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# Investigation of Parameters Affecting the Nanoinjection of HeLa 229 Cancer Cells

Tyler E. Lewis
*Brigham Young University - Provo*

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Tyler E. Lewis

A thesis submitted to the faculty of
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Master of Science

Brian D. Jensen, Chair
Sandra H. Burnett
Larry L. Howell

Department of Mechanical Engineering

Brigham Young University

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ABSTRACT

Investigation of Parameters Affecting the Nanoinjection
of HeLa 229 Cancer Cells

Tyler E. Lewis
Department of Mechanical Engineering, BYU
Master of Science

The ability to deliver sequences of DNA and other molecular loads across the membrane of a cell and into its nucleus is an area of interest in the medical community. One of its many applications is that of gene therapy. In contrast to other forms of treatment, gene therapy seeks to treat diseases at the cellular level. The success of these treatments depends on the technologies for cell transfection that are available. Physical methods are sometimes able to overcome poor efficiencies of chemical methods and the safety concerns of viral methods, but are usually impractical due to the limited number of cells that are able to be transfected at a time, isolation, and immobilization of the cells. Nanoinjection is capable of using millions of small lances in an array to inject hundreds of thousands of cells simultaneously with relatively high efficiencies and viabilities. The solid nature of the lances also allows them to be smaller than their hollow-needle counterparts, which results in higher cell viability. Propidium Iodide (PI), a dye whose fluorescence increases greatly when bound to nucleic acids, was used as an injection molecule for testing the efficacy of the nanoinjection process on HeLa 229 cancer cells in a portion of the experiments, with a GFP plasmid of DNA being used in the rest. After injection, flow cytometry was used to detect the concentration of PI or the expression of the GFP in the injected cells. Since PI cannot normally penetrate the membrane of living cells, those found with high concentrations of PI were either successfully injected or dead, which can be determined by the flow cytometry. Investigation of the parameters that affect the efficiency of the nanoinjection process will help improve it for further research. Some of these parameters that were investigated include the force of injection, the material used for the lances (silicon versus carbon nanotubes), and the injection speed of the lance arrays. An injection device capable of small changes in deflection was designed to ensure accurate increments in force for testing, as well as a pulsed current control injection system. Results for injections of varying forces indicate a slow rise in PI uptake from 0 to 1.8 Newtons where it reaches a maximum uptake of 4.11 when normalized to the PI uptake of the positive controls. The PI uptake then remains relatively level as the force continues to increase, averaging an uptake of approximately 3.1. The slow rise is likely due to more of the cells being punctured as the force increases until most have been punctured and the PI uptake levels off. The viability of the injected cells was close to that of the controls with no clear trend. A comparison of lance arrays made from silicon and carbon nanotubes using DNA as the molecular load shows little difference between materials. Different injection speeds tested show that only 1-5% of the cells in the injection process are lost for speeds in the range of 0.08-0.16 mm/sec, whereas 49-69% of the cells are lost using speeds between 0.6-3 mm/sec.

Keywords: nanoinjection, parameters, lance array, propidium iodide, GFP, gene therapy

# ACKNOWLEDGMENTS

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# NOMENCLATURE

| | |
|---|---|
| *ABS* | Acrylonitrile Butadiene Styrene |
| *CNT* | Carbon Nanotube |
| *CRISPR* | Clustered Regularly Interspaced Short Palindromic Repeats |
| *DNA* | Deoxyribonucleic Acid |
| *DRIE* | Deep Reactive Ion Etch |
| *GFP* | Green Fluorescing Protein |
| *HBSS* | Hanks Balanced Salt Solution |
| *IPA* | Isopropyl Alcohol |
| *NC* | Negative Control |
| *PBS* | Phosphate Buffered Saline |
| *PC* | Positive Control |
| *PI* | Propidium Iodide |
| *US* | Untreated Sample |

# CHAPTER 1. INTRODUCTION AND BACKGROUND

Gene therapy and tissue engineering have become important areas of research with many practical applications and possibilities. The modification of tissue and individual cells to overcome diseases and undesirable genetic functions at the cellular level is actively investigated for possible treatments. These include the treatment of glaucoma [1, 2], viral infections [3, 4], cardiovascular disease [4–6], sickle cell anemia [4, 7, 8], cancer [4, 9, 10], cystic fibrosis [4, 11, 12], immune deficiency disorders [4, 8], and even help in cartilage repair [13–15], bone regeneration [16–18], and ligament healing [19]. These treatments all depend on the technologies and methods available for cell transfection. Improving these technologies will help further these efforts by providing access to injection processes with higher cell transfection yields.

Being able to deliver DNA and other desired particles across the cell membrane while having high cell viability is critical to having a successful delivery method. The difficulty with some methods that are currently in use is the inability to transfect many cells at a time and still have a high viability of the cell culture. There tends to be a compromise between the number of cells transfected and the viability of those cells. This must be overcome to have a delivery method that is both cost effective and practical.

Current gene delivery methods for cells can generally be placed into three different categories– chemical methods, viral methods, and physical methods:

- **Chemical** - Chemical methods have very poor transfection rates in comparison to physical methods of gene delivery, although cell viability is much higher. Large doses of the chemical vector can be used to overcome these poor efficiencies, but a high concentration is toxic to many cells, especially if it is a sensitive cell population. [20]

- **Viral** - Many viral vectors have been developed that have very high transfection rates, but they can be expensive and pose significant safety concerns. [21] Some of these concerns include toxicity, oncogenesis from insertional mutagenesis (the creation of cancer cells from

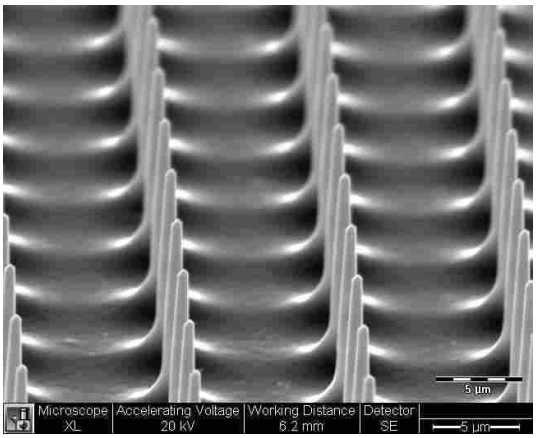


Figure 1.1: A close-up SEM view of the lance array fabricated using standard MEMS manufacturing techniques. The lances are approximately 1-2 $\mu$m in diameter and 8-10 $\mu$m tall. Image courtesy of Jason Lund and Nicholas Gregory.

the mutation of DNA due to insertion of additional base pairs), and immunogenicity. [20] Inherent to the use of viral vectors is the limitation of restricted gene sequence sizes. Additionally, viral delivery methods are usually designed for a specific cell population, so their use on other cell populations is limited. [22]

- **Physical** - Physical methods of gene delivery avoid some of the safety concerns of viral vectors and the low efficiencies of chemical vectors, as well as being able to transfect cells that are very difficult to transfect. Electroporation and microinjection are the most widely used of these methods, although other methods used include sonoporation [23,24], photoporation [25, 26], biolistics (gene gun) [27–29], lipofection [30, 31], magnetofection [32, 33], and electric field induced molecular vibrations [34]. Some of the practical difficulties that arise from using physical methods are the isolation and immobilization of cells as well as the limited number of cells that are able to transfect, sometimes only one at a time. [20, 35]

In the category of physical methods, a nanoinjection lance array has been developed that has approximately four million needles on a 2 cm square silicon chip, each approximately one micron in diameter (See Figure 1.1). [36, 37] The chip is placed into a well that contains a culture of cells to be injected and is pressed down causing the lances to puncture the cells using an automated injection device. Once the cells have been punctured by the lances, the chip and injection device are removed. For the process to work, the cells being injected must adhere to the bottom of the

container that they are cultured in. For this reason, HeLa 229 cancer cells were the cells that were used and tested.

Using a lance array containing millions of needles allows hundreds of thousands of cells to be injected simultaneously. [37] This is important for applications requiring a large numbers of cells and for making the delivery method cost effective. To make the injection process even more effective, key parameters were studied to understand their effects on the nanoinjection process.

## 1.1 Nanoinjection Process

### 1.1.1 Overview

The nanoinjection process consists of a 2 cm square lance array with millions of MEMS-scale lances and an automated injector that can press the lance array down onto a culture of HeLa cells. The molecular load of interest, such as propidium iodide (PI) or a DNA sequence, is placed in a solution with the cells and can be passively diffused into the cells following injection, or can be assisted with pulsed voltage. [38,39] This is able to occur due to the lances piercing the membranes of the cells and creating temporary pores. Localized electroporation may also create pores to allow the molecular load to pass through the membrane and into the cell.

### 1.1.2 Injection Device and Lance Array Fabrication

One of the automated injection devices can be seen in Figures 1.2 and 1.3. A stepper motor that produces a linear displacement can be programmed and drives a spring downward onto the lance array that is being used for injections. The lance arrays are created using standard MEMS manufacturing techniques. More detail will be given in the following chapters on the design of the injection system and the fabrication of the lance arrays.

### 1.1.3 Propidium Iodide

Propidium Iodide (PI) is a fluorescent dye that can be used as a molecular load to test the efficiency of the nanoinjection process. When the cells are pierced, the molecular load diffuses

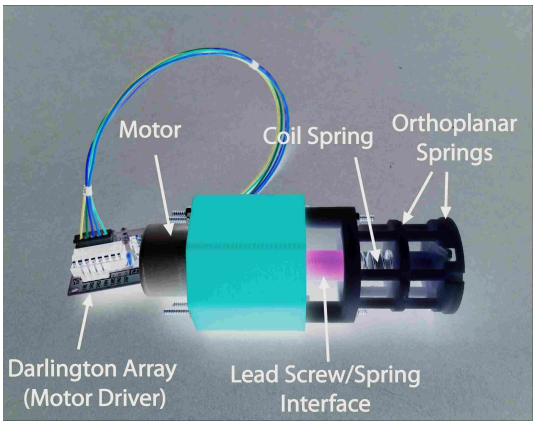


Figure 1.2: Injection device with motor attached.

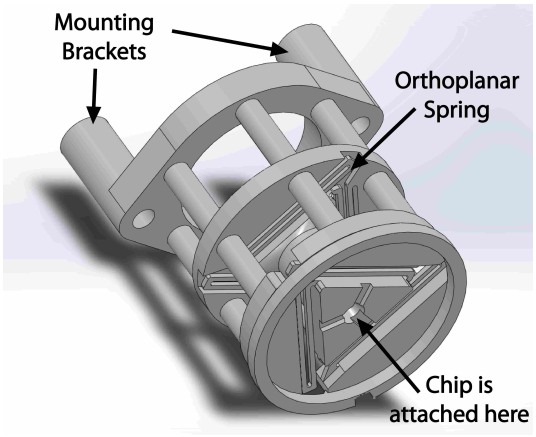


Figure 1.3: CAD model of injection device.

into the cells (and can also be assisted with pulsed voltage) and the number of cells taking up the molecular can be measured using flow cytometry. [37, 40]

## 1.2 Factors Affecting the Nanoinjection Process

Essential to being able to make the nanoinjection process more effective and efficient is studying the effects of different parameters of interest. Such parameters must be both practical and within our ability to control. Different parameters can affect the process in different ways, but they are each valuable in gaining a greater understanding of how to make the process more effective and efficient.

### 1.2.1 Propidium Iodide Concentration

In the analysis of the data presented, each well injected is normalized to controls to account for clustering of the data. It is therefore necessary to find an optimum concentration of PI resulting in the highest ratio when the data is normalized to the controls. This will allow for a better sensitivity.

### 1.2.2 Injection Force

Another parameter of interest to the nanoinjection process is the force which is placed upon the lance array chip during the injections of the HeLa cancer cells and how it affects the injection efficiency. When cultured properly, HeLa cells naturally form a relatively confluent monolayer. The orientation of the cells relative to the lances, however, differs from cell to cell. As the injection device presses the lances down, some cells are pierced by the lances before others. The force increases until most of the cells are likely punctured by the lances.

### 1.2.3 Lance Array Material

The material that the lance array is made from can likely affect the efficiency of the injection process, especially when dealing with molecules larger than PI, such as DNA sequences. The two materials of interest are silicon and carbon-infiltrated carbon nanotubes (CNTs). An array of lances can be made from both of these materials. The surface of the lances made from silicon tends to be relatively smooth, while the surface of lances made from CNTs are much more rough. It is possible that the surface roughness of the CNT lance arrays could trap more of the molecules of interest to be injected, especially if those molecules are large strands of DNA.

### 1.2.4 Injection Speed

For the nanoinjection process to work properly, the cells must be adhered to a surface upon which they can be injected. Our research with 10 $\mu$m lance spacing currently shows that cells are often pulled off of the surface as the lance array is pulled back up following injection. This means there are possibly a large number of cells that are unaccounted for in the current data. Testing

different injection speeds is desired to reduce the number of cells that are pulled off the injection surface and incorporate them into the available data. Slower injection speeds will likely remove fewer cells, but to what extent and how much it will affect the PI uptake of the injection process is of interest.

### 1.3 Research Objectives

The objectives of my research were to investigate the following parameters and how they affect the efficiency of the nanoinjection process:

- **Propidium Iodide Concentration** - This is the concentration of the dye used during the injection process that is used to determine how many cells were successfully injected.
- **Injection Force** - How much force is pressing the lance array down onto the cells being injected.
- **Lance Array Material** - The material the lance array is made from, namely silicon or CNTs.
- **Injection Speed** - The speed of the lance array that presses down on the cells during the injection process.

Experiments for testing each of these parameters and their results will be found in the following chapters. The results of each parameter will be discussed and recommendations will be made for further work and testing.

# CHAPTER 2. PROPIDIUM IODIDE CONCENTRATION TESTING

## 2.1 Propidium Iodide

Propidium Iodide is a fluorescent molecule that intercalates between the base pairs of nucleic acids when in their presence. Once bound to a nucleic acid, such as DNA, the fluorescence of PI increases substantially and can be detected through the use of flow cytometry. PI is often used as a cell viability stain because it cannot normally penetrate the membrane of living cells. When stained, the only cells that contain PI are dead cells because they are not able to actively remove the PI from their system. As a result of these characteristics, PI works well as a "dummy" molecular load that can be used for testing the effectiveness of different parameters in the nanoinjection process. One of these parameters is the concentration of PI itself in the solution used for injection. Different concentrations may change how well the PI works for the purposes we're using it for, so it is important to test various concentrations of PI to find the most effective one. I believe that a lower concentration of PI will likely have better sensitivity when compared to the controls because there is less residual PI in the cell solution after preparing the cells for flow cytometry.

## 2.2 Methods

### 2.2.1 Nanoinjection Process

#### Overview

Nanoinjection is a process that incorporates an array of millions of MEMS-scale lances on a 2 cm square chip to simultaneously inject hundreds of thousands of cells. This is done by culturing cells in plates with 6 wells and using a device containing an Arduino-controlled linear displacement stepper motor to press the square chip with the lances down on to the cells. The lance array is pressed down with a specified amount of force to puncture the cells. This puncturing of

the cells allows PI to cross over the cell membrane when it would not normally be able to, and can be detected by the use of flow cytometry. The cells used for this injection process are HeLa 229 cancer cells, an immortal cell line. One of the most important characteristics to this cell line other than the ability to continue growing and dividing is their adherence to the surface of the container they are cultured in. This immobilization of the cells allows them to be successfully punctured by the lances and injected with the molecular load.

**Lance Array Fabrication**

The silicon lance array used in the PI injections was fabricated using standard MEMS manufacturing techniques. Photolithography was used to create a grid of photoresist dots 5 $\mu$m in diameter and spaced 10 $\mu$m apart. The wafer was then etched with different processes to create an array of needles. This process will be described in more detail in Section 3.2.1.

**Injection Device**

The device used to press the lance array down onto the cell culture was fabricated from ABS using rapid prototyping techniques and can be seen in Figure 2.1. The main body of the device is pressed down to the bottom of the well with a recess in the middle of the device for the culture of cells. The lance array is held on the bottom of the device with the lances facing downward toward the culture of cells contained on a glass slip at the bottom of the well. It is able to traverse up and down via two orthoplanar springs connected in parallel to the main body of the injection device. This section that the lance array is attached to is driven by a linear displacement stepper motor. The motor is controlled by a custom programmable Arduino box, designed and built by Zachary Lindstrom. The wiring for the box can be seen in Figure 2.2. The control box has optional inputs for power supplies if a voltage is desired during the injection process. No voltage was used for the PI concentration injections, so they were simply left unattached. All injections (with the exception of the force injections in Chapter 3) were performed at an approximate force of 8 Newtons. The method for calculating this force can be found in detail in Section 3.2.2. Although the motor described is different, the method of calculation is the same.

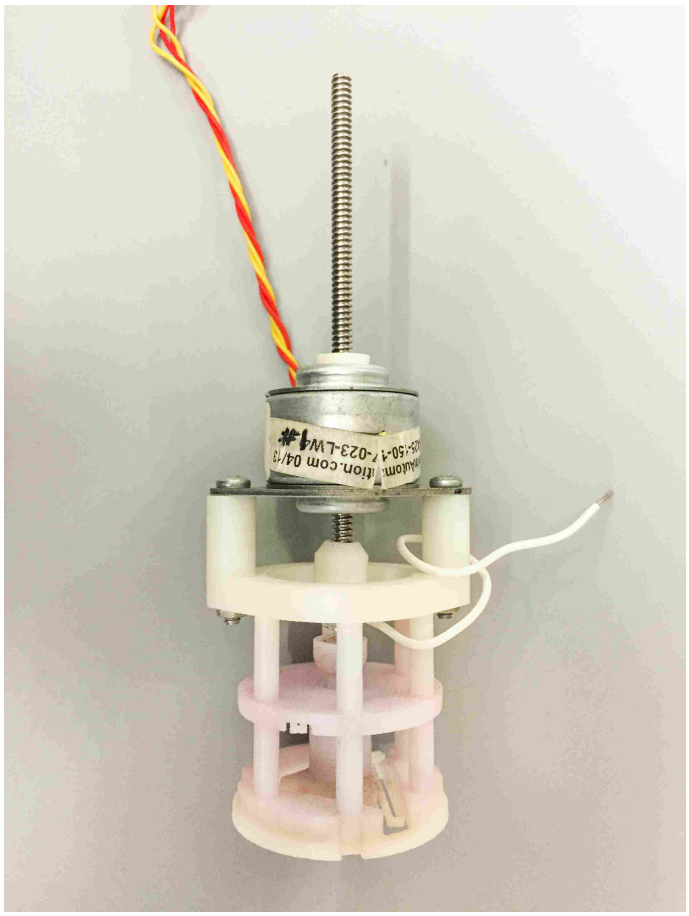

Figure 2.1: ABS injection device used for PI concentration testing.

### 2.2.2 Injection Methods

#### Key Parameters

A sample size of at least 8 was performed for each PI concentration. The concentrations tested were 0.02, 0.03, 0.04, 0.05, 0.1, and 0.15 mg/mL Hanks Balanced Salt Solution (HBSS). Increments were smaller on the lower end of the PI concentration because that is the usual range of concentration used for injection. All other parameters we kept the same.

#### Cell Testing Plate Preparation

Dulbeccos Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum and 5% gentamicin was used to culture the HeLa 229 cancer cells in an incubator at 37 degrees Celsius and

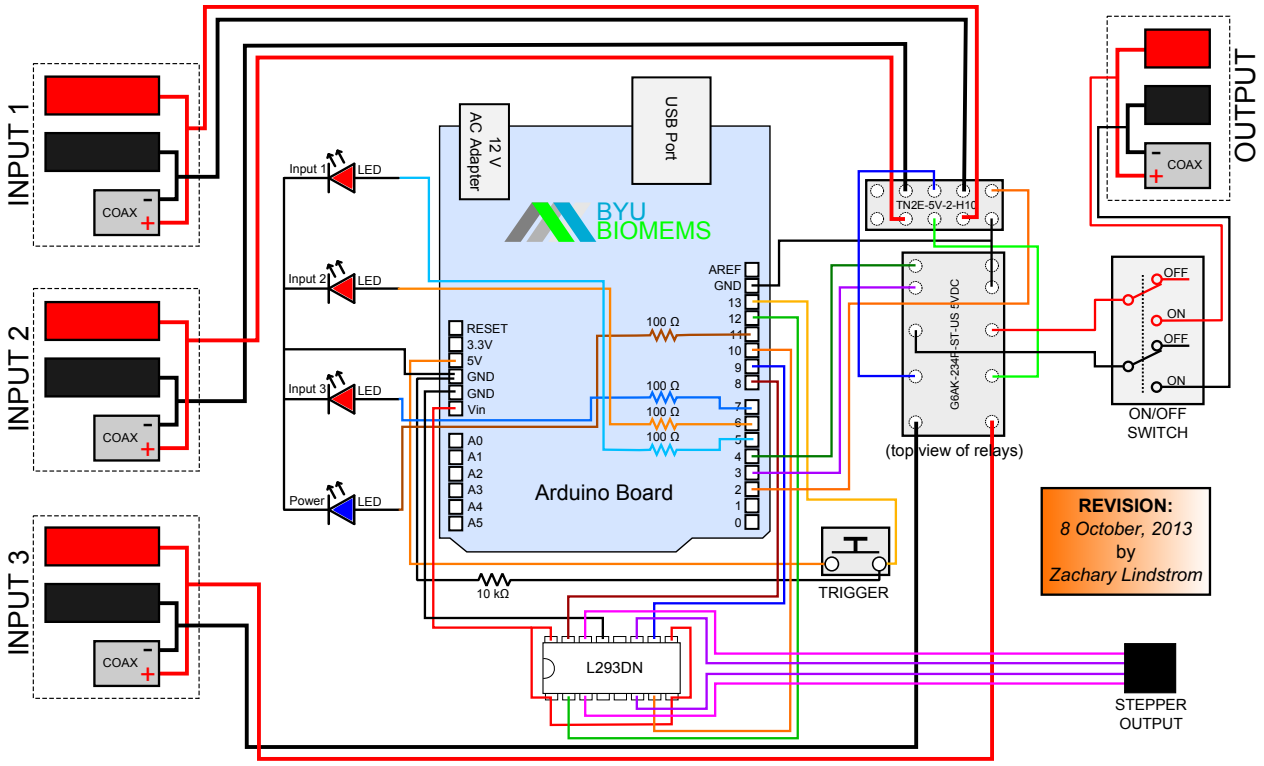


Figure 2.2: Wiring for the automated stepper motor controller box, courtesy of Zachary Lindstrom.

