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Design and Experimental Testing of Nanoinjection Protocols for Delivering Molecules into HeLa Cells with a Bio-MEMS Device

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A thesis submitted to the faculty of
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ABSTRACT

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Delivering foreign molecules into living cells is a broad and ongoing area of research. Gene therapy, or delivering nucleic acids into cells via non-viral or viral pathways, is an especially promising area for pharmaceutics. All gene therapy methods have their respective advantages and disadvantages, including limited delivery efficiency and low viability. Nanoinjection, or delivering molecules into cells using a solid lance, has proven to be highly efficient while maintaining high viability levels. In this thesis, an array of solid silicon lances was tested by nanoinjecting tens of thousands of HeLa cancer cells simultaneously. Several molecule types were injected in different tests to understand cell uptake efficiency and cell viability. Voltage was used to determine the impact of an electric field on molecule delivery. Propidium iodide, a dye that fluoresces when bound to nucleic acids and does not fluoresce when unbound, was delivered into cells using the lance array. Results show that the lance array delivers propidium iodide into up to 78% of a nanoinjected HeLa cell culture, while maintaining 78%-91% viability. Using similar protocol as in propidium iodide experiments, plasmid DNA containing the code for a fluorescent protein was nanoinjected into HeLa cells, resulting in an average expression rate of up to 0.21%. Since gene expression only occurs in cells which have integrated DNA into the genome in the nucleus, a different DNA detection method was developed to determine total DNA count in cells following nanoinjection. DNA strands tagged with a radioactive isotope were nanoinjected into HeLa cells. Liquid scintillation was employed to quantify and discriminate between DNA delivered to cells and DNA that remained in solution around cells following nanoinjection. The largest average amount of DNA delivered to cells was 20.0 x 10^3 DNA molecules per cell. Further development of the radioactive nanoinjection process is needed to more fully understand the parameters that affect DNA delivery efficiency. In all experiments with propidium iodide and DNA molecules, low accumulation voltage, coupled with a short pulsed release voltage, resulted in the greatest molecule delivery efficiencies when compared to tests without voltage or with a constant voltage only. Lastly, an automated nanoinjection system was developed to eliminate variability in user applied nanoinjection force. The automated system was found to reduce variability in average propidium iodide uptake values by 56%. In conclusion, experimental testing of the multi-cell nanoinjection process has shown promising molecule delivery results into human cells, suggesting that further optimization of the process would have positive implications in the field of academic and clinical gene therapy.

Keywords: nanoinjection, propidium iodide, HeLa cells, pCAG-GFP DNA, radioactively labelled DNA, automated nanoinjection system

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NOMENCLATURE

^{32}P	Phosphorus 32 isotope
CPM	Counts per minute
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
FP	Full plasmid
GFP	Green fluorescent protein
HBSS	Hanks' balanced salt solution
PI	Propidium iodide
REDP	Restriction enzyme digested plasmid

Subscripts, superscripts, and other indicators

$[\,]^{*}$	Indicates statistically significant difference in population averages ($p<0.05$)
$[\,]^{**}$	Indicates no statistically significant difference in population averages ($p>0.05$)
$[\,]^{\Psi}$	Indicates statistically significant difference in population averages ($p<0.001$)
$[\,]^{\Psi\Psi}$	Indicates statistically significant difference in population averages ($p<0.01$)

CHAPTER 1. INTRODUCTION AND BACKGROUND

Many methods to deliver foreign material into living animal cells are common in research and clinical studies. By delivering certain particles into cells, diseases can be treated or even cured. Gene therapy is possibly the most common field of foreign material delivery into cells, and has great implications for curing human genetic diseases. Certain nucleic acids, when delivered into cell nuclei, can suppress undesirable cellular processes or supplement certain processes that have been limited due to genetic disease. For example, two monkeys colorblind from birth were given gene therapy treatment in a recent experiment. As a result of gene therapy, both animals had their color vision restored with no apparent side effects [1].

Traditionally, gene therapy has been divided into two categories: biological (viral) vectors and chemical or physical (non-viral) approaches [2–4]. Although viral vectors are currently the most effective approach to delivering DNA into cells, they have certain limitations, including immunogenicity, toxicity, and limited capacity to carry DNA [2, 5–9].

