diameter of 0.90mm (QT120-90-7.5; Sutter Instrument Co.). The capillary was pulled using a P-2000 laser puller (Sutter Instrument Co.) programmed with a two-line program to fabricate nanopipettes.

Acknowledgements

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