5% carbon dioxide. The cells were placed onto 22mm glass slips in 6-well testing plates to ensure a flat surface. The cells were placed in the plates 24 hours before injection to allow sufficient time for them to adhere to the glass. Each plate had 1 untreated sample, 1 negative control with PI added, and 4 test samples. For a detailed protocol of this process, see Appendix A.2.

### Injection Protocol

The injections were performed with a different PI concentration for each plate. Each plate had an untreated sample and a negative control with PI added to allow normalization of the 4 test samples. Once the plates had been prepared for testing, they were removed from the incubator, rinsed with HBSS, and then given fresh HBSS after they had been rinsed. Propidium idodide was then added to the negative controls and test sample wells to bring the concentration to the desired amount for each plate. Once the PI had been added, the injection device was used in each test

sample well to inject the cells for 5 seconds with the silicon lance array using 8 Newtons of force. For a detailed protocol of this process, see Appendix A.3.

#### Untreated Sample Protocol

The untreated samples were cultured exactly the same as the test samples, but no PI was added and no injections were performed. Flow cytometry preparation and flow cytometery measurements were performed exactly the same as with the test samples.

#### Negative Control Protocol

The negative controls were cultured exactly the same as the test samples and PI was added, but no injections were performed. Flow cytometry preparation and flow cytometery measurements were performed exactly the same as with the test samples.

### 2.2.3 Flow Cytometry

#### Preparation

After the injections were completed, trypsin was used to remove the cells from the well and they were centrifuged at 2000 rpm for 10 minutes. The supernatant was then removed and 0.25 mL of HBSS was added to each sample. Once the HBSS was added, each sample was vortexed to prepare them for flow cytometry. For a detailed protocol of this process, see Appendix A.3.

#### Measurements

Once the flow cytometry preparation was finished, the viability and PI concentration of the cells in each sample were measured using flow cytometry. During the analysis, 10,000 events were taken from each sample to ensure statistical accuracy. Using Attune Cytometric Software, gates were placed on a scatter plot of the forward and side scatter of the cells to determine how many cells were alive and which cells had PI. The PI concentration (noise) in the untreated samples were used to determine what concentration of PI was considered to be PI positive. Background PI noise is usually about 50 PI positive cells per sample (both live and dead).

### Statistical Analysis

Statistical analysis was used to determine the PI uptake and cell viability. To do this, the cell viability in the test samples were normalized to the untreated samples, and the PI uptake measurements were normalized to the negative controls to prevent bias from clustered data. For cell viability, the fraction of living cells in each test sample was divided by the average fraction of living cells in the untreated samples. The PI uptake was calculated by normalizing to the negative control PI concentration in the same way. Cells were considered to have PI uptake if they were both living and PI positive. More details for these methods will be given in Section 3.2.4.

Since this experiment involves more than two population means, an ANOVA table for both the PI uptake and the cell viability was calculated to determine if there was a statistically significant difference between the population means. If a staistically significant difference was found, the student's t-test was performed between each population pair to determine where the differences were.

## 2.3 Results and Discussion

### 2.3.1 PI Uptake

Table 2.1: ANOVA Table for normalized PI uptake with varying concentrations of PI, ranging from 0.02 to 0.15 mg/mL HBSS. The table is calculated as a single factor, five level, multi-replicate experiment at $\alpha = 0.05$. Since F is larger than $F_{crit}$, this means that there is a statistically significant difference between two or more population means.

ANOVA

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Groups | 155.2166 | 5 | 31.04332 | 95.51553 | 9.43E-24 | 2.408514 |
| Within Groups | 15.60039 | 48 | 0.325008 | | | |
| | | | | | | |
| Total | 170.817 | 53 | | | | |

From the PI uptake results shown in Figure 2.3, it is obvious that the concentration has an effect on the uptake (see Figure 2.4 for a linear scale). The results of the ANOVA table for the PI

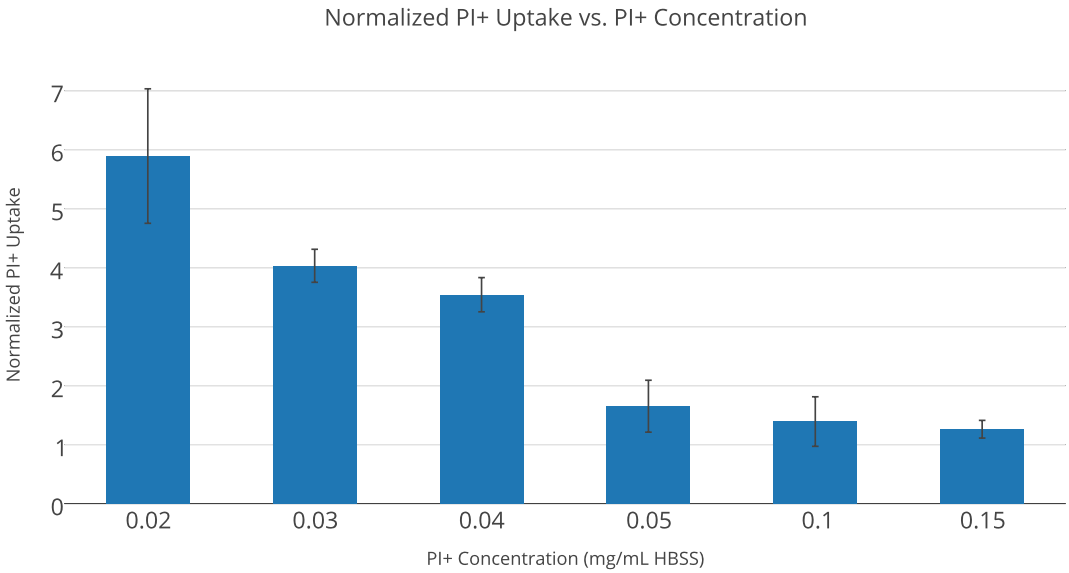


Figure 2.3: Normalized PI uptake using varying concentrations of PI in the injection solution.

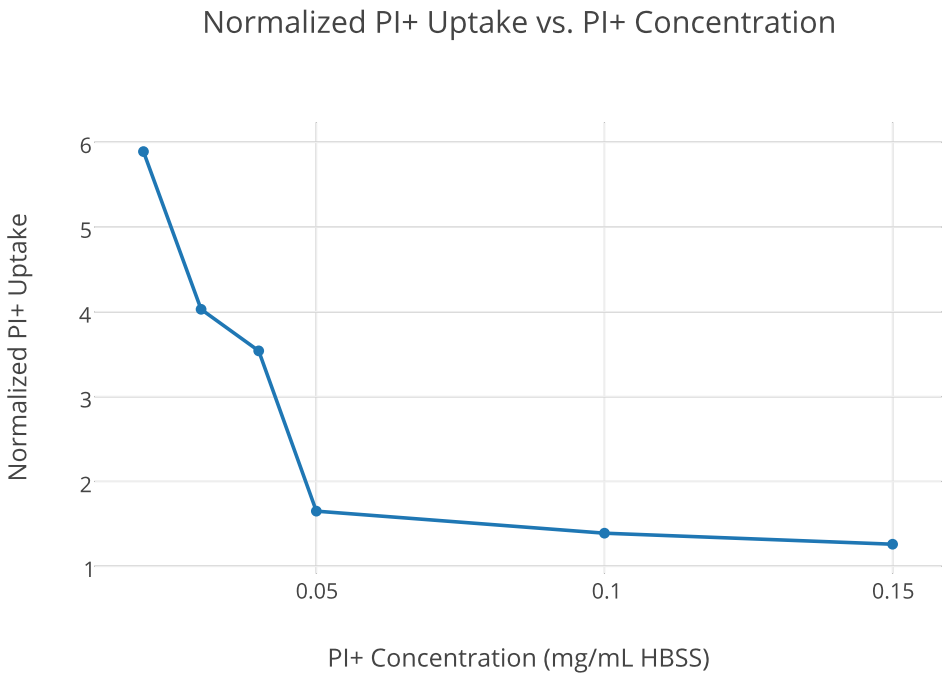


Figure 2.4: Normalized PI uptake using varying concentrations of PI in the injection solution (Linear Scale).

Table 2.2: P-values for normalized PI uptake with varying concentrations of PI, ranging from 0.02 to 0.15 mg/mL HBSS. The student's t-test was performed assuming a two-tailed distribution and two-sample unequal variance. P-values of less than 0.05 were considered statistically significant.

| | **0.02** | **0.03** | **0.04** | **0.05** | **0.1** | **0.15** |
|---|---|---|---|---|---|---|
| **0.02** | – | 4.91E-04 | 7.66E-05 | 1.45E-07 | 8.90E-08 | 3.13E-07 |
| **0.03** | | – | 1.10E-03 | 2.90E-09 | 2.92E-10 | 1.20E-13 |
| **0.04** | | | – | 4.17E-08 | 3.40E-09 | 4.93E-12 |
| **0.05** | | | | – | 2.20E-01 | 3.12E-02 |
| **0.1** | | | | | – | 3.95E-01 |
| **0.15** | | | | | | – |

uptake can be seen in Table 2.1. Since the $F$ value is larger than $F_{crit}$ in the analysis of variance, there is a statistically significant difference between two or more of the population means. To determine which population means were different, the student's t-test was performed between all population pairs. The p-values from the student's t-tests of each population mean comparison of PI uptake can be seen in Table 2.2. All of the population means were found to be different from each other except the concentrations of 0.05 compared to 0.1 mg/mL, and 0.1 compared to 0.15 mg/mL. These statistical findings agree with the visual representation of the data seen in Figure 2.3. The lowest concentration of PI tested has the highest average PI uptake at 5.89 when normalized to the negative controls, and thus the best sensitivity. This agrees with our hypothesis that the lower PI concentrations would have better sensitivity, likely because of less residual PI in the cell solution. The lowest concentration of PI also has the highest variation, with a standard deviation of 1.14. Since the PI uptake at 0.02 mg/mL HBSS is so much larger than the other concentrations, this is the concentration that was used for the investigation of other parameters in the nanoinjection process.

### 2.3.2 Cell Survival

The cell survival data can be seen in Figure 2.5 (see Figure 2.6 for a linear scale). Viability had no clear trend when compared to the concentration of PI in the HBSS. The analysis of variance (Figure 2.3) shows that there is a difference, however, between at least two of the population means. The p-values of each population mean comparison of cell viability can be seen in Table 2.4. The viability of the cells at a concentration of 0.15 mg/mL is statistically different from all

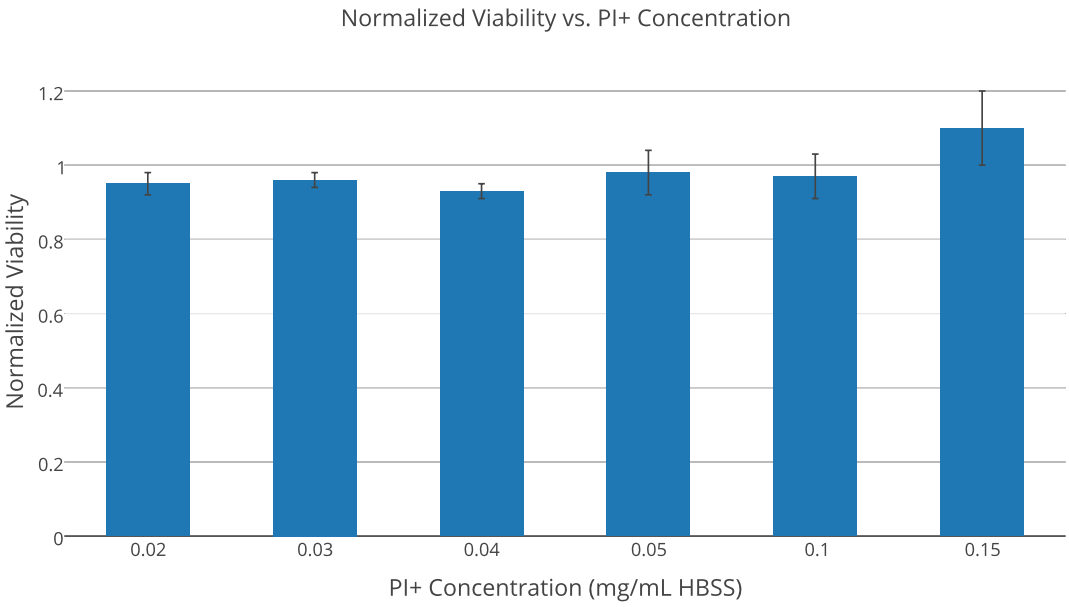


Figure 2.5: Normalized cell viability using varying concentrations of PI in the injection solution.

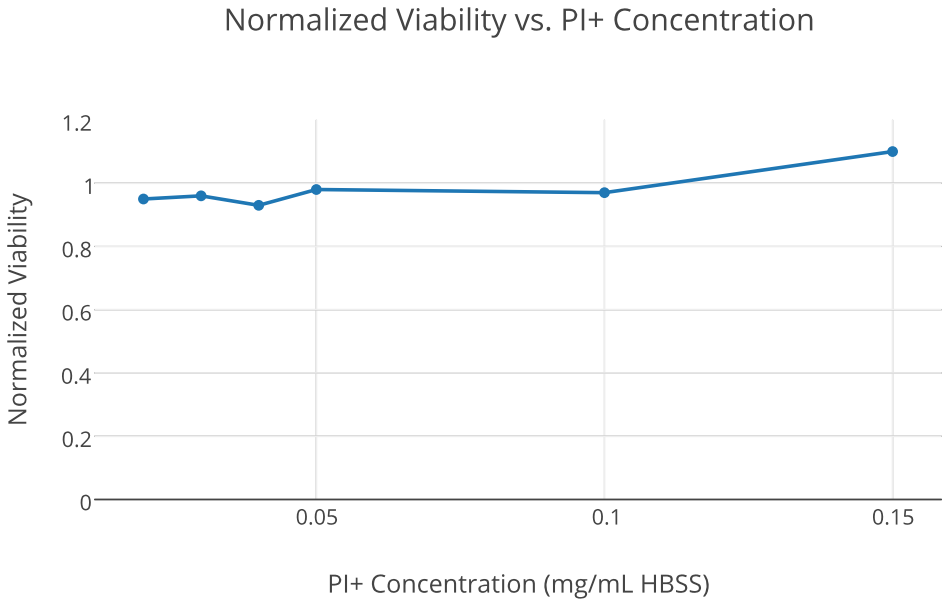


Figure 2.6: Normalized cell viability using varying concentrations of PI in the injection solution.

Table 2.3: ANOVA Table for normalized cell viability with varying concentrations of PI, ranging from 0.02 to 0.15 mg/mL HBSS. The table is calculated as a single factor, five level, multi-replicate experiment at $\alpha = 0.05$. Since F is larger than $F_{crit}$, this means that there is a statistically significant difference between two or more population means.

ANOVA

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Groups | 0.163453 | 5 | 0.032691 | 10.7297 | 5.83E-07 | 2.408514 |
| Within Groups | 0.146243 | 48 | 0.003047 | | | |
| | | | | | | |
| Total | 0.309696 | 53 | | | | |

Table 2.4: P-values for normalized cell viability with varying concentrations of PI, ranging from 0.02 to 0.15 mg/mL HBSS. The student's t-test was performed assuming a two-tailed distribution and two-sample unequal variance. P-values of less than 0.05 were considered statistically significant.

| | **0.02** | **0.03** | **0.04** | **0.05** | **0.10** | **0.15** |
|---|---|---|---|---|---|---|
| **0.02** | – | 0.1651 | 0.1622 | 0.1173 | 0.3038 | 0.0017 |
| **0.03** | | – | 0.0015 | 0.3177 | 0.6806 | 0.0033 |
| **0.04** | | | – | 0.0323 | 0.0982 | 0.0010 |
| **0.05** | | | | – | 0.6612 | 0.0094 |
| **0.10** | | | | | – | 0.0053 |
| **0.15** | | | | | | – |

other population means as well as a few other sets. There doesn't appear to be a clear trend, however, and the viability at 0.02 mg/mL HBSS is acceptable.

# CHAPTER 3. FORCE INJECTIONS

*Note: The following chapter has been accepted as a stand-alone paper for the ASME 2015 International Design Engineering Technical Conference in Boston, Massachusetts. Consequently, some of the material may be a restatement of material given in previous chapters because it was needed to provide context to the audience of this paper.

## 3.1 Introduction

Using a lance array containing millions of needles allows hundreds of thousands of cells to be injected simultaneously. This is important for applications requiring a large numbers of cells and for making the delivery method cost effective. To make the injection process even more effective, key parameters are currently being researched such as saline solution type, the use of voltage (both DC and pulsed), lances made from carbon-infiltrated nanotubes, etc. In this experiment, the force used to inject the cells was studied to determine its effects on the efficacy of the process and the viability of the cells. I expect there to be a slow rise in PI uptake as the force increases, and then a more dramatic increase when the cells are pierced. After reaching a maximum, it will likely level off once the cells have all been pierced by the lances. Cultures were injected with PI using different forces, and the results were measured using flow cytometry.

## 3.2 Methods

### 3.2.1 Nanoinjection Process

#### Overview

The nanoinjection process allows hundreds of thousands of cells to be injected at once. This is done using silicon chips that have been fabricated with millions of MEMS-scale lances in

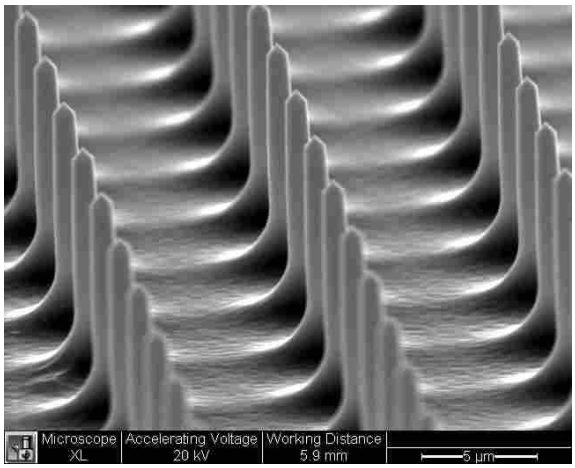


Figure 3.1: Typical silicon lance array created using MEMS manufacturing techniques. Image courtesy of Nicholas Gregory.

an array. The chips are placed onto an injection device which utilizes a stepper motor with a lead screw in conjunction with two orthoplanar springs in parallel to generate linear motion downward. With the lance array chip attached to the bottom of the injector, this downward motion causes the lances to penetrate the HeLa cell membranes. The injection of these cells is done in Hanks Balanced Salt Solution (HBSS) with propidium iodide added so that the results of the injections can be seen in flow cytometry. During these tests, the amount of force used to press the lance array down onto the cells was varied to see how it affects the injection efficiency.