Microinjection, or injecting DNA solution through a micropipette into a cell, is a highly efficient delivery method. However, microinjection is limited to *in vitro* applications and injecting a single cell at a time, which can be time consuming [3, 7, 10]. Electroporation techniques use an electric field to increase permeability of the plasma membrane, allowing foreign particles to enter the cells through membrane openings. Although electroporation can be an efficient gene delivery method, toxicity from the electric field limits the viability of the process [3, 6, 7, 11, 12]. Other studies have used chemicals to deliver foreign particles into cells [6, 11, 13–15]. Chemical reagents can be efficient at delivering DNA into cells, but studies have found that these chemicals can also be toxic to cells [16]. Numerous other methods exist, and can be reviewed in [2–4].

1.1 Nanoinjection Technique

Genetic-based therapy is a promising area of drug delivery, although there are currently many limitations and advantages to all gene therapy methods in practice. In this thesis we discuss a new mechanical approach to deliver foreign particles into cells. Nanoinjection techniques have the potential for high material delivery efficiency while maintaining high viability. Previous nanoinjection studies have designed and used a single polysilicon lance mechanism to inject mouse zygotes with DNA. These mouse zygote injections have proven more efficient and less damaging to mouse zygotes, when compared to parallel studies using a microinjection technique [17–20].

Nanoinjection is a new gene delivery technique that uses a solid silicon lance to penetrate the cell membrane and deliver foreign material [18]. Two methods can be used to disperse particles into the cell: 1) particles can either diffuse into the cell through pores opened by the lance, or 2) electric charge, applied to the lance, can assist in particle entry. When including an electric charge on the lance array chip in the nanoinjection method, charged particles are accumulated on lances through application of voltage. Lances then penetrate the cell membrane, the charge is reversed, and the particles are released inside cells. The diffusion method is nearly identical, without the application of an electric charge to lances.

A nanoinjection lance array has been developed for injecting thousands of culture cells simultaneously [21,22]. Propidium iodide, DNA plasmids, and radioactively labelled DNA are used in this thesis as the injection molecules to measure molecule uptake efficiency and cell viability when using the nanoinjection lance array. The nanoinjection lance array penetrates cell membranes in an entire cell culture, opening up pores in the membranes where injection particles can enter cells. Lances are small enough, relative to the size of the cells, that damage to the cell is minimal. This thesis presents the proposed nanoinjection method and discusses results from injecting several molecule types into HeLa culture cells. This method could have tremendous implications as an injection technique in academic or clinical research, including the gene therapy field.

Three different molecules were delivered into HeLa cancer cells for the purpose of this thesis: (1) propidium iodide (Chapter 2), a stain that fluoresces when bound to nucleic acids, (2) pCAG-GFP (Chapter 3), a DNA plasmid containing the code to express green fluorescent protein, and (3) radioactively labelled DNA strands (Chapter 4), for detection by liquid scintillation.

1.1.1 Propidium Iodide

A fluorescent dye, propidium iodide (PI), is frequently used as an injection particle when performing preliminary experiments with new gene therapy methods. In one study, optical injection (photoporation with a laser) was used to deliver PI molecules into living HEK293 cells in a microfluidic device. At a PI concentration of 1 mg/mL solution, an average PI delivery efficiency and viability of 42% and 67% were obtained, respectively [23]. Another study used highly localized electroporation with nanostraws to deliver PI into small amounts of HEK293 cells. With PI concentrations at 0.1 mg/mL solution, >95% PI uptake and >98% viability were achieved [24].

Propidium iodide (Sigma) was used in some experiments as an injection particle to determine the efficiency and viability of the lance array nanoinjection method. When propidium iodide (PI) comes into contact with genetic material, PI molecules intercalate between base pairs of DNA and RNA [25–31]. PI molecules attached to nucleic acids emit enhanced (20x-30x) fluorescent light waves when excited by certain lamps, making PI positive cells detectable with flow cytometry. PI does not penetrate the membrane of viable cells [26, 30–32], so PI positive cells in this experiment must be either successfully injected, dead, or both. In previous experiments, no cytotoxicity has been seen in cells for at least one hour after the addition of PI to islet cells [33]. In theory, the double positive charge on a PI molecule [34] allows PI to be manipulated in the presence of an electric field. We performed tests to determine if an electric charge would increase or decrease PI uptake in cells. All the attributes of propidium iodide listed in this section make PI molecules good candidates for testing the effectiveness of the nanoinjection lance array system.