**Lance Array Fabrication**

The lance arrays used were patterned using standard photolithography techniques. A 2 $\mu$m film of AZ 3330 positive photoresist is spun onto a silicon wafer and then aligned and exposed to form a grid of photoresist dots that are 5 $\mu$m in diameter and 10 $\mu$m from center to center. Once the photoresist has been developed, an isotropic plasma etch using sulfur hexafluoride ($SF_6$) is then performed, which undercuts the photoresist, producing the start of the lance tips. A 5 minute Bosch anisotropic deep reactive ion etch (DRIE) then utilizes etching and passivation (alternating between the two) to create the tall lances. Once this has been done, acetone and a 2 minute oxygen plasma etch are used to strip the remaining photoresist from the wafer, followed by a 30 second hydrofluoric acid wet etch to remove any residual silicon dioxide that may have formed during the

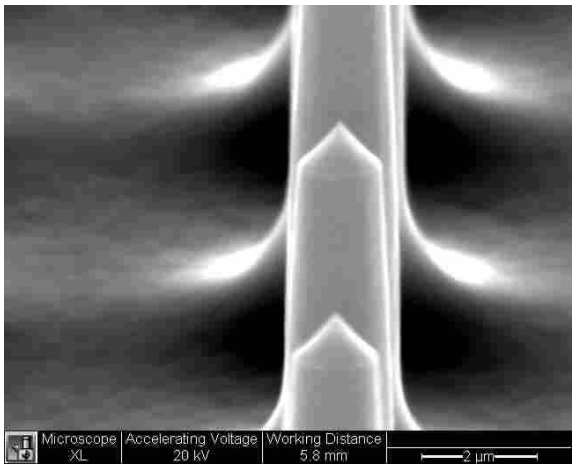


Figure 3.2: Individual lances. Image courtesy of Nicholas Gregory

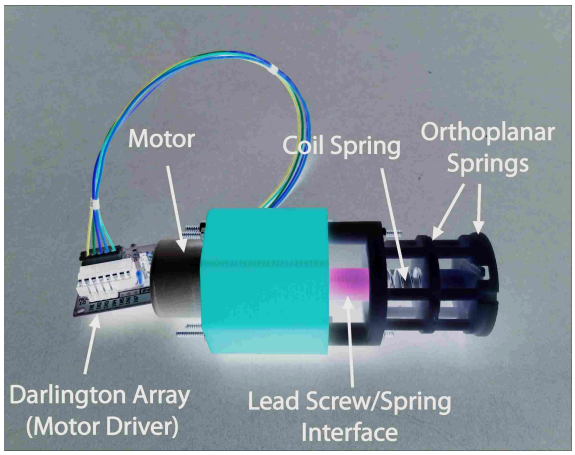


Figure 3.3: Incremental force injection device with motor attached.

oxygen plasma etch. Lastly, the lance diameter is measured and the appropriate time is determined for the final $SF_6$ etch ($\sim$ 35 seconds). This makes the final tip on the lance, removes the scalloping from the DRIE, and brings the final diameter and height of the lances to 1-2 $\mu$m and 8-10 $\mu$m, respectively. The wafer is then diced into 2 cm square chips that each have an estimated 4 million lances.

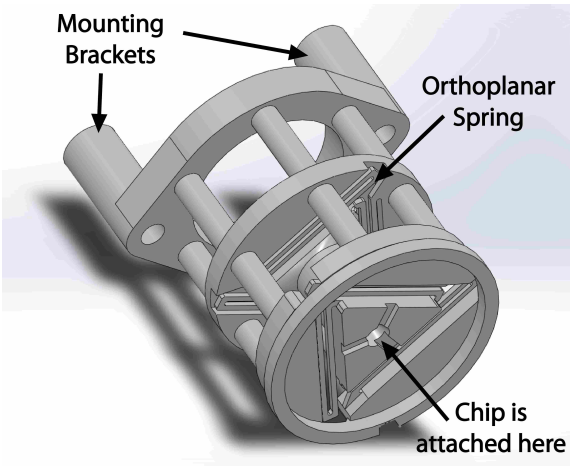


Figure 3.4: CAD model of the incremental force injection device.

**Injection Device**

The injection device that was used to inject the HeLa cells was constructed with ABS using rapid prototyping techniques. It was designed to have a high resolution of displacement so that the force during the injection process could be accurately controlled and would be repeatable. To achieve this, a stepper motor with 64 steps per revolution and a gear ratio of 64:1 was used, giving an effective 4096 steps per revolution of the motor output. As can be seen in Figure 3.3, the stepper motor (driven with an Arduino) was mounted to a base, and the output of the motor was attached to an 8-32 threaded rod. This rod acted as a lead screw, moving a coupling nut up and down through a shaft that didn't allow the coupling nut to turn. This coupling nut was then attached to the top end of a coil spring used to press down the injection device. A coil spring was used rather than connecting the lead screw directly to the orthoplanar springs, so that when the chip contacted the glass slip the force would continue to increase slowly. A resolution of 4096 steps per revolution and 32 threads per inch resulted in a resolution of 0.19 $\mu$m per step. To avoid backlash in the system, a second spring was used that resisted the displacement of the coupling nut relative to the base. This did not affect the displacement of the coupling nut, since it was a displacement load and not a force load as a result of the high mechanical advantage.

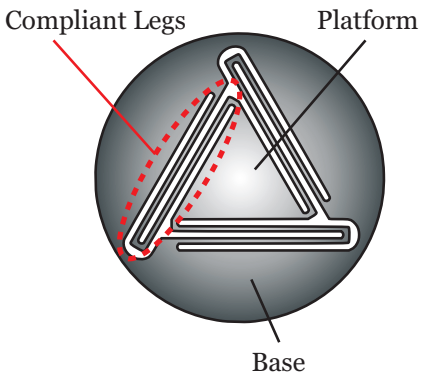


Figure 3.5: Orthoplanar spring on the bottom of the injection device.

### Propidium Iodide

Propidium iodide (PI) is an intercalating agent that can be used to stain cells and detect cell viability. When PI is in the presence of DNA, it binds to it by intercalating between the base pairs. [41–47] Once bound to the DNA, the fluorescence of PI increases greatly, which allows it to be detected through the use of flow cytometry. These characteristics, combined with the fact that PI doesn't penetrate living cells, make PI a good candidate molecule to monitor the effects of varying the force of injection on the HeLa 229 cancer cells using the nanoinjection lance array. Any cells found with a high enough concentration of PI were either successfully injected with the lance array or are no longer alive, which can be determined using the flow cytometry cell cross scatter between granularity and size (forward scatter vs. side scatter).

## 3.2.2 Calculation of Injection Force

As the stepper motor lead screw moves the coupling nut down, the spring becomes compressed and transfers force to the lance array through the orthoplanar spring and onto the HeLa cell culture. The spring in the injection device has a stiffness of 2.85 N/mm, which is appropriate for the range of the deflection and the force desired. Because such small deflections of the spring are used, it is considered to act linearly throughout its range of motion. The high resolution of deflection control from the stepper motor allows the force to be controlled and calculated very accurately. The number of steps from the stepper motor controls the deflection, from which the force can be calculated using the spring stiffness.

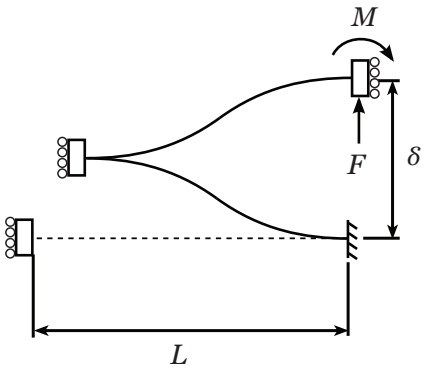


Figure 3.6: Side-view of a single leg from the orthoplanar spring on the injection device.

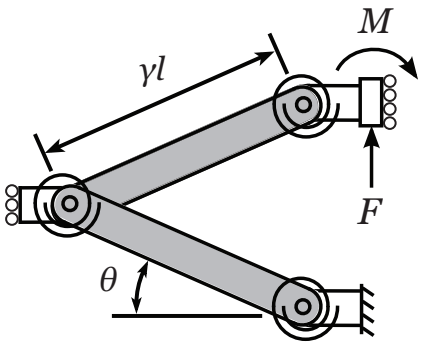


Figure 3.7: Pseudo-rigid-body model of a single leg of the orthoplanar spring.

The orthoplanar spring must also be factored in, however. It prevents rotation of the lance array during the injection of the HeLa cells, but it also counteracts a portion of the force during this process as it is displaced downward. Therefore it must be taken into account when calculating the total injection force from the displacement. As can be seen in Figure 3.5 and 3.6, the orthoplanar spring consists of three legs, each made from 2 flexible segments in series with each other, that connect a moveable plate to the main injection device. Two of these orthoplanar springs are implemented in parallel to keep the lance array parallel to the cell culture during injection.

To determine the force-deflection relationship, the orthoplanar spring can be modeled using a pseudo-rigid-body model (see Figure 3.7). This model is comprised of rigid links held together with revolute joints and torsional springs to represent the stiffness of the spring. [48, 48] The force for each leg can be calculated using:

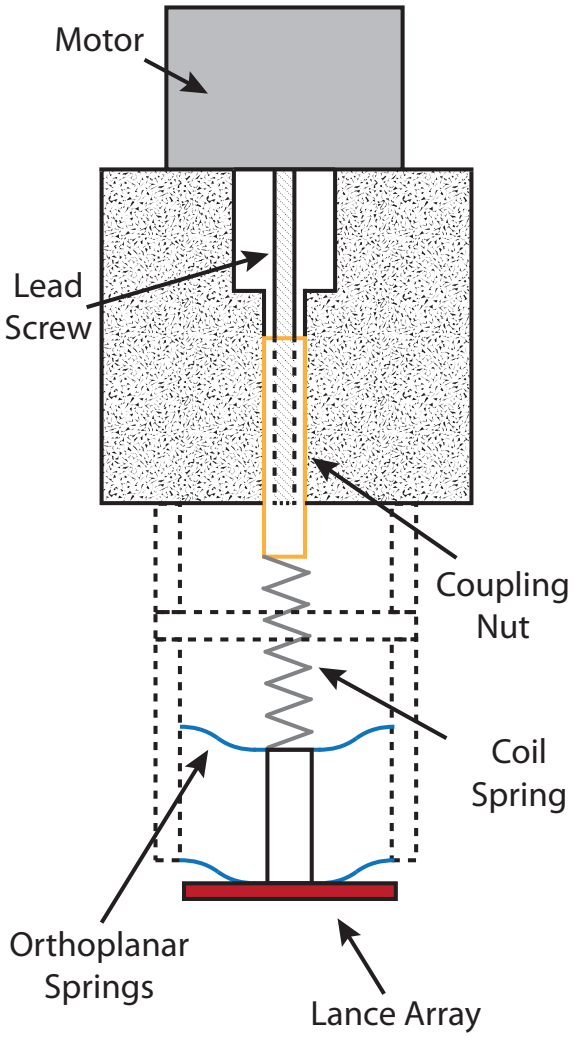


Figure 3.8: Cross section view of the Injection Device.

$$F_{leg} = \frac{4K_\theta EI\theta}{L^2 cos\theta} \tag{3.1}$$

and

$$\theta = sin^{-1}\left(\frac{\delta_{chip}}{2\gamma L}\right) \tag{3.2}$$

where $K_\theta$ is the stiffness coefficient, L is the length of each of the individual flexible segments, E is Young's modulus, I is the area moment of inertia, $\theta$ is the angular displacement, $\delta_{chip}$ is the deflection of the chip from its original position, and $\gamma$ is the characteristic radius factor. In this case,

$K_\theta \approx 2.65$ and $\gamma \approx 0.85$. [48,49] Due to the geometry of the injection device, the silicon lance array has 1 mm of clearance before it reaches the glass slip. During this time, the resistance force from the deflected orthoplanar springs is equal to the force pressing down from the lead screw and the force exerted on the chip is zero because it hasn't yet made contact with the glass slip. The force from the orthoplanar springs resisting deflection stops increasing once the chip has made contact with the glass, and remains constant. Since we are only concerned with the force when the chip is pressing down on the glass slip containing the cells, we will consider $\delta_{chip}$ to be a constant of 1 mm (0.001 m).

The force transferred by the coil spring as the lead screw moves downward continues to increase, even after the chip has made contact with the glass and the resisting force from the orthoplanar springs has reached a maximum and remains constant. The total force can be calculated by using the coil spring stiffness and deflection, and then subtracting the resisting force from the orthoplanar springs. It should be noted that the deflection of the coil spring is equal to the difference between the deflection of the lead screw and the deflection of the chip (or orthoplanar spring). This can be written as

$$\delta_{coil} = \delta_{lead} - \delta_{chip} = \delta_{lead} - 1 \tag{3.3}$$

where $\delta_{coil}$ and $\delta_{lead}$ are in mm. The total force exerted on the chip (in Newtons), then, is

$$F_{chip} = \delta_{coil} k_{coil} - n\frac{4K_\theta EI\theta}{L^2 cos\theta} \tag{3.4}$$

where n is the number of legs from the orthoplanar springs. Inserting the symbolic values of the coil spring deflection and the compliant leg forces from Equations 3.1, 3.2, and 3.3 we get

$$F_{chip} = (\delta_{lead} - 1)k_{coil} - \frac{24K_\theta EIsin^{-1}\left(\frac{0.001}{2\gamma L}\right)}{L^2 cos\left(sin^{-1}\left(\frac{0.001}{2\gamma L}\right)\right)} \tag{3.5}$$

$$= 2.855\delta_{lead} - 2.98 \tag{3.6}$$

where $\delta_{lead}$ is the deflection of the lead screw downward in mm. This equation is only valid after the chip has made contact with the glass. At any point before this, the force exerted on the chip is zero. To find the deflection necessary from the lead screw to get a specified force, the equation can be rearranged to get

$$\delta_{lead} = 0.35F_{desired} + 1.042 \tag{3.7}$$

where $\delta_{lead}$ is in mm and $F_{desired}$ is in Newtons.

### 3.2.3 Injection Methods

#### Key Parameters

A sample size of about 8 per force magnitude is statistically acceptable. However, variation is expected due to the nature of biological research and outliers would doubtless exist. Therefore, 16 samples per force magnitude were injected with the expectation that we would have a statistically significant sample after outliers were removed.

The forces that were examined were from 0.2 to 2.2 Newtons in increments of 0.2 Newtons, as well as 3, 4, and 5 Newtons. A higher resolution of data was desired in the lower range of force where the silicon lances first start to puncture the cells. This means that 14 different forces were tested with 16 samples per force increment, resulting in a total of 224 samples, not including the untreated samples and negative controls.

#### Cell Testing Plate Preparation

Dulbeccos Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum and 5% penicillin and streptomycin was used to culture the HeLa 229 cancer cells in an incubator at 37 degrees Celsius and 5% carbon dioxide. The cells were placed onto 22mm glass slips in 6-well testing plates to ensure a flat surface. The cells were placed in the plates 24 hours before injection to allow sufficient time for them to adhere to the glass. Each plate had 1 untreated, 1 negative control with PI added, and 4 test samples.

#### Injection Protocol

The injections were performed in sets of 4 plates with a different injection force for each plate, producing 4 untreated samples, 4 negative controls, and 4 sets of injection force magnitude samples. Once the plates had been prepared for testing, they were removed from the incubator, rinsed with HBSS, and then given fresh HBSS after they had been rinsed. Propidium idodide was then added to the negative controls and test sample wells to bring the concentration to 0.02 mg PI/mL HBSS. Once the PI had been added, the injection device was used in each test sample well to inject the cells with the silicon lance array using the specified force.

#### Untreated Sample Protocol

The untreated samples were cultured exactly the same as the test samples, but no PI was added and no injections were performed. Flow cytometry preparation and flow cytometery measurements were performed exactly the same as with the test samples.

#### Negative Control Protocol

The negative controls were cultured exactly the same as the test samples and PI was added, but no injections were performed. Flow cytometry preparation and flow cytometery measurements were performed exactly the same as with the test samples.

### 3.2.4 Flow Cytometry

#### Preparation

After the injections were completed, trypsin was used to remove the cells from the well and they were centrifuged at 2000 rpm for 10 minutes. The supernatant was then removed and 0.25 mL of HBSS was added to each sample. Once the HBSS was added, each sample was vortexed to prepare them for flow cytometry.

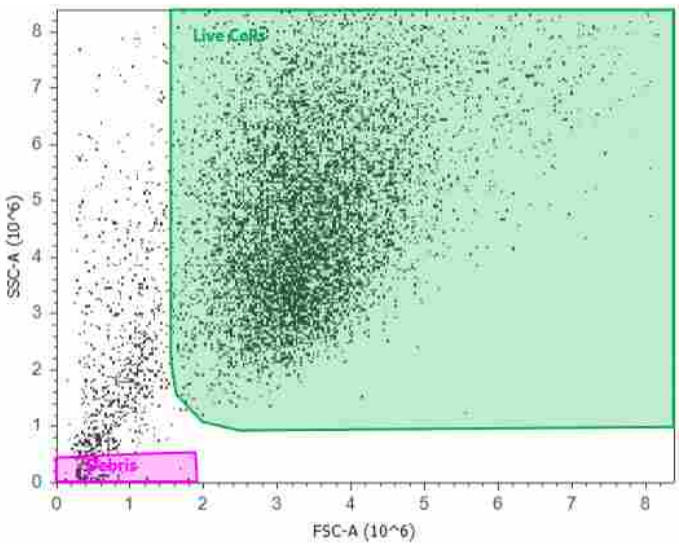


Figure 3.9: Typical cross scatter using flow cytometry.

**Measurements**

Once the flow cytometry preparation was finished, the viability and PI concentration of the cells in each sample were measured using flow cytometry. During the analysis, 10,000 events were taken from each sample to ensure statistical accuracy. Using Attune Cytometric Software, the forward and side scatter of the cells could be seen (see Figure 3.9) and quantified, as well as the propidium iodide concentration. The PI concentration (noise) in the untreated samples was used to determine what concentration of PI was considered to be PI positive.

**Statistical Analysis**

Statistical analysis was then used to determine PI uptake and cell viability. To do this, the cell viability in the test samples were normalized to the untreated samples, and the PI uptake measurements were normalized to the negative controls. After normalizing the data to the controls, a modified Thompson Tau test was performed to remove any outliers in the data.

Since the data in each of the test samples are being normalized to the controls, it was important that there were as many controls of each type as there were test samples so that the values obtained from them are as statistically significant as the test samples. Therefore, the viabilities of

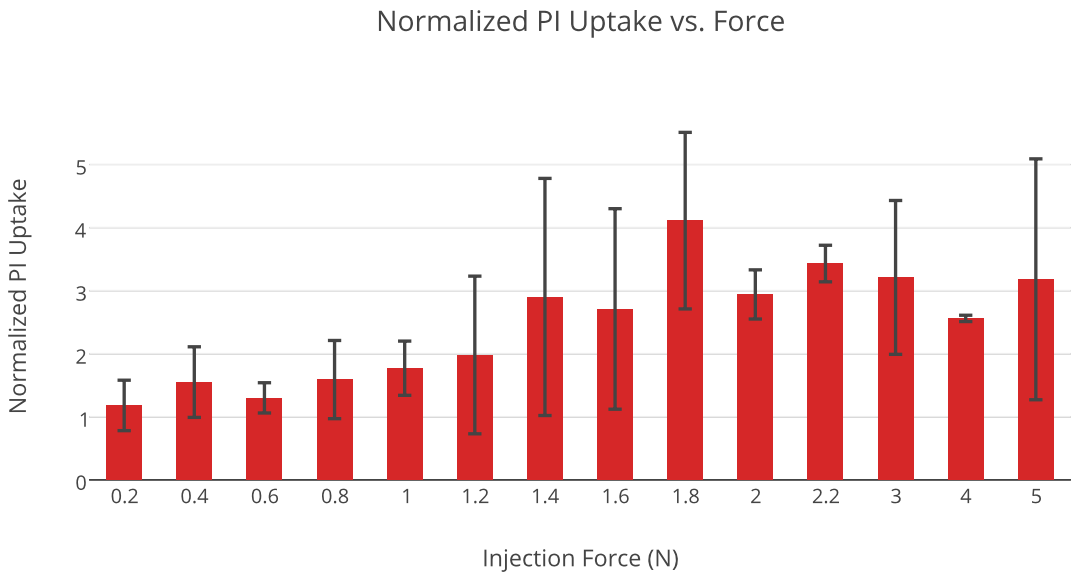


Figure 3.10: Normalized PI uptake after injection.

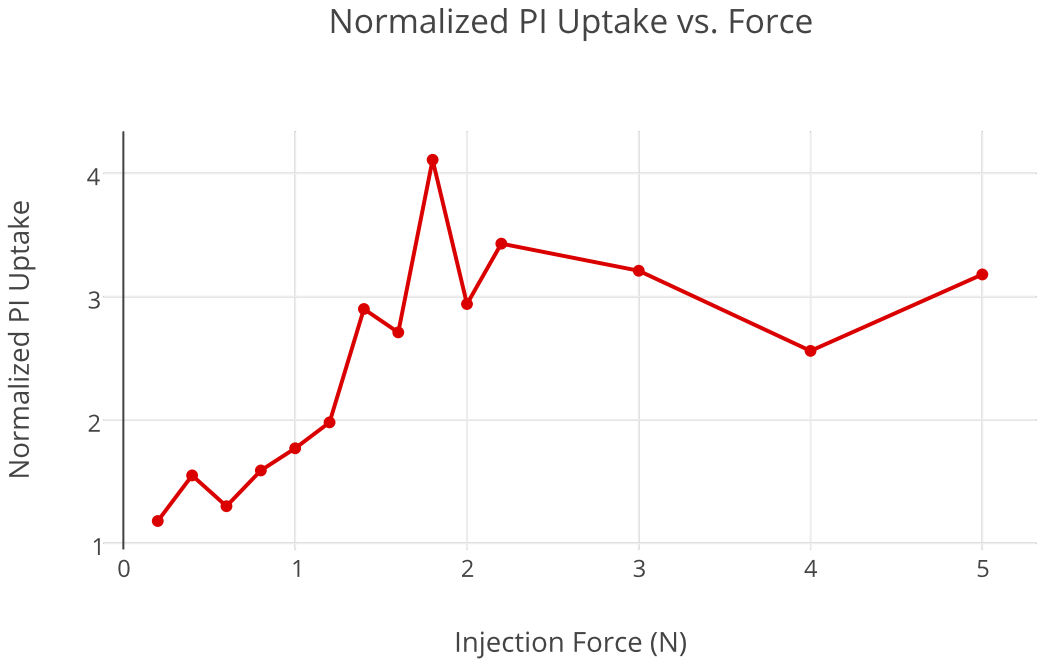


Figure 3.11: Normalized PI uptake after injection (Linear Scale).

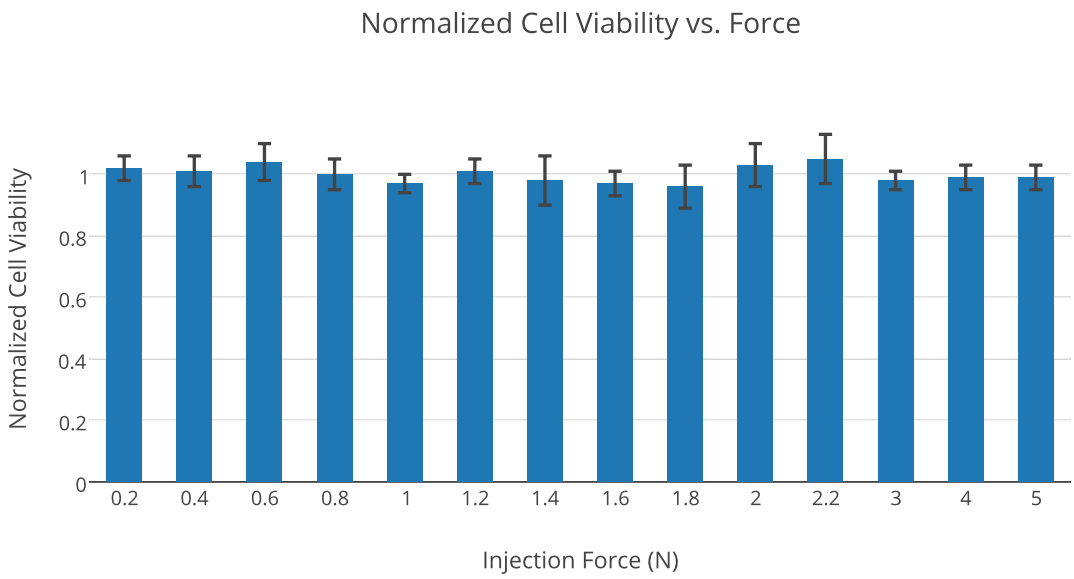


Figure 3.12: Cell viability after the injection process.

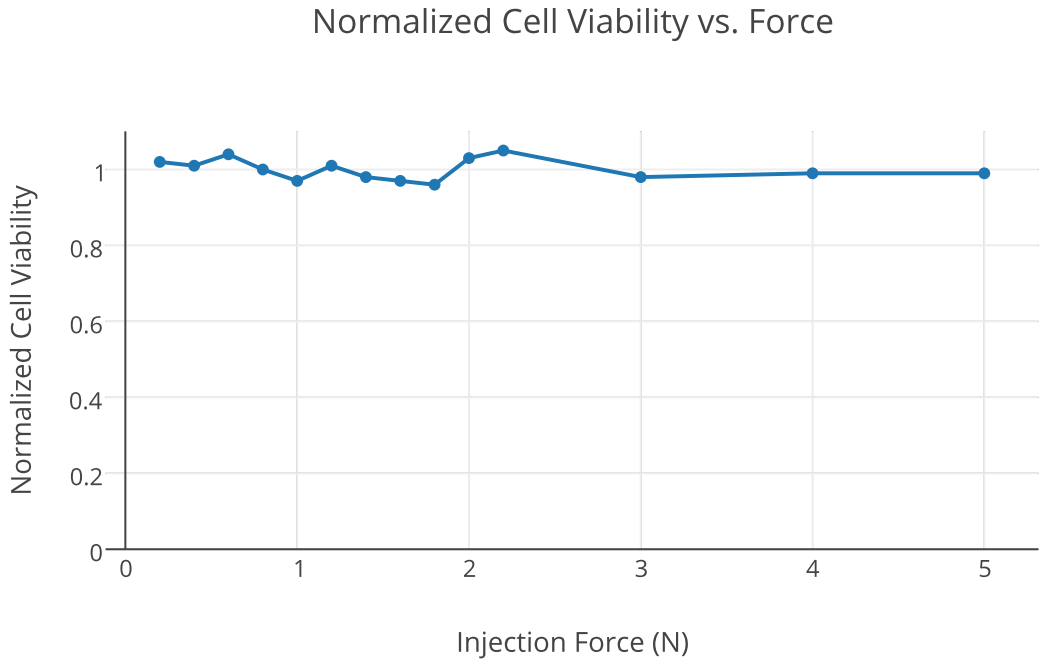


Figure 3.13: Cell viability after the injection process (Linear Scale).

Table 3.1: P-values for normalized PI uptake after injection. The student's t-test was performed assuming a two-tailed distribution and two-sample unequal variance. P-values of less than 0.05 were considered statistically significant.

| | **0.4** | **0.6** | **0.8** | **1** | **1.2** | **1.4** | **1.6** | **1.8** | **2** | **2.2** | **3** | **4** | **5** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **0.2** | 0.09 | 0.50 | 0.04 | 0.02 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **0.4** | – | 0.18 | 0.85 | 0.34 | 0.18 | 0.02 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| **0.6** | | – | 0.08 | 0.03 | 0.02 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| **0.8** | | | – | 0.39 | 0.20 | 0.02 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.02 |
| **1** | | | | – | 0.53 | 0.04 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.03 |
| **1.2** | | | | | – | 0.11 | 0.16 | 0.00 | 0.00 | 0.00 | 0.01 | 0.04 | 0.07 |
| **1.4** | | | | | | – | 0.77 | 0.05 | 0.94 | 0.32 | 0.62 | 0.49 | 0.71 |
| **1.6** | | | | | | | – | 0.02 | 0.61 | 0.13 | 0.38 | 0.73 | 0.50 |
| **1.8** | | | | | | | | – | 0.01 | 0.10 | 0.08 | 0.00 | 0.17 |
| **2** | | | | | | | | | – | 0.07 | 0.49 | 0.01 | 0.67 |
| **2.2** | | | | | | | | | | – | 0.57 | 0.03 | 0.67 |
| **3** | | | | | | | | | | | – | 0.09 | 0.97 |
| **4** | | | | | | | | | | | | – | 0.28 |
| **5** | | | | | | | | | | | | | – |

the untreated samples (NC) were averaged, as well as the PI uptake measurements for the negative controls (NC).

For cell viability, the fraction of living cells in each test sample was divided by the average fraction of living cells in the untreated samples:

$$US\ Viability = \frac{Number\ of\ Living\ US\ Cells}{Total\ Number\ of\ US\ Cells} \tag{3.8}$$

$$Sample\ Viability = \frac{Number\ of\ Living\ Sample\ Cells}{Total\ Number\ of\ Sample\ Cells} \tag{3.9}$$

$$Normalized\ Viability = \frac{Sample\ Viability}{US\ Viability} \tag{3.10}$$

The PI uptake was calculated by normalizing to the negative control PI concentration in the same way. Cells were considered to have PI uptake if they were both living and PI positive:

$$NC\ PI\ = \frac{Number\ of\ Living\ AND\ PI^{+}\ NC\ Cells}{Total\ Number\ of\ Living\ NC\ Cells} \tag{3.11}$$

$$Sample\ PI\ = \frac{Number\ of\ Living\ AND\ PI^{+}\ Sample\ Cells}{Total\ Number\ of\ Living\ Sample\ Cells} \tag{3.12}$$

$$Normalized\ PI\ = \frac{Sample\ PI}{NC\ PI} \tag{3.13}$$

## 3.3 Results and Discussion

### 3.3.1 PI Uptake

From the data seen in Figure 3.10, a slow rise in PI uptake can be seen from 0.2 to 1.8 Newtons, starting at an uptake of 1.18 and reaching a maximum of 4.11 (refer to Figure 3.11 for a linear scale). The slow rise in PI uptake to a maximum was consistent with our hypothesis, but no dramatic increase was seen. This is because so many cells are being pierced at different times that it is statistically averaged and is a much more steady increase. The injections at higher forces drop slightly in PI uptake and become relatively level, averaging an uptake of approximately 3.1. This is also consistent with what we believed would occur. Table 3.1 shows p-values for the student's t-test for each of the injection forces. These values show that almost all of the injection forces of 1.4 Newtons and higher have a statistically significant difference from all of the injection forces of 1 Newton and below. The slow rise in PI uptake in the lower forces is most likely because the number of cells that are being penetrated by the lances increases due to the increasing injection force. The cell membrane is very elastic by nature, and the force at which a particular lance will puncture a specific cell varies depending on the position of the cell and the lance. The lances are 10 microns from center to center while the size of HeLa cancer cells vary from approximately 5-8 microns in width, 15-30 microns in length, and 5-6 microns in thickness. This means that anywhere from approximately 1 to 3 lances are pressing on each individual cell. More force results in more cells punctured until the lances are essentially all pressing on the glass slip that the cells are adhered to. It is possible that the elastic variation between cells contributes to some of the larger variations seen in portions of Figure 3.10. Once the lance array is removed from the cell culture, the holes left in the cell membrane allow PI in the saline solution to diffuse into the cells. Over time, the holes in the cell membrane will close, or the cell will die.

Using the total force from the injection process and the approximate number of lances interacting with the cells, we can also estimate the force required per lance to inject the cells. The number of lances must first be approximated. This can be done by multiplying by the total number of lances on one chip by the fractional area of the cell culture relative to the area of the entire chip. The cultures average 1 cm in diameter, giving an area of 0.78 $cm^2$. This means that 19.6 percent of the chip area is interacting with the cells, corresponding to 784,000 lances. The force at which the PI uptake appears to stop increasing is 1.8 Newtons, which likely means that almost all of the lances are pressed down against the glass slip under the cells at that force. This gives us an approximate force of 2.3 $\mu$N per lance to puncture the HeLa cells. It should also be noted from Table 3.1 that the PI uptake at 1.8 Newtons has a statistically significant difference from all of the other forces tested except 1.4 Newtons.

### 3.3.2 Cell Survival

Cell survival is critical in testing to having a successful transfection process. High efficiencies won't matter if most of the cells have died. As can be seen from the data in Figure 3.12, there is no observable trend in the HeLa cell viability with the varying injection force (see Figure 3.13 for a linear scale). Even in forces much higher than the first section of data, the viability is very comparable. Furthermore, the viability of the HeLa cells using this injection process are extremely close to that of the untreated samples. This means that cells that are lost are a result of natural or environmental causes rather than the injection process itself, which is very promising. Injecting the HeLa cells with solid lances rather than hollow lances allows them to be approximately one tenth of the size of hollow lances. The small size of the lances might be the reason for a much smaller loss of viability when the cells are punctured.

## 3.4 Conclusion

From the data it is obvious that the force of injection has an effect on the PI uptake and efficacy of the injection process. The uptake at 1.8 Newtons of force is nearly 4 times that of 0.2 Newtons of force and higher than any of the other forces tested. There is, however, large variability near this location. Therefore, if a high injection efficiency is desired and variability is

not a concern, this would be the ideal force to inject with this specific injection system. It should be kept in mind that changes in the fabrication of the lance array or the injection system may result in changes of the performance at different forces.

It should be noted that the results of the PI uptake represent how many whole cells have taken up PI, but they do not show how much of that PI has specifically entered into the nucleus of the cell. Further, they do not indicate how much DNA would be taken up by the cell, since DNA is much a much larger and more complex molecule than PI. Further investigation would be needed to determine these quantities.

Possible future work may include a higher resolution in the data for the injection force, or more samples. The rate of injection could also be a parameter that would affect the PI uptake, and may be worth testing. The investigation into the causes of the high variation at certain forces could be even more valuable. Based on the number of cells being injected, the force per lance may also be able to be calculated and used in the spherical Hertz model to estimate mechanical properties of the cell membrane, such the elastic modulus or stiffness.

### 3.5 Acknowledgment

This material is based upon work supported by the National Science Foundation under Grant No. ECCS-1055916.

# CHAPTER 4. COMPARISON OF SILICON AND CNT LANCE INJECTIONS

To gain insight into the effects of the lance material, a direct comparison is made between silicon and carbon nanotube (CNT) nanoinjection needles when injecting HeLa cells with DNA in 6-well plates. The experiment was done using pulsed voltage during the injection process and all variables remained constant except the chip type used. We expected the carbon nanotube lance arrays would yield a higher transfection rate because more DNA gets into the cells due to the surface roughness of the nanotube lance arrays. There are more places for the DNA to get stuck as it is attracted to the needles, so more of it can be transferred into the cell. The CNT lance arrays used in the experiments found in this chapter were fabricated by another student, Jason Lund. SEM images of the two different lance array types can be seen in Figure 4.1.

## 4.1 Methods

### 4.1.1 Summary of Key Parameters

A summary of the key parameters for the comparison between the silicon and CNT chips can be seen in Table 4.1. After these injections were performed, preliminary data using chloroquine with the different chips was desired to determine if further study would be beneficial. Chloroquine can be used to help prevent lysosomal degradation of the DNA as it travels inside the cell. Current control during the attraction and final repulsion process was used to prevent variation of current. A summary of parameters from these injections can be seen in Table 4.2.

### 4.1.2 Sample Size

A sample size of about 8 per chip type is statistically acceptable. However, significant variation is expected for our current injection protocols and outliers will doubtless exist. Therefore, for the injections with only the GFP plasmid 12 samples per chip type were done with the expectation

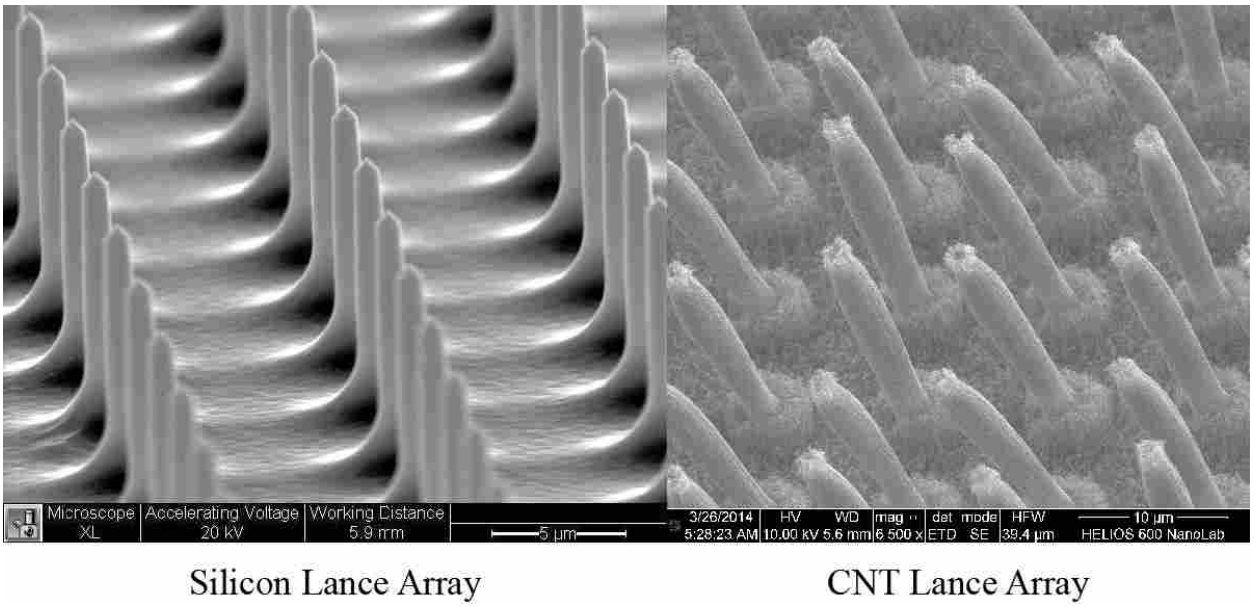


Figure 4.1: SEM images of the silicon and CNT lance arrays, courtesy of Jason Lund.

Table 4.1: Summary of critical experimental parameters for GFP plasmid injections.

| **Parameter** | **Value** | |
|---|---|---|
| Sample Size | Silicon | 12 |
| | CNT | 12 |
| Controls | Untreated | 12 |
| | Negative | 0 |
| Voltage | Silicon | +9V attract, -1.5V to -9V pulsed repel, -9V repel |
| | CNT | Match current (+2.7V), -1.5V to -2.7V pulsed repel, Match current (-2.7V) repel |
| Injection Solution | | Phosphate Buffered Saline (PBS) |
| DNA Concentration | | 2 $\mu$g/mL PBS |
| Molecular Load | | pCAG-GFP plasmid |

that we will have a statistically significant sample after outliers have been removed. Thus, a total of 6 plates were prepared and injected that have 2 untreated samples each. This resulted in a total of 12 silicon chip samples, 12 CNT samples, and a total of 12 untreated samples. See Figure 4.3.

Table 4.2: Summary of critical experimental parameters for GFP injections with chloroquine to inhibit lysosomal degradation of protein products.

| **Parameter** | **Value** | |
|---|---|---|
| Sample Size | Silicon | 8 |
| | CNT | 8 |
| Controls | Untreated | 8 |
| | Negative | 0 |
| Voltage | Silicon | +3 mA attract, -1.5V to -9V pulsed repel, -1 mA repel |
| | CNT | +3 mA attract, -1.5V to -9V pulsed repel, -1 mA repel |
| Injection Solution | | Phosphate Buffered Saline (PBS) |
| DNA Concentration | | 2 $\mu$g/mL PBS |
| Molecular Load | | pCAG-GFP plasmid + Chloroquine |

### 4.1.3 Chips

Silicon and CNT lance array chips were used for both injection types in this experiment. Each injection set consisted of 2 plates, one injected with a silicon lance array and one with a CNT lance array. This helps to ensure that any environmental factors affecting injections with one chip type at a specified time will also affect the other chip type.

### 4.1.4 GFP Plasmid

Previous experiments in this thesis have used PI as the molecular load for testing, but plasmids with DNA code for a green fluorescing protein (GFP) are also commonly used for experiments in gene therapy. If properly delivered to and integrated in the nucleus, the cell will produce fluorescing proteins that can also be detected through the use of flow cytometry. A plasmid was desired for testing because it more closely mimics experiments for inserting DNA into cells than PI. The CNT chips were also chosen to test to determine the effect of the surface roughness on large molecules such as DNA. The plasmid used was the pCAG-GFP plasmid (see Figure 4.2) and is approximately 5500 base pairs.