1.1.2 Green Flourescent Protein Plasmid DNA

Plasmids coded for green fluorescent protein (GFP) are commonly used in gene therapy research to monitor gene expression in cells [35–37]. When a DNA plasmid has been integrated into the nucleus of a cell, the fluorescent protein manufactured by the cell can easily be viewed under fluorescent microscopy or detected with flow cytometry. In the presence of an electric field, DNA molecules can be manipulated due to their negative charge. Previous experiments have shown that it is possible to accumulate DNA to a charged lance, insert the lance into the nucleus of a mouse

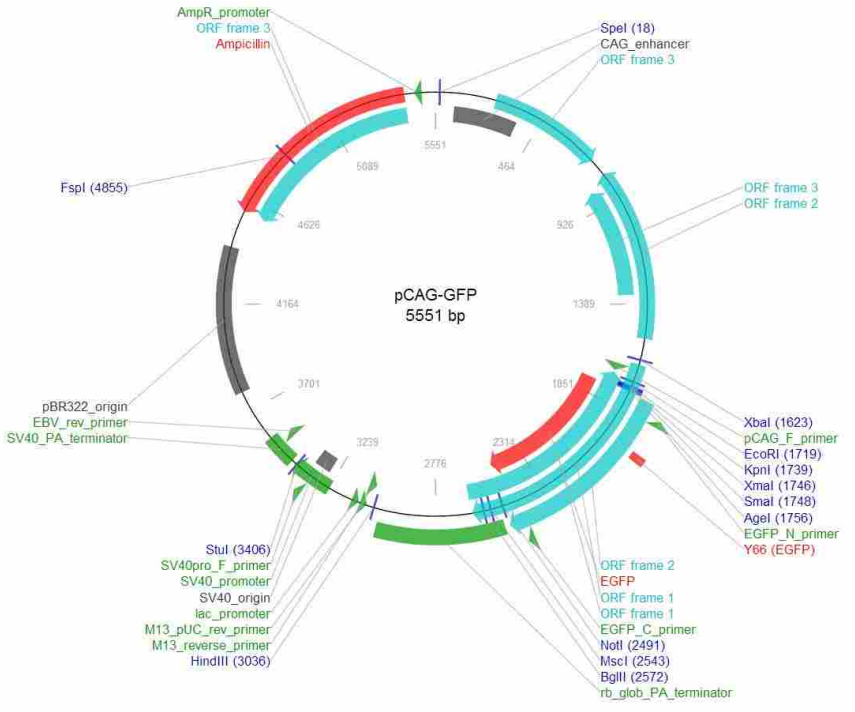

Figure 1.1: Schematic of the pCAG-GFP plasmid, showing SpeI and HindIII sites. Image courtesy http://www.addgene.org/11150/

egg cell, and successfully release the DNA inside the cell [17, 18]. Figure 1.1 shows the gene map of the pCAG-GFP plasmid used in these experiments.

1.1.3 Radioactively Labelled DNA

The same plasmid used for GFP expression can be tagged with radioactive molecules. After a plasmid is restriction enzyme digested, radioactive nucleotides can be attached to the ends of the newly cut linear DNA strands. Following nanoinjection of the DNA into cells, the radioactively labelled DNA can be quantified using liquid scintillation.

1.2 Lance Array Fabrication

Lance arrays are fabricated using standard photolithography and etching methods for silicon wafers outlined in [21, 22]. A bare silicon wafer is patterned with a grid of photoresist dots. The locations of the photoresist dots determine where lances will be located on the wafer by the end of the fabrication process. A combination of plasma etches at specific etch times results in an