### 4.1.5 Plate Setup and Untreated Samples

Due to the nature of the DNA injection test, a negative control (DNA plasmid added, no injection) is not useful for the experiment itself. This is because the DNA cannot permeate the

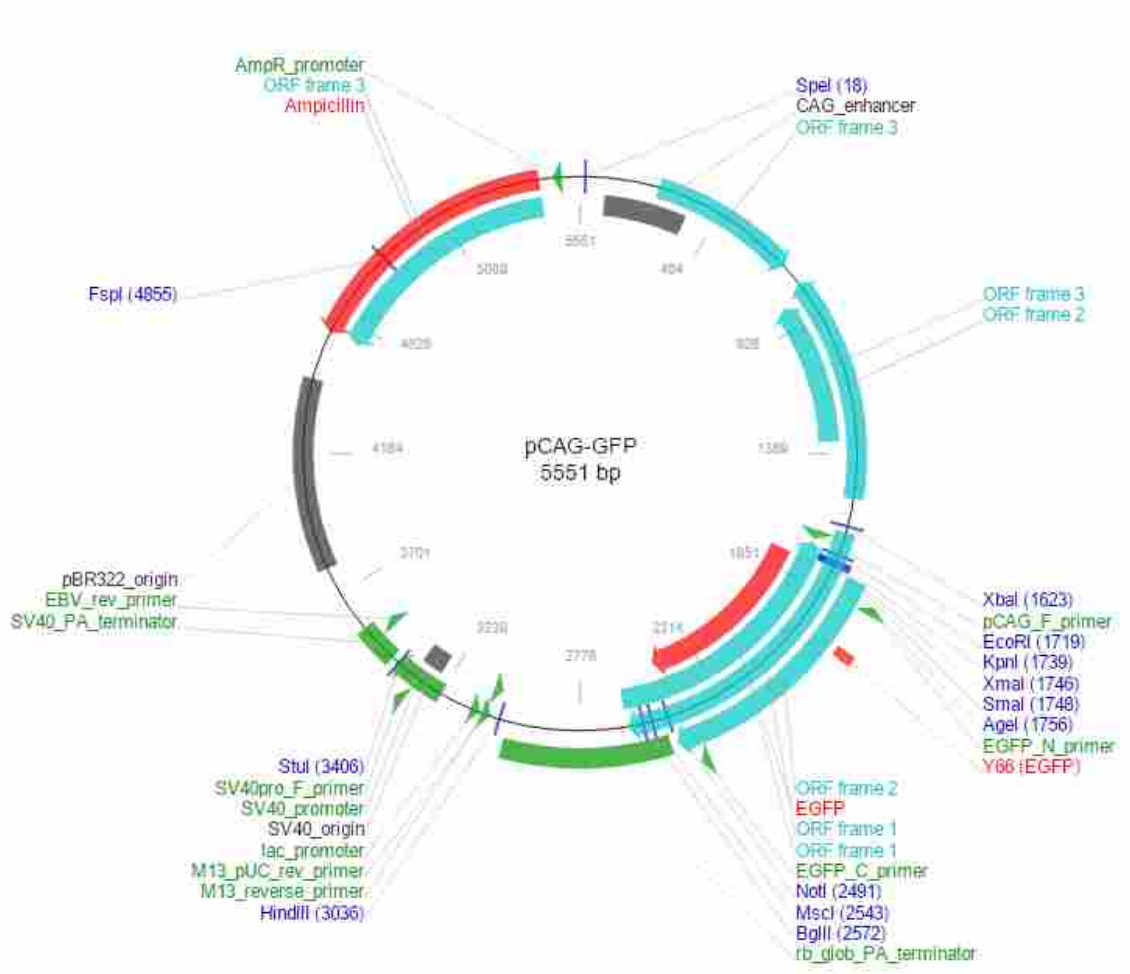


Figure 4.2: Schematic of the pCAG-GFP plasmid injected into the HeLa cells. Image courtesy of http://www.addgene.org/11150/

membrane of the cells by itself to cause gene expression, and any residual DNA stuck to the cells will not affect the flow cytometry analysis. A negative control for the instrument, however, is valuable and necessary for the flow cytometer approximately once a month. Therefore 2 untreated samples were used per 6-well plate as shown in Figure 4.3. Since the injections with silicon and CNT lances were side-by-side, the untreated samples were used for normalizing both chip types. Two 6-well plates were injected at a time, one with silicon chips and the other with CNT chips, resulting in 4 untreated samples total and 4 injected samples from each chip type. This will maintain the statistical significance of our data as we normalize to the untreated samples.

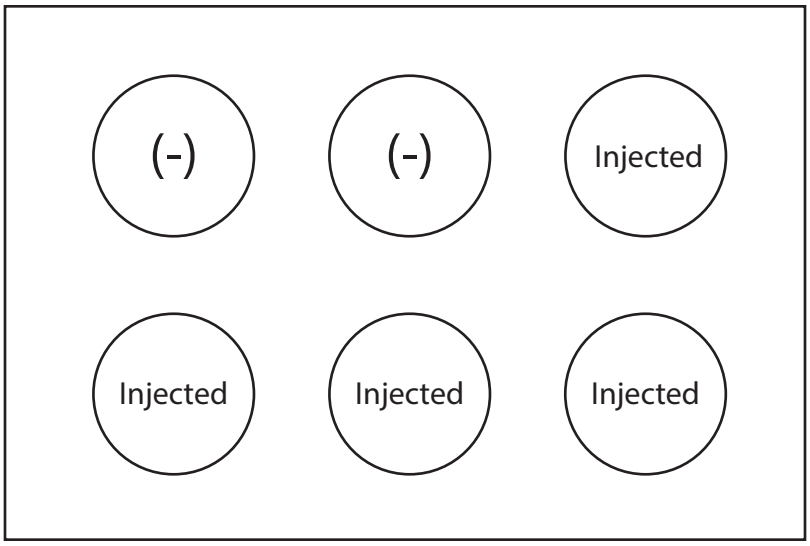


Figure 4.3: 6-Well Plate Setup

The HeLa cells were cultured in 6-well plates for approximately 24 hours before injecting them to give them enough time to adhere to the bottom of the wells. They were placed into the wells using the plate preparation protocol found in Appendix A.2. The bottom of the well contains a stainless steel plate with a wire coming up the side of the well so it can be used as an electrode during the injection process. A thin glass microscope plate was then placed on top of the stainless steel electrode and the cells were placed on the glass plate. Flow cytometry was performed on the untreated samples exactly the same as the injected samples.

### 4.1.6 Voltage and Current

The electrical conductivity of the silicon and CNT lances are very different, meaning that the current at a given voltage will vary greatly between the two materials. A current controlled system for pulsing would be ideal, but as such a system was unavailable at this time, a voltage controlled pulsed system was used for the injections with only the GFP plasmid. [38, 39] A set of tests was performed to develop an open loop control of the current using voltage. To do this, 6-well plates were prepared and injections were performed with silicon lance arrays at various voltages while measuring the current. A +9 V attraction for the silicon lance arrays used, and +2.7 V was required on the CNT chips to match the average current of 9 mA that we got on the silicon lance arrays.

For the injections with the GFP plasmid and chloroquine, a current controlled system for the constant attraction (+3 mA) and repulsion (-1 mA) was used. A voltage controlled system was still used for pulsed repulsion due to the lack of a current controlled system with an appropriate response time for the pulsing period.

The pulsed voltage that was used once the lance array was been pressed down onto the cells was a square wave with a period of 2 milliseconds and a duty cycle of 50%. For the GFP injections, it switched back and forth between -1.5 V and -9 V for the silicon lances, and for the CNT lances switched between -1.5 V and -2.7 V to approximately match the silicon lance array current. The injections with GFP and chloroquine pulsed between -1.5 and 9 V for both chips types since current for such short time periods won't negatively affect the cell viabilities. The pulse voltage lasted only for 20 milliseconds, for a total of 10 pulses per sample. After 10 pulses, -9 V was used on the silicon chips and -2.7 V was used for the CNT chips for repulsion. A step by step visualization process of the voltage and current parameters during injection can be seen in Figure 4.4.

The power supplies were hooked up to the black Arduino control box designed by Zachary Lindstrom. The attraction voltage/current supply was attached to input 1, the pulsed voltage signal from the function generator was attached to input 2, and the constant repel voltage was attached to input 3. The power supplies and control box can be seen in Figure 4.5.

### 4.1.7 DNA Concentration

A DNA concentration of 2 $\mu$g/mL was used in the injected cells wells.

### 4.1.8 Injection Methods

The injections of the HeLa cells were performed inside a clean hood to keep the injection equipment sterile and the cells viable. Power supplies were attached as previously described and a different injector device was used that has a normal stepper motor resolution. Once the power supplies were attached, the injections themselves were performed largely in the same manner as the force injections with the exception that a GFP plasmid and was used instead of PI. When the chloroquine was used in conjunction with the GFP, it was simply added before the GFP and

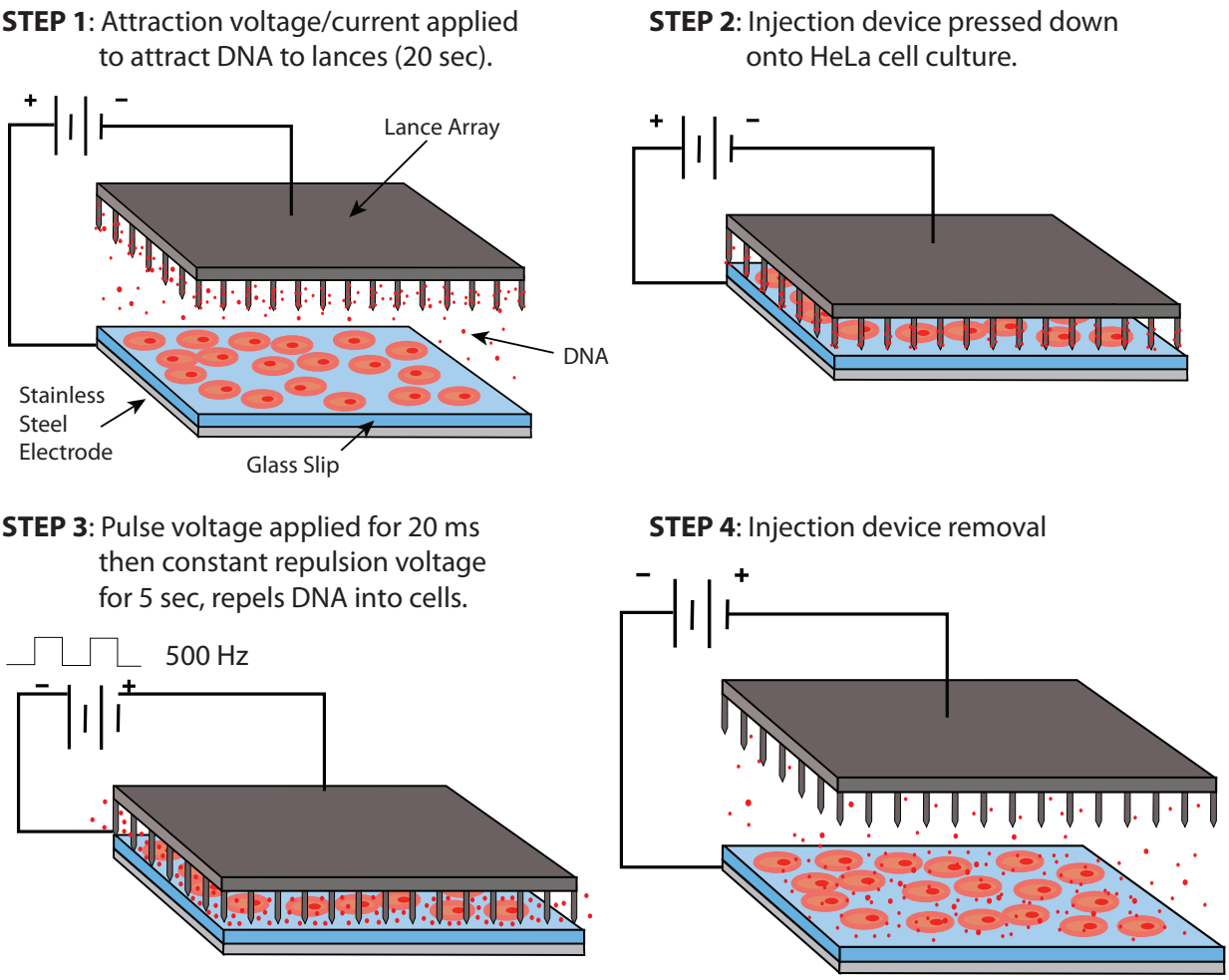


Figure 4.4: Attraction and repulsion process during injection. The device is first placed into the well to be injected and a voltage or current is applied to electrically attract the DNA molecules to the lances for 20 seconds. The lance array is then pressed down onto the HeLa cell culture. Once pressed down, a pulsed repulsion is applied for 20 ms followed by a constant repulsion voltage or current for another 5 seconds. The injection device then retracts the lance array and it is removed from the well.

allowed to sit for 15 min before adding the GFP and then injecting. Detailed protocols can be found in Appendix A.5.

### 4.1.9 Flow Cytometry

After injecting, the HeLa cells were incubated for a period of 24 hours before preparing them for flow cytometry. This gives the cells time to start producing fluorescent proteins if the

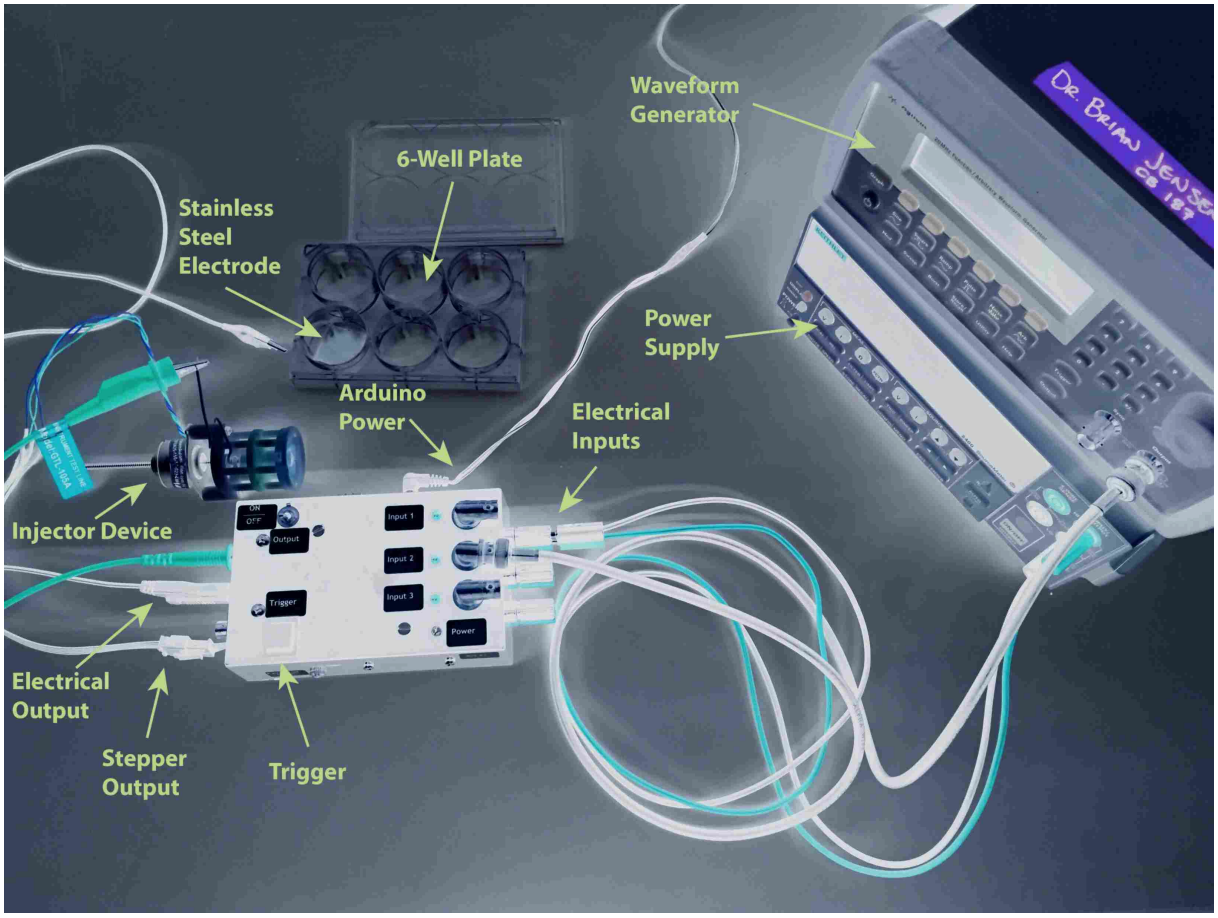


Figure 4.5: Power supply and injection device setup. The attraction voltage power supply is connected to input 1 of the Arduino controller box, the waveform generator is connected to input 2, and the repel voltage power supply is connected to input 3. The electrical output of the box is attached to the injection device and stainless steel electrode. The lance array is attached to the bottom of the injection device facing downward with carbon tape.

DNA successfully integrates into the nucleus. Additionally, this will ensure that only cells that have successfully integrated the DNA into the nucleus (not just the cytoplasm) are fluorescing.

### Preparation

The cells were prepared for flow cytometry by using trypsin to remove the cells from the well centrifuging the samples at 2000 rpm for 10 minutes. The supernatant was then removed and 0.25 mL of PBS was added to each sample. Once the PBS was added, each sample was vortexed to prepare them for flow cytometry. A detailed protocol can be found in Appendix A.5.

#### Measurements

Once the flow cytometry preparation was finished, the viability and PI concentration of the cells in each sample were measured using flow cytometry. During the analysis, 10,000 events were taken from each sample to ensure statistical accuracy. Using Attune Cytometric Software, the forward and side scatter of the cells was seen and quantified, as well as the number of cells that were fluorescing from the GFP protein.

#### Statistical Analysis

Normalized cell viability was determined using the same methods described in Section 3.2.4 of Chapter 3. The expression of GFP in the injected cells, however, cannot be normalized to the untreated samples since many of them have 0 % expression of the GFP. The GFP expression is therefore simply reported as a percentage, showing both the untreated samples and the injected wells.

### 4.2 Results and Discussion

#### 4.2.1 GFP Plasmid Injections (No Chloroquine)

#### GFP Plasmid Expression

Table 4.3: P-values for normalized GFP expression injected with silicon and CNT lance arrays. The student's t-test was performed assuming a two-tailed distribution and two-sample unequal variance. P-values of less than 0.05 were considered statistically significant.

| Student's T-Test (P-Values) | | |
|---|---|---|
| | Expression | Viability |
| Plasmid Only | 0.065 | 0.831 |
| Plasmid + Chloroquine | 0.587 | 0.192 |

The percent of GFP expression of the injected cells can be seen in Figure 4.6. The CNT lance arrays would appear to have performed much better than the silicon lances, but a student's

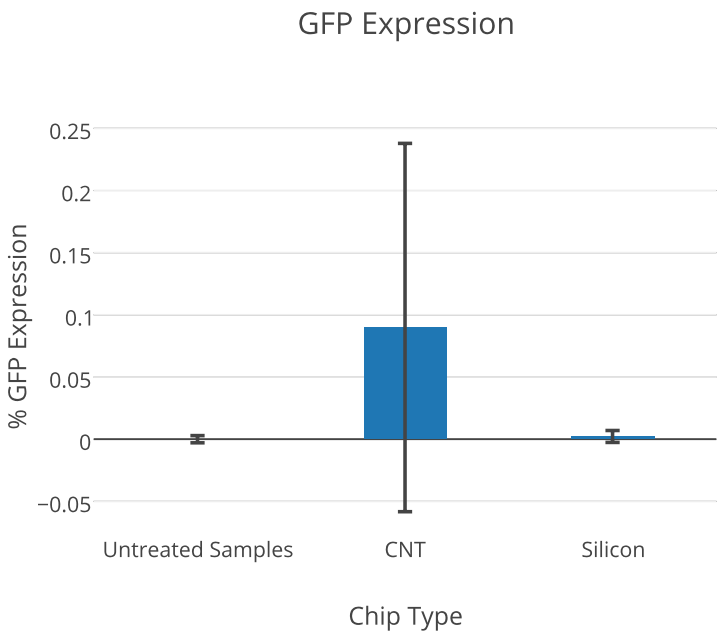


Figure 4.6: Percent GFP expression for injections with silicon and CNT lance arrays. Data was not normalized to the untreated samples because they were zero.

t-test gives a p-value of 0.065 (see Table 4.3), revealing that there is not a statistically significant difference between the two population means. This shows that our hypothesis that the CNT lance arrays would perform better was incorrect, or possibly needs further investigation. Most of the injected wells had little or no cell expression. The majority of the expressing cells, however, were found in only a few wells. This is why the variation in the CNT injections was so high.

### Cell Survival

As can be seen in Figure 4.7, the average normalized viability is very close for both the silicon and the CNT lance arrays. The student's t-test also confirms that there is no statistical difference between the population means. The variation is very large, even more so in the CNT lance arrays. Using voltage to assist in the transfection process is likely the reason for the higher variation. Localized electroporation can sometimes be detrimental to critical cellular biological functions, decreasing viability.

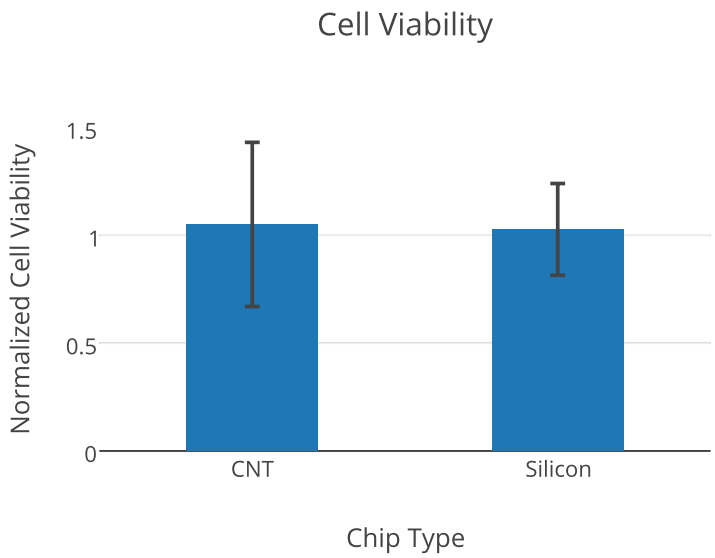


Figure 4.7: Normalized cell viability for silicon and CNT lance arrays.

### 4.2.2 GFP Plasmid and Chloroquine Injections

#### GFP Plasmid Expression

Looking at the preliminary data results from Figure 4.8, the silicon lance arrays appeared to have performed slightly better than the CNT lance arrays. A t-test gives us a p-value of 0.587 (see Table 4.3), showing that there is no statistical difference between the two population means. Once again, the data is not normalized to the untreated samples because they have an average value of zero. Since the percent of cells that is expressing is so small, it is difficult to determine if the slightly higher GFP expression from the silicon lance arrays was due to the chloroquine used in the experiment or just normal variation. Further tests would be needed to determine which is the case.

#### Cell Survival

The normalized cell viability was much lower in the injections using chloroquine than those without (see Figure 4.9), suggesting that is likely that the chloroquine had a negative impact on

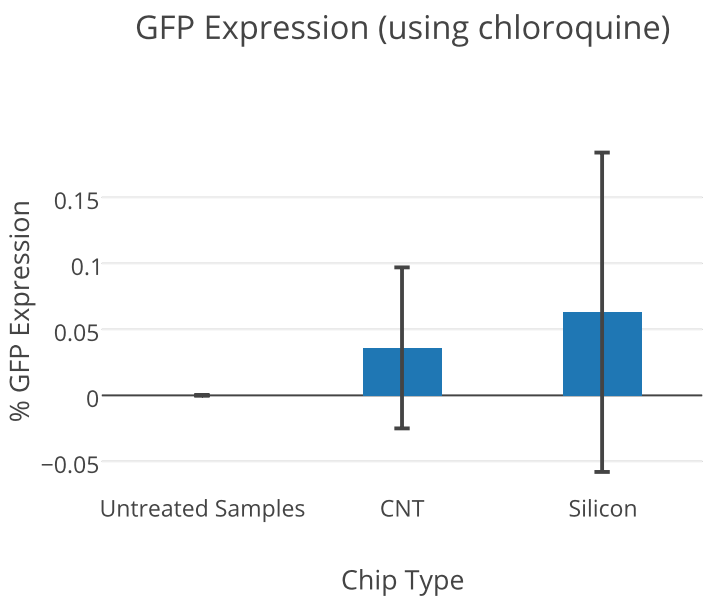


Figure 4.8: Precent GFP expression for injections with silicon and CNT lance arrays. Data was not normalized to the untreated samples because the they were zero.

the viability of the cells. A t-test in Table 4.3 indicates that there is not a statistically significant difference between the population means. The viabilities are, however, noticeably lower than that of the untreated samples.

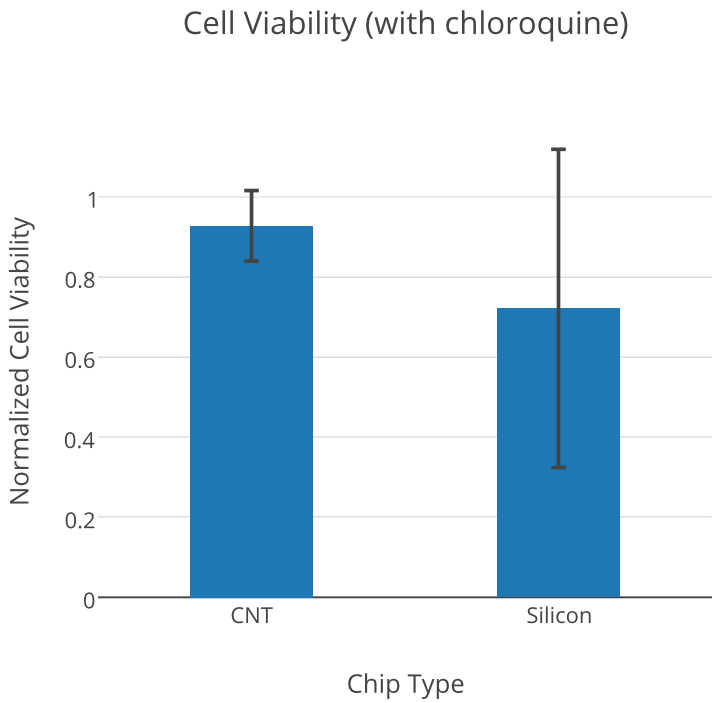


Figure 4.9: Normalized cell viability for silicon and CNT lance arrays.

# CHAPTER 5. INJECTION SPEEDS AND CURRENT CONTROL DEVICE

During the injection process, the contact resistance from chip to chip varies, resulting in potentially large differences in magnitudes of current. These large magnitudes of current can be disruptive and even fatal to the cells being tested, negatively affecting the injection process. To prevent this, the automated injection system was redesigned to allow current control for attraction, pulsing, and repulsion. The previous injection system would allow current control, but only with constant sources (no pulsing). This is because the voltage pulsing from the original injection system is performed by a function generator connected to the second input, which can only generate a voltage function, not a current function. A new design was therefore needed to perform pulsed current control.

Additionally, a design that can control the speed of injection will allow slower rates of injection, possibly resulting in fewer cells being pulled off of the glass slide when the motor retracts the lance array. After the injection system was designed and built, both the new and the older injection system used in previous experiments were tested at different speeds to see how this affects the number of cells pulled off during the injection process. Both systems were tested because each system had non-overlapping ranges of speeds that the motors would allow, and it was also desirable to compare the new system with the old. This was done by injecting different wells with each system and comparing how many cells remained relative to a control.

## 5.1 Control Box Design

The new injection system was designed to be controlled using a programmable Arduino. The program written for the control box allows the user to specify the distance the motor travels out, the distance at which input 1 is shut off, whether pulsing for input 2 is desired, the period of pulsing, and the length of each input during the injection process. The arduino sketch for this program can be found in Appendix B.4. A stepper motor driver which can control either a 4-wire

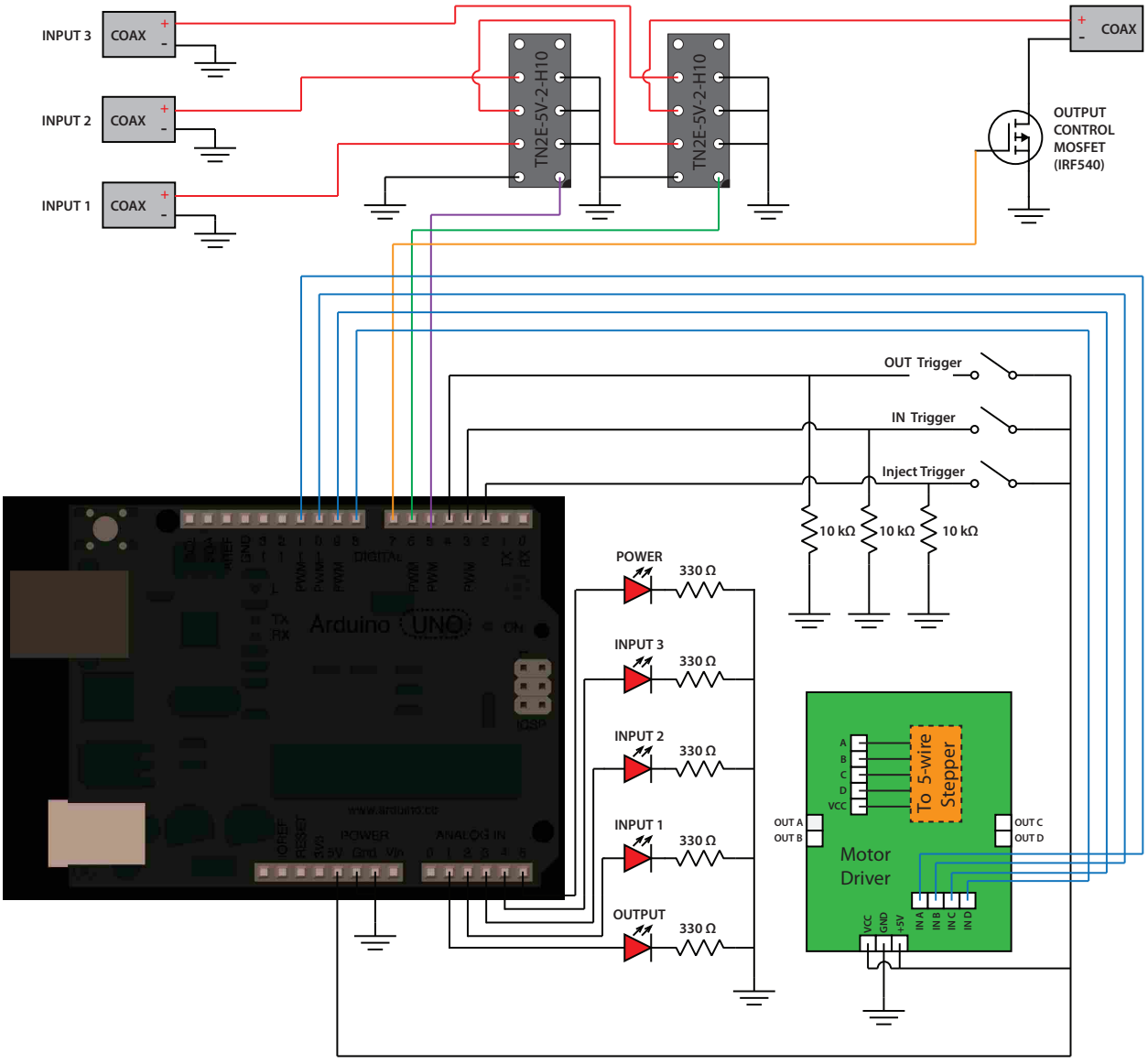


Figure 5.1: Electrical schematic for the current control box. An Arduino was used to control two relays and a stepper motor driver for the injection process. Five LEDs are used as indicators for power, output, and which input being passed through the box.

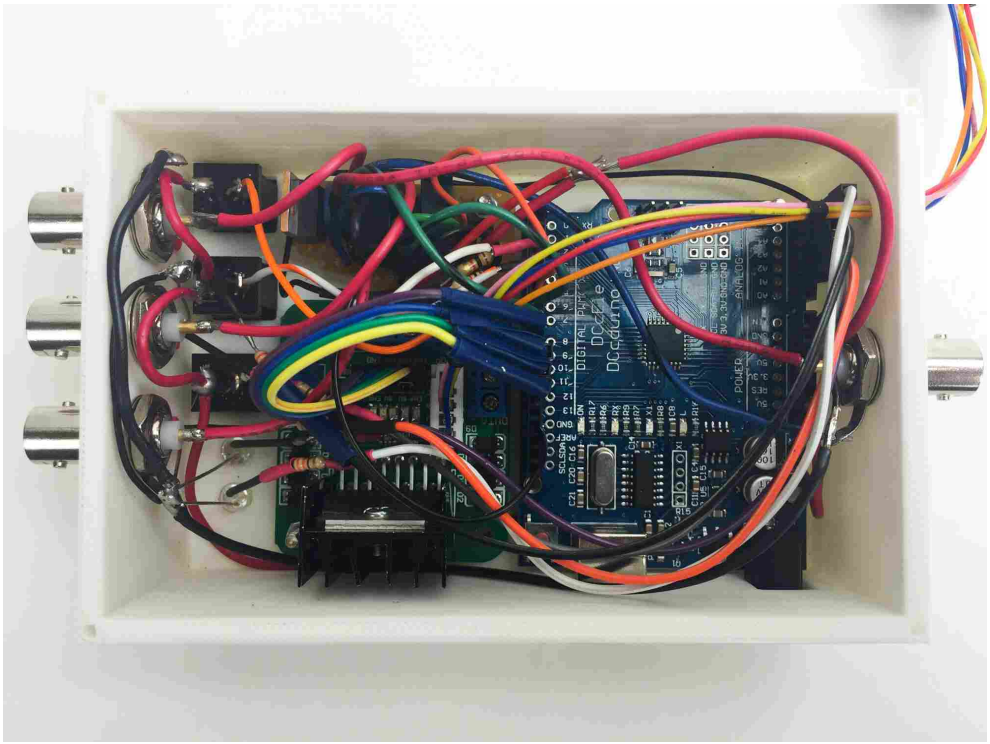

Figure 5.2: Inside view of the control box for the new injection system.

or 5-wire stepper motor for future flexibility was utilized. The control box was created using rapid prototyping techniques to allow users to easily change the design if needed using a CAD model.

A full electrical schematic of the control box can be seen in Figure 5.1. The circuit built from the schematic is shown in Figure 5.2. The electrical inputs to the control box were run through two relays in series. The first relay was used to choose whether to allow input 1 or 2 to pass through, while the second relay was used to choose whether to allow the input from the previous relay or input 3 to pass through. This allowed one input at a time to pass through using two digital pins (one to control each relay, using 5V or GND). The output was controlled using a MOSFET. This allow allows input 2 to be pulsed during the injection process. The power supplies used for constant current sources were tested and successfully verified to be able to sufficiently respond to a 2 millisecond period for pulsing.

The control box has 3 button controls available to the user (See Figure 5.3). An injection button, once pressed, will perform the injection programmed by the user. An IN and an OUT button allow for manual calibration of the starting location of the motor in the event that the power

Figure 5.3: User interface on the automated injection control box.

is disconnected during the injection process or if the starting location of the motor has drifted for any reason over time. Five LEDs indicate power to the device, whether or not the output is active, and which input is currently active.

## 5.2 Motor and Injection Device

The motor and injection device used with the new current control box are the same as those used for the force injection testing in Chapter 3. The full injection system can be seen in Figure 5.4.

## 5.3 Injection Methods

### 5.3.1 Key Parameters

The speed of the injection process was the variable tested in this experiment, and a sample size of 10 injected wells per injection speed was used. The injection speeds that were examined were the native speeds of the injection devices, to ensure the stepper motors would not skip steps by being driven too quickly. This was from 0.08 to 0.16 mm/sec for the new system, and from 0.6

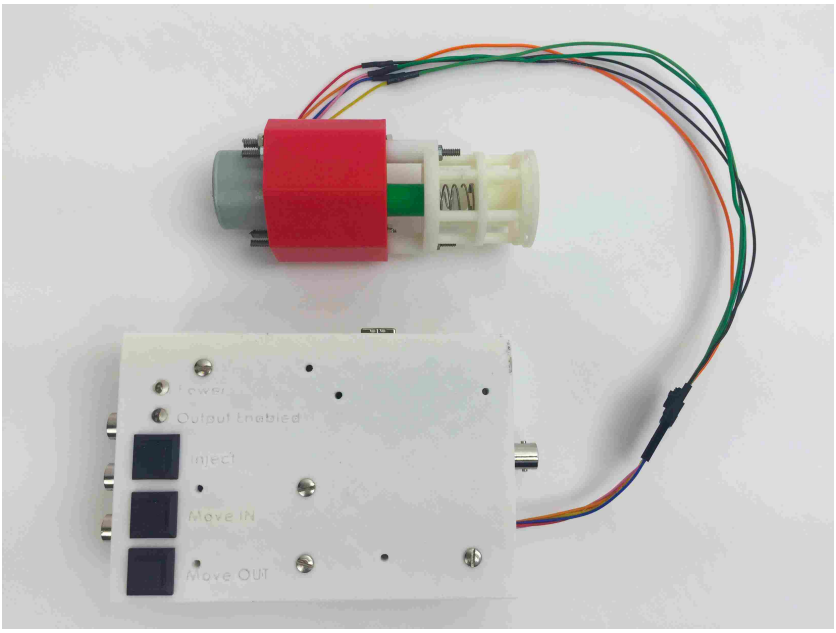


Figure 5.4: Injection system setup.

to 3 mm/sec for the old injection system. As is mentioned in Chapter 3, the slower speed of the new injection system occurs because the new stepper motor is geared down by a ratio of 64.

### 5.3.2 Cell Testing Plate Preparation

Dulbeccos Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum, 5% penicillin and streptomycin, and 5% L-glutamine was used to culture the HeLa 229 cancer cells in an incubator at 37 degrees Celsius and 5% carbon dioxide. The cells were placed onto 22mm glass slips in 6-well testing plates to ensure a flat surface. The cells were placed in the plates 24 hours before injection to allow sufficient time for them to adhere to the glass. Each plate had 2 untreated samples and 4 test samples.

### 5.3.3 Untreated Sample Protocol

The untreated samples were cultured exactly the same as the test samples, but no injections were performed. Hemocytometry preparations and cell counting were performed the same as the test samples.

### 5.3.4 Injection Protocol

The injections were perform in sets of two plates. In addition to 2 controls, each plate had 2 wells injected by the old injection system and 2 injected by the new system, for a total of 4 test samples. Once the plates had been prepared for testing, they were removed from the incubator, rinsed with PBS, and then given fresh PBS after they had been rinsed. Once the fresh PBS was added, the test samples were injected with their respective injection systems using a silicon lance array.

## 5.4 Hemocytometry

After the injections were completed, trypsin was used to remove the cells from the well and they were centrifuged at 2000 rpm for 10 minutes. The supernatant was then removed and 0.25 mL of HBSS was added to each sample. Once the HBSS was added, each sample was vortexed to prepare them for hemocytometry.

### 5.4.1 Statistical Analysis

After the cells were counted, each well was normalized to the average of the controls. Since all the test wells were prepared with the same amount of thoroughly mixed cell concentration fluid, they have approximately the same number of cells. A modified Thompson Tau test was used to remove any statistical outliers and then an analysis of variance was performed on the population samples to determine if there was a statistically significant difference between them. If a significant difference was found, a student t-test was performed between each population to determine which pairs had a significant difference.

## 5.5 Results and Discussion

The results from the injections at different speeds can be seen in Figure 5.5 (for a linear scale, refer to Figure 5.6). An analysis of variance found in Table 5.1 reveals that there is a statistically significant difference between at least two of the population means. A student's t-test (Table 5.2) confirms the appearance that the cells remaining after injection using the slower speeds

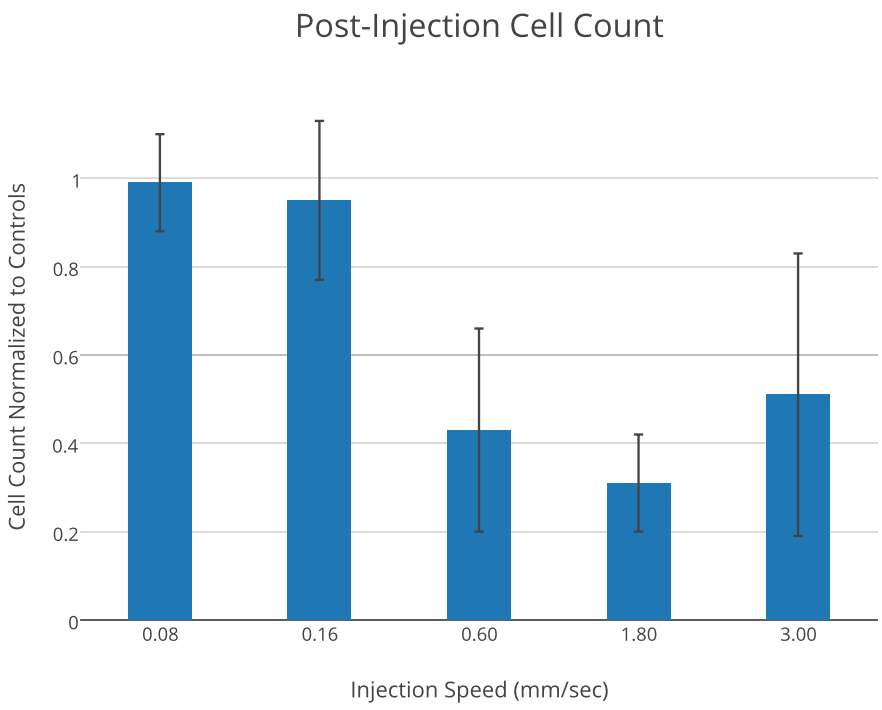


Figure 5.5: Cell count using a hemocytometer after injections at various speeds.

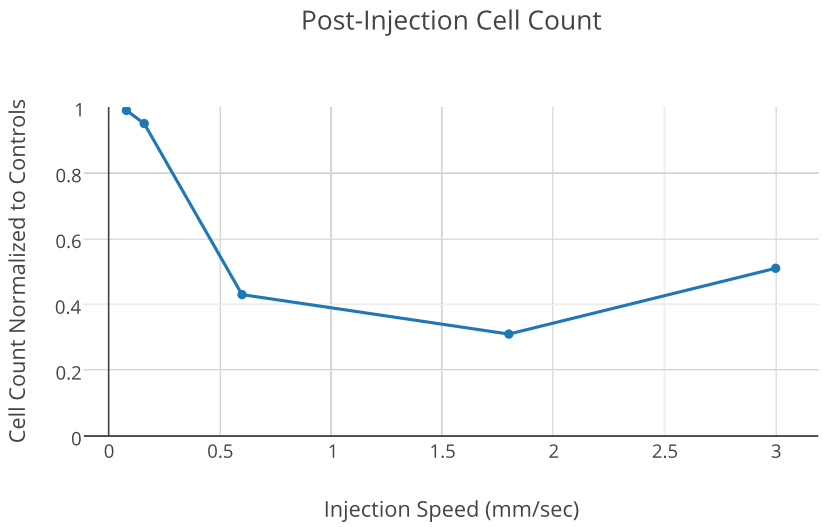


Figure 5.6: Cell count using a hemocytometer after injections at various speeds (Linear Scale).

of 0.08 and 0.16 mm/sec are much larger than that of the higher speeds. This means that fewer cells are being pulled from the injection surface upon retraction of the lances at slower speeds. Without being able to see a microscopic view of the injection process taking place, the mechanics behind this loss of cells cannot be definitively stated easily. It is possible that the strain rate with the faster injection speeds is simply too great for the dendritic adherence of the cells to the injection surface, or it could be too large for the cell membrane itself, causing it to tear apart. It should also be noted from looking at Figure 5.6 that the number of cells removed during the injection process increases dramatically between 0.16 and 0.6 and stays relative high at even greater speeds.