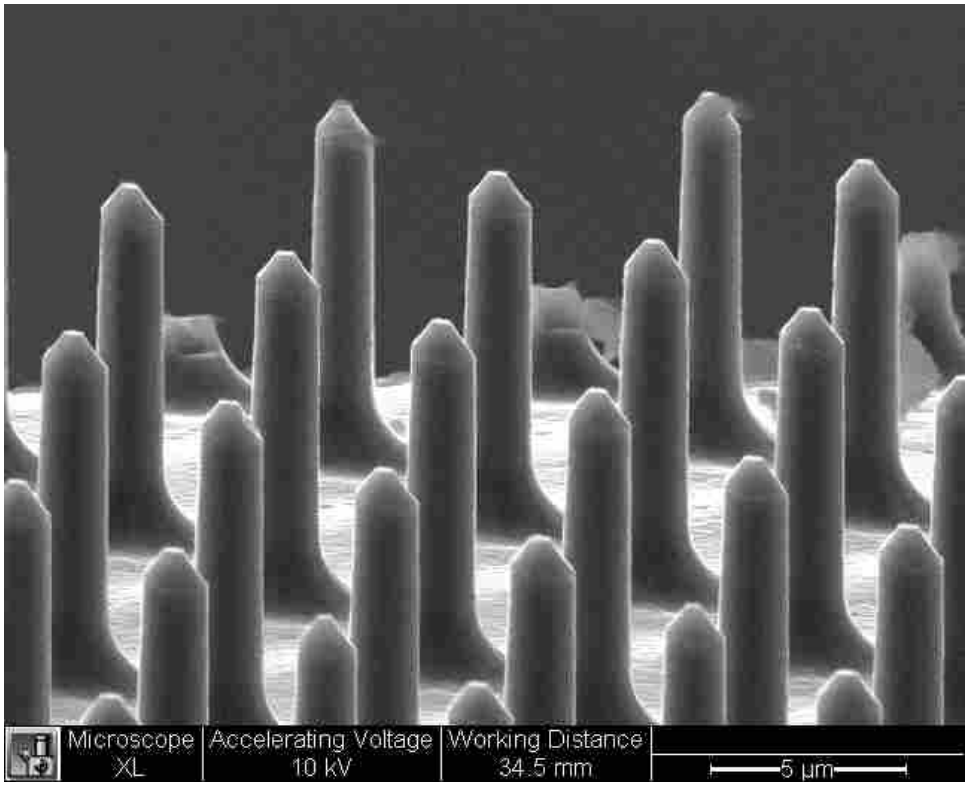

Figure 1.2: Scanning electron micrograph showing the edge of a lance array chip.

array of high-aspect ratio lances on the surface of the wafer. The lances are 1-1.5 μm in diameter and 8-10 μm in length (see Figure 1.2). The silicon wafer is diced into square pieces 2 cm by 2 cm. With the space between lances being 10 μm, each 2x2 cm silicon chip contains approximately four million lances on the surface. Lance geometry and spacing have been optimized for testing on a culture of HeLa 229 cells [21].

1.3 Injection Mechanism Design

For injection experiments, the lance array was mounted on an injection device to control lance motion. The injection device was designed to fulfill several purposes: 1) provide the motion necessary to plunge the lances through cell membranes and withdraw them during the injection process, 2) constrain lance movement to the vertical direction only, 3) fit firmly into each well of the culture dish, and 4) contain conductive sections of material that allow voltage to be applied to the lance array chip [22, 38]. The injection device was fabricated from ABS plastic with rapid prototyping technology (Figure 1.3). Two parallel ortho-planar springs ensure injection motion is

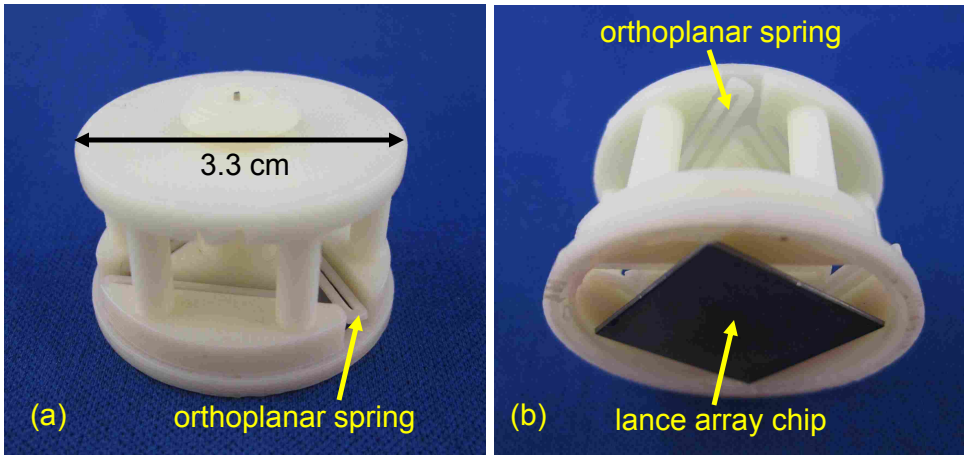

Figure 1.3: Images of the injection device from (a) above and (b) below.

strictly vertical [39]. Voltage was required for some of the tests to determine if an electric charge on lances increases molecule uptake into cells. A combination of stainless steel wire, conductive epoxy, and gold contacts results in a conductive section of the device necessary for voltage application [38].

1.4 Research Objectives and Thesis Outline

There were three main objectives of this research project:

1. Design and test multi-cell nanoinjection protocols with PI as the injection molecule to determine efficiency and cell viability

2. Using knowledge gained from the first objective, design and test nanoinjection protocols with DNA as the injection molecule and determine efficiency

3. Design and test an automated nanoinjection system that reduces variability caused by multiple users

The second chapter describes the protocol developed to nanoinject PI into HeLa cells. Several PI concentrations were used to determine the impact of PI concentration on uptake efficiency. It was shown that PI concentration does affect the amount of PI taken up by cells during nanoinjection. Other experiments showed that voltage has a positive impact on delivering PI molecules into

cells, while maintaining low cell death. The results discussed in Chapter 2 have been submitted as an article to a peer reviewed journal.