Table 5.1: ANOVA Table for normalized post-injection cell count with varying speeds of injection, ranging from 0.08 to 3 mm/sec. The table is calculated as a single factor, five level, multi-replicate experiment at $\alpha = 0.05$. Since F is larger than $F_{crit}$, this means that there is a statistically significant difference between two or more population means.

ANOVA

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Groups | 3.438005 | 4 | 0.859501 | 12.69772 | 2.19E-07 | 2.539689 |
| Within Groups | 3.722917 | 55 | 0.067689 | | | |
| | | | | | | |
| Total | 7.160922 | 59 | | | | |

Table 5.2: P-values for normalized post-injection cell count with varying speeds of injection, ranging from 0.08 to 3 mm/sec. The student's t-test was performed assuming a two-tailed distribution and two-sample unequal variance. P-values of less than 0.05 were considered statistically significant.

| | **0.08** | **0.16** | **0.6** | **1.8** | **3** |
|---|---|---|---|---|---|
| **0.08** | – | 5.66E-01 | 1.08E-05 | 1.32E-06 | 8.43E-04 |
| **0.16** | | – | 3.26E-05 | 4.55E-06 | 1.93E-03 |
| **0.6** | | | – | 7.16E-01 | 5.25E-01 |
| **1.8** | | | | – | 3.43E-01 |
| **3** | | | | | – |

## 5.6 Conclusion

It is obvious from the data that slower speeds dramatically decrease the loss of cells during injection process. The use of these slower speeds for injection will allow new experiments with HeLa cells that might be able to exploit the advantage of fewer cells being lost. Preliminary data from research designed and performed by fellow Bio-MEMS research team member John Sessions has shown that slower injections allow the cells to be injected multiple times to increase transfection rates as much as three or four times.

# CHAPTER 6. CONCLUSION

## 6.1 Importance of Nanoinjection Parameters

The nanoinjection process provides a valuable opportunity to develop a process that is likely both practical and effective at transfecting large numbers of cells with little loss in cell viability. Finding efficient parameters and methods for these injections is critical to fulfilling this goal. Some parameters appear to have little or no effect on the injection process, while others can have a very large effect. Results from the previous chapters are summarized below and recommendations are made as to how much each parameter affects the injection process.

## 6.2 PI Concentration

From the results seen in Chapter 2, the PI concentration had a large impact on the sensitivity of the experiment. The lowest concentration of PI tested (0.02 $\mu$g/mL HBSS) gave the highest sensitivity when normalized to the controls and had no negative impact on cell viability. These tests allowed a determination of the concentration to use for the rest of the experiments in this thesis when PI was used.

## 6.3 Force Injections

The force of injection (if below 1.8 N) can have a large impact on the injection efficiency. The PI uptake increases from 1 (at 0 N of force) to 4.11 (at 1.8 N of force) when normalized relative to the positive controls. Above 1.8 N, any force produces an average of approximately 3.1. This is likely due to the lances penetrating all of the cells, and any additional force produces no additional PI uptake. From the results, it is recommended to inject at or above 1.8 N of force to obtain the best efficiency of the injection process.

## 6.4 Chip Material

The material that the lance arrays were made from appeared to have little effect on the outcome of the GFP expression. Since the expression percentages were so small, it is difficult to determine whether the difference in results was really due to the different material, or if it was due to variation. This variation could have possibly come from the use of pulsed voltage to assist in the transfection of the cells. The viability of the cells varied more with the use of voltage, and appeared to be negatively impacted by the use of chloroquine. At this point both materials work with similar efficacy, and further testing would be required to make definitive recommendations on the lance array material.

## 6.5 Injection Speeds and Current Control Injection Device

The injection speed had a large impact on the loss of cells during the injection process. Slower injection speeds resulted in fewer cells lost (only 1-5%), while faster injection speeds dramatically increased the number of cells that were lost (49-69%). It is recommended to use slower speeds so that more cells will live through the injection process and serial injections may be used.

## 6.6 Possible Future Work

Future work may include testing different parameters, such as chip types and voltages, with the use of a CRISPR CAS9 for effective insertion of a specific molecular load.

## 6.7 Concluding Remarks

The investigation and improvement of technologies for cell transfection is important to opening new doors and methods of treatment for previously untreatable diseases and undesirable cellular functions. Future work and possibilities for gene therapy and tissue engineering depend on these technologies to perform treatments at the cellular level, fixing not only the symptoms but also the cellular functions that cause those symptoms.

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# APPENDIX A. MATERIALS AND PROTOCOLS

## A.1 HeLa Cell Passaging

### A.1.1 Materials

- Sterile Dulbeccos Modified Eagle Medium (DMEM)(with 10% FBS and penicillin/streptomycin)
- Sterile Hanks Balanced Salt Solution (HBSS)
- Sterile 5x Trypsin ($\approx$ 2.5 mL)
- Flask of cells to be passaged
- Pipette-aid
- Waste beaker
- (x2) 25 mL serological pipettes
- (x2) 10 mL serological pipettes
- (x1) 5 mL serological pipette

### A.1.2 Protocol

1. Place all items (except liquids and flask of cells) into the clean hood and turn on the UV for at least 15 minutes
2. Spray hands, arms, and other items with ethanol before placing in the hood
3. Avoid touching pipettes against hands and surfaces
4. Loosen caps on all containers
5. Using a 25 mL pipette, remove the old media from the flask and discard into waste beaker
6. Using a 10 mL pipette, add 10 mL Hanks solution to the flask, then rinse the flask bottom and discard the Hanks into waste beaker
7. Using the 5 mL pipette, add 2.5 mL Trypsin to the flask

8. Make sure the Trypsin entirely covers the cells on the flask bottom
9. Place the flask in the incubator for 5 minutes
10. Remove flask from incubator and swirl until all cells are no longer adhered (if needed, hit flask against countertop to further break up cells)
11. Place flask back in hood (spray arms, hands, and flask with ethanol first)
12. Using a 10 mL pipette, add 7.5 mL DMEM to the flask
13. Pipette the solution up and down several times to thoroughly mix the contents, and make sure to rinse any remaining cells from the bottom of the flask
14. Pull all the cell solution back into the 10 mL pipette
15. Add desired amount of cell solution back to the flask (i.e. for a 1:20 ratio, put 0.5 mL back; for a 1:2 ratio, put 5 mL back)
16. Discard remaining cell solution into waste beaker
17. Using a 25 mL pipette, add enough DMEM to the flask to bring the total amount of solution to $\approx$ 20–25 mL
18. Place flask back into incubator, being sure to loosen the lid to allow $CO_2$ flow
19. Clean up materials and wipe down hood with ethanol

## A.2 HeLa Cell Passaging Plate Preparation

### A.2.1 Materials

- Sterile Dulbeccos Modified Eagle Medium (DMEM)(with 10% FBS and penicillin/streptomycin)
- Sterile Hanks Balanced Salt Solution (HBSS)
- Sterile 5x Trypsin ($\approx$ 2.5 mL)
- Flask of cells to be passaged
- Pipette-aid
- Waste beaker
- One 15 mL conical tube (red cap)
- (x2) 25 mL serological pipettes
- (x2) 10 mL serological pipettes

- (x2) 5 mL serological pipettes
- (x1) 1 mL serological pipette
- Micropipette (green, 2-200 $\mu$L size) set at 150 $\mu$L, and a pipette tip
- Desired number of 6-well plates with glass cover slips covering well-bottom (for wells that will be nanoinjected, place a stainless steel plate underneath the glass cover slip)

### A.2.2 Protocol

1. Place all items (except liquids and flask of cells) into the clean hood and turn on the UV for at least 15 minutes
2. Spray hands, arms, and other items with ethanol before placing in the hood
3. Avoid touching pipettes against hands and surfaces
4. Loosen caps on all containers
5. Using a 25 mL pipette, remove the old media from the flask and discard into waste beaker
6. Using a 10 mL pipette, add 10 mL Hanks solution to the flask, then rinse the flask bottom and discard the Hanks into waste beaker
7. Using the 5 mL pipette, add 2.5 mL Trypsin to the flask
8. Make sure the Trypsin entirely covers the cells on the flask bottom
9. Place the flask in the incubator for 5 minutes
10. Remove flask from incubator and swirl until all cells are no longer adhered (if needed, hit flask against countertop to further break up cells)
11. Place flask back in hood (spray arms, hands, and flask with ethanol first)
12. Using a 10 mL pipette add 7.5 mL DMEM to the flask
13. Pipette the solution up and down several times to thoroughly mix the contents, and make sure to rinse any remaining cells from the bottom of the flask
14. Pull all the cell solution back into the 10 mL pipette
15. Add desired amount of cell solution back to the flask (i.e. for a 1:20 ratio, put 0.5 mL back; for a 1:2 ratio, put 5 mL back)
16. Add cell solution (0.5 mL cell solution per six-well plate prepared) to the 15 mL tube
17. Discard remaining cell solution into waste beaker

18. Using a 25 mL pipette, add enough DMEM to the flask to bring the total amount of solution to $\approx$ 20–25 mL
19. Using a 5 mL pipette, add the same amount of DMEM to the 15 mL tube as cell solution from step 16
20. Using the micropipette, place 150 $\mu$L cell solution from the 15 mL tube into each well-bottom
21. Using the 1 mL pipette, spread the cell solution in each well into a circle roughly 2 cm in diameter
22. Place flask and 6-well plates into incubator, being sure to loosen the lid to allow $CO_2$ flow
23. Clean up materials and wipe down hood with ethanol

**1–2 Hours Later:**

1. Turn on the clean hood and wipe down with ethanol
2. Spray arms and hands with ethanol
3. After spraying with ethanol, place a pipette-aid, 25 mL pipettes (you need one pipette per two six-well plates), and a bottle of DMEM in the hood
4. Using the 25 mL pipettes, add 3 mL DMEM to each well containing a stainless steel electrode, and 2 mL to wells without a stainless steel electrode, being sure to cover the cells
5. Place the plates back in the incubator
6. Clean up materials and wipe down hood with ethanol

## A.3 PI Concentration Injections

### A.3.1 Materials

- Cells that have been cultured in 6-well plates for $\approx$ 24 hours
- Normal resolution force injection device
- (x4) 10 mL serological pipettes
- (x4) 5 mL serological pipettes
- (x24) FACS tubes
- Pipette-aid

- Non-sterile Hanks Balanced Salt Solution (HBSS)
- Non-sterile Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + gentamicin)
- Propidium iodide solution
- 12 mL of 5x Trypsin
- Micropipette (green, 2-200 $\mu$L size) and a pipette tip
- Micropipette (blue, 100-1000 $\mu$L size) and multiple pipette tips
- Waste beaker
- Arduino controller box and injection device

### A.3.2 Injection Protocol

1. Program the Arduino using the Arduino sketch found in Appendix B.1
2. Using a 10 mL pipette, remove media from wells and discard into waste beaker
3. Using a new 10 mL pipette, rinse each well with Hanks ($\approx$ 10 mL per plate) and discard
4. Using the same 10 mL pipette, add 2 mL Hanks to each well
5. Using the green micropipette, add desired amount of PI solution to each well to obtain the desired PI concentration
6. Inject the test wells by doing the following:
   (a) Lower injection device and attached chip into well
   (b) Press the button to activate the injection process from the Arduino
   (c) Continue holding lance array until the injection device has retracted the lance array completely
7. Remove injection device from well (make sure the glass slip is not attached to the chip)
8. Repeat steps 6-7 for each well to be injected, rising the injection device between plates

### A.3.3 Flow Cytometry Preparation Protocol

1. Using a 10 mL pipette, add 0.5 mL Trypsin to each well
2. Place plates in incubator for 5 minutes
3. While Trypsin is incubating, label FACS tubes 1-24

4. Using a 10 mL pipette, add 1 mL DMEM to each well to deactivate Trypsin once the plates have been in the incubator for 5 minutes
5. Transfer contents of each well to their respective FACS tubes (use a new micropipette tip for each well)
6. Put tubes in centrifuge for 10 minutes at 2000 RPM (turn brake off, set timer to "hold time." To stop the centrifuge, turn brake on "high," set time to "off," and open the lid)
7. Remove the supernatant from each tube by pouring it down the drain
8. Break up cell pellet in each tube by hitting tube base against a table top several times
9. Add 250 $\mu$L Hanks to each tube using the blue micropipette
10. Take tubes up to RIC facility for flow cytometry analysis

$\Rightarrow$ If you need to wait longer than 5-10 minutes to take your samples to flow, put them in the fridge or on ice

11. Following flow cytometry, discard FACS tubes and clean up work area.

## A.4 Force Injections

### A.4.1 Materials

- Cells that have been cultured in 6-well plates for $\approx$ 24 hours
- High resolution force injection device
- (x4) 10 mL serological pipettes
- (x4) 5 mL serological pipettes
- (x24) FACS tubes
- Pipette-aid
- Non-sterile Hanks Balanced Salt Solution (HBSS)
- Non-sterile Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + gentamicin)
- Propidium iodide solution
- 12 mL of 5x Trypsin
- Micropipette (green, 2-200 $\mu$L size) and a pipette tip

- Micropipette (blue, 100-1000 $\mu$L size) and multiple pipette tips
- Waste beaker
- Arduino controller hooked to computer and injection device

### A.4.2 Injection Protocol

1. Program the Arduino using the Arduino sketch found in Appendix B.2 to the desired force and upload it to the Arduino
2. Using a 10 mL pipette, remove media from wells and discard into waste beaker
3. Using a new 10 mL pipette, rinse each well with Hanks ($\approx$ 10 mL per plate) and discard
4. Using the same 10 mL pipette, add 2 mL Hanks to each well
5. Using the green micropipette, add desired amount of PI solution to each well to obtain the desired PI concentration
6. Inject the test wells by doing the following:
   (a) Lower injection device and attached chip into well
   (b) Press the button to activate the injection process from the Arduino
   (c) Continue holding lance array until the injection device has retracted the lance array completely
7. Remove injection device from well (make sure the glass slip is not attached to the chip)
8. Repeat steps 6-7 for each well to be injected, rising the injection device between plates

### A.4.3 Flow Cytometry Preparation Protocol

1. Using a 10 mL pipette, add 0.5 mL Trypsin to each well
2. Place plates in incubator for 5 minutes
3. While Trypsin is incubating, label FACS tubes 1-24
4. Using a 10 mL pipette, add 1 mL DMEM to each well to deactivate Trypsin once the plates have been in the incubator for 5 minutes
5. Transfer contents of each well to their respective FACS tubes (use a new micropipette tip for each well)
6. Put tubes in centrifuge for 10 minutes at 2000 RPM (turn brake off, set timer to "hold time." To stop the centrifuge, turn brake on "high," set time to "off," and open the lid)

7. Remove the supernatant from each tube by pouring it down the drain
8. Break up cell pellet in each tube by hitting tube base against a table top several times
9. Add 250 $\mu$L Hanks to each tube using the blue micropipette
10. Take tubes up to RIC facility for flow cytometry analysis

$\Rightarrow$ If you need to wait longer than 5-10 minutes to take your samples to flow, put them in the fridge or on ice

11. Following flow cytometry, discard FACS tubes and clean up work area.

## A.5 Silicon and CNT Pulsed Injections

### A.5.1 Injection Materials

- Cells that have been cultured in 6-well plates for $\approx$ 24 hours
- Automated injection device and lance array chip
- (x3) 10 mL serological pipettes
- (x2) 25 mL serological pipettes
- Pipette-aid
- Sterile Phosphate Buffered Saline (PBS)
- Sterile Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + gentamicin)
- Tube of DNA in solution
- Micropipette (red, 2-20 $\mu$L size) and a pipette tip
- Waste beaker
- Power supplies
- Function generator
- Black box Arduino switch
- Coaxial cable (connect from function generator output to input 2 of black box)
- Red and black cables (connect from power supply to input 1 of black box)
- Red/black alligator clips (connect to output of black box)

### A.5.2 Injection Protocol

1. Be sure the correct Arduino sketch is uploaded to the Arduino
2. Place all items (except for fluids) in the clean hood under UV light for 15 minutes
3. Spray solution bottles with ethanol and add to hood
4. Using a 10 mL pipette, remove media from wells and discard into waste beaker
5. Using a new 10 mL pipette for each plate, rinse each well with PBS ($\approx$ 10 mL per plate) and discard
6. Using a 25 mL pipette, add 2 mL PBS to each well
7. If no chloroquine is being used, skip to the next step. If chloroquine is being used with the injection process, add the desired amount of chloroquine to each well and allow to incubate for 15 minutes before continuing.
8. Using the micropipette, add desired amount of DNA solution to each well to obtain the desired concentration (2 $\mu$g/mL PBS)
9. Inject the test wells by doing the following:
   (a) Set desired settings on power supplies and attach power supplies to the Arduino control box (input 1 is the attraction voltage, input 2 is the pulsed voltage repel, and input 3 is the constant repel voltage)
   (b) Attach positive alligator clip (red) to upper electrode wire on the injection device
   (c) Lower injection device and attached chip into well
   (d) Attach negative clip (black) to lower electrode plate wire
   (e) Flip black box toggle switch to "on" and press the trigger button
   (f) Hold the injection device firmly in place and wait for the lance array to be pressed down and retracted by the device
   (g) Flip black box toggle switch to "off" (this turns off the output from the box to the dvice)
   (h) Remove black alligator clip from wire
10. Release chip from cells and remove injection device from well (make sure the glass slip is not attached to the chip)
11. Repeat steps 9-10 for each well to be injected
12. Using the remaining 25 mL pipette, add 2 mL DMEM to each well (add 3 mL DMEM to wells with a stainless steel plate)
13. Place culture plates in the incubator
14. Clean up workspace

### A.5.3 Flow Cytometry Preparation Materials

- Cells cultured in 6-well plates that have been nanoinjected and incubated for the desired time (to express fluorescent protein)
- (x1) 5 mL serological pipettes
- (x3) 10 mL serological pipettes
- (x1) 25 mL serological pipettes
- Micropipette (blue, 100-1000 $\mu$L size) and a pipette tip
- (x12) FACS tubes
- Pipette-aid
- Non-sterile Phosphate Buffered Saline (PBS)
- Non-sterile Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + penicillin/streptomycin)
- 6 mL of 5x Trypsin
- Waste beaker

### A.5.4 Flow Cytometry Preparation Protocol

1. Using a 10 mL pipette, remove media from wells and discard into waste beaker
2. Using a new 10 mL pipette, rinse each well with Hanks ($\approx$ 8 mL per plate) and discard
3. Using the same 10 mL pipette, add 1 mL Hanks to each well (if using stainless steel plates for a base electrode, you need to add 2 mL Hanks to cover the cells adequately)
4. Using a new 10 mL pipette, add 0.5 mL Trypsin to each well
5. Place plates in incubator for 5 minutes
6. While Trypsin is incubating, label FACS tubes 1-12
7. Swirl the plates to knock cells off the glass slips
8. Using a 25 mL pipette, add 1 mL DMEM to each well to deactivate Trypsin
9. Using a 5 mL pipette, transfer contents of each well to their respective FACS tubes (use a new micropipette tip for each well)
10. Put tubes in centrifuge for 10 minutes at 2000 RPM (turn brake off, set timer to "hold time." To stop the centrifuge, turn brake on "high," set time to "off," and open the lid)

11. Remove the supernatant from each tube by pouring it down the drain
12. Break up cell pellet in each tube by hitting tube base against a table top several times
13. Add 250 $\mu$L PBS to each tube using the micropipette
14. Take tubes up to RIC facility for flow cytometry analysis

$\Rightarrow$ If you need to wait longer than 5-10 minutes to take your samples to flow, put them in the fridge or on ice

15. Following flow cytometry, discard FACS tubes and clean up work area.

## A.6 Automated Current Control Device Injections

### A.6.1 Injection Materials

- Cells that have been cultured in 6-well plates for $\approx$ 24 hours (4 plates)
- Automated injection device(s) and lance array chip
- (x7) 10 mL serological pipettes
- (x2) 25 mL serological pipettes
- Pipette-aid
- Phosphate Buffered Saline (PBS)
- Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + gentamicin)
- Micropipette (blue, 100-1000 $\mu$L size) and a pipette tip
- Waste beaker
- Injection Control Box

### A.6.2 Injection Protocol

1. Be sure the correct Arduino sketch is uploaded to the Arduino
2. Using a 10 mL pipette, remove media from wells and discard into waste beaker
3. Using a new 10 mL pipette, rinse each well with PBS ($\approx$ 10 mL per plate) and discard
4. Using a 25 mL pipette, add 2 mL PBS to each well
5. Lower injection device and attached chip into the first well

6. Make sure the injection control box is powered and and press the inject button
7. Hold the injection device firmly in place and wait for the lance array to be pressed down and retracted by the device
8. Release chip from cells and remove injection device from well (make sure the glass slip is not attached to the chip)
9. Repeat steps 5-8 for each well to be injected
10. Using a 10 mL pipette, remove the PBS from each well and discard into waste beaker
11. Using a new 10 mL pipette, lightly rinse each glass slide off with 2 mL of PBS and discard
12. Using a new 10 mL pipette, add 1 mL of PBS to each well
13. Using a 10 mL pipette, add 0.5 mL Trypsin to each well
14. Place plates in incubator for 5 minutes
15. While Trypsin is incubating, label FACS tubes
16. Using a 10 mL pipette, add 1 mL DMEM to each well to deactivate Trypsin once the plates have been in the incubator for 5 minutes
17. Transfer contents of each well to their respective FACS tubes (use a new micropipette tip for each well)
18. Use the protocol found in section A.6.4
19. Clean up workspace

### A.6.3 Cell Counting Materials

- 15mL tube of cell solution
- Hemocytometer
- Hand counter/clicker
- Microscope
- 25x25mm glass cover slip
- Micropipette 2-20L and tip(1x)
- Kimwipes

### A.6.4 Cell Counting Protocol for Hemocytometer

1. Rinse hemocytometer and dry with a Kimwipe
2. Place the glass cover slip on the center of the hemocytometer
3. Vortex or invert the 15mL conical tube several times to ensure the cell solution is well mixed
4. Using the micropipette, slowly place 14L of cell solution in the groove at the edge of the hemocytometer, capillary action will draw the solution up beneath the glass cover slip uniformly
5. Place the hemocytometer on the microscope and adjust the location and focus until you find the grid that contains cells
6. Using the counter, count and record the number of cells in each of the 16-square corners of the hemocytometery
7. The hemocytometer is most accurate when the cell count is near 100, counts higher greater than 150 or less than 15 cells per 16-square corner will not yield accurate cell counts
8. If the count is too high (more than 150 per 16-square corner), dilute the solution and repeat the counts
9. Find the average count of cells per 16-square corner
10. Multiply the average number of cells per 16-square corner by 104 average number of cells per 1mL of cell solution to compute the average number of cells per 1mL of cell solution
11. If a dilution was performed to reduce the cell count, remember to account for this dilution by multiplying by the dilution factor
12. Multiply the approximate number of cells per mL by the amount solution placed in each well to obtain the approximate number of cells per well
13. Record the cell count per well.
14. When finished, dispose of the glass slide, rinse the hemocytometer, and dry it with a Kimwipe

## APPENDIX B. ARDUINO SKETCH CODES FOR DIFFERENT INJECTIONS

*Note: The stepper.h library is required for the PI concentration testing program. No libraries are needed for for the rest of the programs.

### B.1 PI Concentration Injections

The PI concentration injections didn't use any voltage, so the power supply inputs of the injection box controller were simply left unattached.

```
// ********************************************** //
// // // // Nanoinjection 10V Pulse Program // // // //
// // // //       USE WITH BOX 1!           // // // //
// ********************************************** //

// Created by Zachary Lindstrom
// Last updated Jan. 27th, 2015 by Tyler Lewis

#include <Stepper.h>

// Input delay times
int input1duration = 20000; //ms of constant DC
int input2duration = 20; //ms 10 V repel pulse time
int input3duration = 5000; //ms Reverse voltage, withdraw lances

// Motor control settings
int mspeed = 10; // motor speed
int steps = 100; // number of steps

// Pin numbers for relay switches
int relay_1_pin = 3;    // Pin 3 goes to input 1 relay
int relay_2_pin = 2;    // Pin 2 goes to input 2 relay
int relay_3_pin = 4;    // Pin 4 goes to input 3 relay

// Pin numbers for LEDs
int input_1_LED_pin = 5;
int input_2_LED_pin = 6;
int input_3_LED_pin = 7;
```

```
int blueLEDpin = 11;

// Pins for stepper control
int in1Pin = 12;
int in2Pin = 10;
int in3Pin = 9;
int in4Pin = 8;
Stepper motor(768, in1Pin, in2Pin, in3Pin, in4Pin);

// Pin number for button
int buttonPin = 13;

// Current state variable for the button
int buttonState = 0;
int previousBtnState = 0;

void setup() {
  // Initialize stepper control pins
  pinMode(in1Pin, OUTPUT);
  pinMode(in2Pin, OUTPUT);
  pinMode(in3Pin, OUTPUT);
  pinMode(in4Pin, OUTPUT);
  motor.setSpeed(mspeed);

  // Initializing relay Pins
  pinMode(relay_1_pin, OUTPUT);
  pinMode(relay_2_pin, OUTPUT);
  pinMode(relay_3_pin, OUTPUT);

  // and LED pins
  pinMode(input_1_LED_pin, OUTPUT);
  pinMode(input_2_LED_pin, OUTPUT);
  pinMode(input_3_LED_pin, OUTPUT);
  pinMode(blueLEDpin, OUTPUT);

  // Button Pin Initialize
  pinMode(buttonPin, INPUT);

  // Turning on blue LED for "Power" and connecting input 1
  analogWrite(blueLEDpin, 10);
  digitalWrite(input_1_LED_pin, HIGH);
  digitalWrite(relay_1_pin, HIGH);
  digitalWrite(input_2_LED_pin, LOW);
  digitalWrite(relay_2_pin, LOW);
  digitalWrite(input_3_LED_pin, LOW);
```

```
  digitalWrite(relay_3_pin, LOW);
}

void loop() {
  buttonState = digitalRead(buttonPin);

  if(buttonState == HIGH && previousBtnState == LOW)
  {
   // delay 20 seconds for DC
    delay(input1duration);

   // Activate stepper to push lances into cells
    motor.step(-steps);

   // Turn off input 1
    digitalWrite(input_1_LED_pin, LOW);
    digitalWrite(relay_1_pin, LOW);

   // Turn on input 2
    digitalWrite(input_2_LED_pin, HIGH);
    digitalWrite(relay_2_pin, HIGH);

   // wait the specified input 2 duration
    delay(input2duration);

   // Turn off input 2
    digitalWrite(input_2_LED_pin, LOW);
    digitalWrite(relay_2_pin, LOW);

   // Turn on input 3
    digitalWrite(input_3_LED_pin, HIGH);
    digitalWrite(relay_3_pin, HIGH);

   // wait the specified input 3 duration
    delay(input3duration);

   // Turn off input 3
    digitalWrite(input_3_LED_pin, LOW);
    digitalWrite(relay_3_pin, LOW);

   // retract the lances
    motor.step(steps);

   // Turn on input 1
    digitalWrite(input_1_LED_pin, HIGH);
```

```
    digitalWrite(relay_1_pin, HIGH);
  }

  previousBtnState = buttonState;
}
```

### B.2 Force Testing Injections

```
// ********************************************** //
//     Nanoinjection Force Testing Program        //
// ********************************************** //

// Based on code created by Zachary Lindstrom
// Last updated Jan. 27th, 2015 by Tyler Lewis

// Stepper Motor Control Pins
int motorPin1 = 8; // Blue   - 28BYJ48 pin 1
int motorPin2 = 9; // Pink   - 28BYJ48 pin 2
int motorPin3 = 10; // Yellow - 28BYJ48 pin 3
int motorPin4 = 11; // Orange - 28BYJ48 pin 4

// Time delay between each step
// This controls the speed of the motor
// (1000 microseconds is the minimum possible)
int timedelay = 1200; // microseconds

// Number of cycles of steps to travel
// (8 steps/cycle, ~131,072 steps/inch, or 0.19 microns/step)
int steps = 758;
// 0.4 N = 815  step cycles
// 0.8 N = 894  step cycles
// 1.2 N = 984  step cycles
// 2.4 N = 1256 step cycles

// Pin number for button
int buttonPin = 13;

// Current state variable for the button
int buttonState = 0;
int previousBtnState = 0;

  void setup() {
    // Initialize stepper motor control pins
    pinMode(motorPin1, OUTPUT);
```

```
  pinMode(motorPin2, OUTPUT);
  pinMode(motorPin3, OUTPUT);
  pinMode(motorPin4, OUTPUT);

  // Initialize button pin
  pinMode(buttonPin, INPUT);
}

void loop()
{
  // Read the button state
  buttonState = digitalRead(buttonPin);

  // If the button state is high, move the stepper motor
  if (buttonState == HIGH && previousBtnState == LOW)
  {
    // Turn motor clockwise to move out for the
    // specified # of steps
    for (int i = 1; i <= steps; i++)
    {
      out();
      resetpins();
    }

    // Hold injection device for 5 seconds
    delay(5000);

    // Turn motor counterclockwise to move back in for
    // the same # of steps
    for (int i = 1; i <= steps; i++)
    {
     in();
     resetpins();
    }
  }
  previousBtnState = buttonState;
}

//////////////////////////////////////////////////////

void in ()
{
  clockwise();
}
```

```
void out ()
{
  counterclockwise();
}

void resetpins ()
{
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
}

void counterclockwise ()
{
    // 8
    digitalWrite(motorPin1, HIGH);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, HIGH);
    delayMicroseconds(timedelay);
    // 7
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, HIGH);
    delayMicroseconds(timedelay);
    // 6
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, HIGH);
    digitalWrite(motorPin4, HIGH);
    delayMicroseconds(timedelay);
    // 5
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, HIGH);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 4
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, HIGH);
    digitalWrite(motorPin3, HIGH);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
```

```
    // 3
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, HIGH);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 2
    digitalWrite(motorPin1, HIGH);
    digitalWrite(motorPin2, HIGH);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 1
    digitalWrite(motorPin1, HIGH);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
}

////////////////////////////////////////////////

void clockwise ()
{
    // 1
    digitalWrite(motorPin1, HIGH);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 2
    digitalWrite(motorPin1, HIGH);
    digitalWrite(motorPin2, HIGH);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 3
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, HIGH);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 4
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, HIGH);
```

```
    digitalWrite(motorPin3, HIGH);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 5
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, HIGH);
    digitalWrite(motorPin4, LOW);
    delayMicroseconds(timedelay);
    // 6
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, HIGH);
    digitalWrite(motorPin4, HIGH);
    delayMicroseconds(timedelay);
    // 7
    digitalWrite(motorPin1, LOW);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, HIGH);
    delayMicroseconds(timedelay);
    // 8
    digitalWrite(motorPin1, HIGH);
    digitalWrite(motorPin2, LOW);
    digitalWrite(motorPin3, LOW);
    digitalWrite(motorPin4, HIGH);
    delayMicroseconds(timedelay);
}
```

### B.3 Silicon vs. CNT Chip Injections

The code used for the silicon and CNT injections was the same as the code used for the PI concentration testing, but the inputs were attached to the appropriate power supplies. See Section B.1 for the code and Section 4.1.6 of Chapter 4 for the power inputs and protocols.

### B.4 Automated Current Control Box

```
// ********************************************** //
//              Nanoinjection Program             //
// ********************************************** //

// Created by Tyler Lewis
// Last updated Apr. 30, 2015 by Tyler Lewis
```

```
//************************************************
//*************   User Parameters   ***************
//************************************************

// Distance travel
int distance = 3;   // Desired travel distance in mm

// Distance at which input 1 turns off
double cutoff_distance = 0.8; // mm

// Input delay times
int input1duration = 20000;   // ms of constant DC
int input2duration = 20;      // ms repel/pulse time
int input3duration = 5000;    // ms Reverse voltage, withdraw lances

// Pulsed input parameters
int pulse = 1;                // 0 = no, 1 = yes
int period_length = 2;        // period (ms) of pulse duration

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


// Time delay between each step
// This controls the speed of the motor
// (1000 microseconds is the minimum possible)
// This can be changed, but usually isn't recommended.
// If modified, DO NOT go less than 1000 or the motor will
// miss steps and the displacement won't be accurate
int timedelay = 1200; // microseconds

// Calculate needed parameters from pulsed input parameters
int num_pulses = input2duration/period_length;  // number of pulses
int pulse_duration = period_length/2;           // Length of the actual
                                                // pulse (Do not edit)

// Define LED pins
int LED_Output = A1;
int LED_Input1 = A2;
int LED_Input2 = A3;
int LED_Input3 = A4;
int LED_Power = A5;
```

```
// Define Input Relays and MOSFET control pins
// To choose Input 1 as the output, make Relay1 = LOW, and Relay 2 = LOW
//   (Relay 2 doesn't matter in this case, it can be anything)
// To choose Input 2 as the output, make Relay1 = HIGH, and Relay 2 = LOW
//   (BOTH matter in this case)
// To choose Input 3 as the output, make Relay1 = LOW, and Relay 2 = HIGH
//   (Relay 1 doesn't matter in this case, it can be anything)
// To enable or disable the output of the control box,
//   use MOSFET = HIGH or LOW, respectively
int Relay1 = 5;
int Relay2 = 6;
int MOSFET = 7;

// Define Stepper Motor Control Pins
int motorPin1 = 8; // Input D on stepper motor driver
int motorPin2 = 9; // Input C on stepper motor driver
int motorPin3 = 10; // Input B on stepper motor driver
int motorPin4 = 11; // Input A on stepper motor driver

// Define injection trigger button pin
int injectionTrigger = 2;

// Define manual in/out calibration pins
int calibrateIN = 3;
int calibrateOUT = 4;

// Current state variables for the injection trigger & calibration
int buttonState_injection = 0;
int prevState_injection = 0;
int buttonState_in = 0;
int buttonState_out = 0;

// Initialize steps variable and Input 1 cutoff flag and cutoff value
int steps = 0;
int input1_cutoff = 0;
int cutoff_value = cutoff_distance*619;  // Cutoff value for flag

void setup()
{

  // Initialize LED control pins
  pinMode(LED_Output, OUTPUT);
  pinMode(LED_Input1, OUTPUT);
  pinMode(LED_Input2, OUTPUT);
  pinMode(LED_Input3, OUTPUT);
```

```
  pinMode(LED_Power, OUTPUT);

  // Turn on Power LED and turn off all others
  digitalWrite(LED_Output, LOW);
  digitalWrite(LED_Input1, LOW);
  digitalWrite(LED_Input2, LOW);
  digitalWrite(LED_Input3, LOW);
  digitalWrite(LED_Power, HIGH);

  // Initialize Input Relays and MOSFET control pins
  pinMode(Relay1, OUTPUT);
  pinMode(Relay2, OUTPUT);
  pinMode(MOSFET, OUTPUT);

  // Set all inputs and output to off using the Input Relay
  // and MOSFET control pins
  digitalWrite(Relay1, LOW);
  digitalWrite(Relay2, LOW);
  digitalWrite(MOSFET, LOW);

  // Initialize stepper motor control pins
  pinMode(motorPin1, OUTPUT);
  pinMode(motorPin2, OUTPUT);
  pinMode(motorPin3, OUTPUT);
  pinMode(motorPin4, OUTPUT);

  // Set all motor control pins to LOW (off)
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);

  // Initialize injection trigger pin
  pinMode(injectionTrigger, INPUT);

  // Initialize calibration control pins
  pinMode(injectionTrigger, INPUT);
  pinMode(injectionTrigger, INPUT);

  // Calculate the number of steps cycles required
  // to go the specified distance (619 steps/mm)
  steps = 619*distance;
}

void loop()
```

```
{

  // Read the trigger button state
  buttonState_injection = digitalRead(injectionTrigger);

  // If the button state is high, start the injection process
  if (buttonState_injection == HIGH && prevState_injection == LOW)
  {
    // Turn on input 1
    digitalWrite(MOSFET, HIGH);

    // Turn on input 1 LED and output LED
    digitalWrite(LED_Input1, HIGH);
    digitalWrite(LED_Output, HIGH);

    // Delay for specified time for DC attraction
    delay(input1duration);

    // Activate stepper to push lances into cells
    for (int i = 1; i <= steps; i++)
    {
      out();
      resetpins();

      // Increment Input 1 cutoff flag
      input1_cutoff = input1_cutoff + 1;

      // If cutoff value is reached turn off input 1 and its LED
      if (input1_cutoff > cutoff_value)
      {
        // Turn off input 1
        digitalWrite(MOSFET, LOW);

        // Turn off input 1 LED
        digitalWrite(LED_Input1, LOW);
      }
    }

    // Turn on input 2
    // (turning off Input 1 at this point isn't necessary because it
    // is disconnected in the first relay when Input 2 is chosen)
    // Turn on input 2 LED
    digitalWrite(Relay1, HIGH);
    digitalWrite(LED_Input2, HIGH);
```

```
// Delay 4 microseconds to allow Relay 1 time to switch
delay(4);

if (pulse == 1)
{
  // If pulse was set to 1, pulse input 2 on and off
  // according to the parameters
  for (int i = 1; i <= num_pulses; i++)
  {
    // Turn on input 2 using output enable MOSFET
    digitalWrite(MOSFET, HIGH);
    delay(pulse_duration);

    // Turn off input 2 using output enable MOSFET
    digitalWrite(MOSFET, LOW);
    delay(pulse_duration);
  }
}
else
{
  // Turn on input 2 using output enable MOSFET
  digitalWrite(MOSFET, HIGH);

  delay(input2duration);

  // Turn off input 2 using output enable MOSFET
  digitalWrite(MOSFET, LOW);
}

// Turn off input 2 LED
digitalWrite(LED_Input2, LOW);

// Turn on input 3
digitalWrite(Relay2, HIGH);
delay(4);
digitalWrite(MOSFET, HIGH);

// Turn on input 3 LED
digitalWrite(LED_Input3, HIGH);

// Hold injection device for the specified time
delay(input3duration);

// Turn off input 3
digitalWrite(MOSFET, LOW);
```

```
    // Turn off input 3 and output LED
    digitalWrite(LED_Input3, LOW);
    digitalWrite(LED_Output, LOW);

    // Reset Relays
    digitalWrite(Relay1, LOW);
    digitalWrite(Relay2, LOW);

    // Activate stepper motor to retract the lances
    for (int i = 1; i <= steps; i++)
    {
     in();
     resetpins();
    }

    // Reset Input 1 cutoff flag
    input1_cutoff = 0;
  }

  // Current trigger state becomes the prevous trigger state
  prevState_injection = buttonState_injection;

  // Read the calibration IN button state
  buttonState_in = digitalRead(calibrateIN);

  // If the button state is high, move the motor IN one increment
  if (buttonState_in == HIGH)
  {
    in();
    resetpins();
  }

  // Read the calibration OUT button state
  buttonState_out = digitalRead(calibrateOUT);

  // If the button state is high, move the motor OUT one increment
  if (buttonState_out == HIGH)
  {
    out();
    resetpins();
  }
}

//////////////////////////////////////////
```

```
//                Functions               //
////////////////////////////////////////////

void in ()
{
  counterclockwise();
}

void out ()
{
  clockwise();
}

void resetpins ()
{
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
}

void counterclockwise ()
{
  // 8
  digitalWrite(motorPin1, HIGH);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, HIGH);
  delayMicroseconds(timedelay);
  // 7
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, HIGH);
  delayMicroseconds(timedelay);
  // 6
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, HIGH);
  digitalWrite(motorPin4, HIGH);
  delayMicroseconds(timedelay);
  // 5
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, HIGH);
```

```
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 4
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, HIGH);
  digitalWrite(motorPin3, HIGH);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 3
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, HIGH);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 2
  digitalWrite(motorPin1, HIGH);
  digitalWrite(motorPin2, HIGH);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 1
  digitalWrite(motorPin1, HIGH);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
}

////////////////////////////////////////////////

void clockwise ()
{
  // 1
  digitalWrite(motorPin1, HIGH);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 2
  digitalWrite(motorPin1, HIGH);
  digitalWrite(motorPin2, HIGH);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 3
```

```
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, HIGH);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 4
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, HIGH);
  digitalWrite(motorPin3, HIGH);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 5
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, HIGH);
  digitalWrite(motorPin4, LOW);
  delayMicroseconds(timedelay);
  // 6
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, HIGH);
  digitalWrite(motorPin4, HIGH);
  delayMicroseconds(timedelay);
  // 7
  digitalWrite(motorPin1, LOW);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, HIGH);
  delayMicroseconds(timedelay);
  // 8
  digitalWrite(motorPin1, HIGH);
  digitalWrite(motorPin2, LOW);
  digitalWrite(motorPin3, LOW);
  digitalWrite(motorPin4, HIGH);
  delayMicroseconds(timedelay);
}
```