Chapter 3 describes protocols and results from preliminary nanoinjection of plasmid DNA into cells. The cells were tested for gene expression rates. Chapter 4 describes a different protocol for delivering DNA in to cells and quantifying the amount of nanoinjected DNA per cell. DNA was tagged with radiaoctive particles, injected into cells, and DNA molecules were counted with liquid scintillation. After further testing with radioactively tagged DNA, the results from Chapter 4 will be submitted to a peer reviewed journal.

The fifth chapter describes the design and testing of an automated nanoinjection system. The system incorporated previous injection device designs, and coupled them with a linear actuator, switching relays, and a microcontroller. The system reduced PI uptake variability by 56%, successfully eliminating some large variability inherent to multiple users performing nanoinjections manually. The Chapter 5 results have been submitted and accepted as a DETC 2014 conference paper.

CHAPTER 2. NANOINJECTION OF PROPIDIUM IODIDE INTO HELA CELLS

To test the efficiency of the multi-cell nanoinjection process, propidium iodide (PI) molecules were added to saline solution around cells prior to nanoinjection. PI is a cell stain common in research and was chosen as the initial molecule for cell delivery. Efficiency and viability data for nanoinjections were gathered from the PI experiments.

2.1 Testing Methods

HeLa 229 cancer cells (Figure 2.1) were nanoinjected with PI in this experiment. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum and gentamicin, and incubated at 37°C with 5.0% CO_2. To prepare for the injection process, cells were cultured in six well culture plates, with approximately 25 x 10^4 cells per well. Cells were cultured on glass microscope cover slips in each well to eliminate any surface variation in the culture plates. If the experiments were performed using voltage, stainless steel plates were placed in the base of the well before adding cells. Cells were spread into a circular shape approximately 2 cm in diameter in the center of the well.

Following incubation in six well plates for 24 hours, media was removed from each well and the cells were rinsed with Hanks Balanced Salt Solution (HBSS). HBSS and PI were added to each well in a pre-determined concentration. Four PI concentrations were used in these experiments to determine trends in cellular PI uptake and cell viability due to PI concentration in solution: 0.01, 0.02, 0.03, and 0.04 mg PI per 1 mL HBSS. A typical injection experiment consisted of negative controls (one with no PI and no membrane penetration with lances, and eleven with PI added but no membrane penetration), and twelve injected samples (PI added and cells punctured with lances).

For each injected sample, the injection device and lance array chip were lowered into a well of the six-well culture dish. A side view schematic of the injection device and lance array chip in

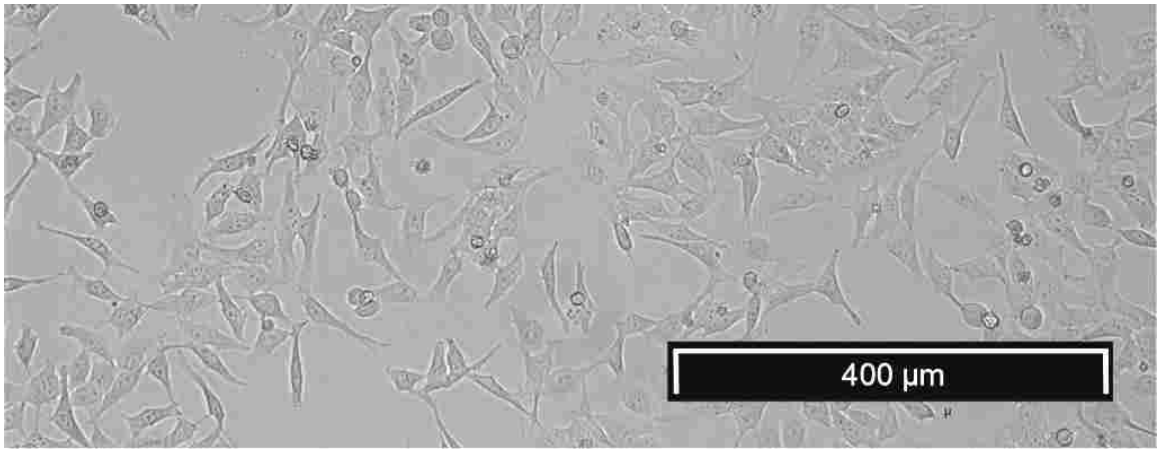

Figure 2.1: Optical micrograph of a HeLa 229 cell culture. The cells are approximately 50% confluent.

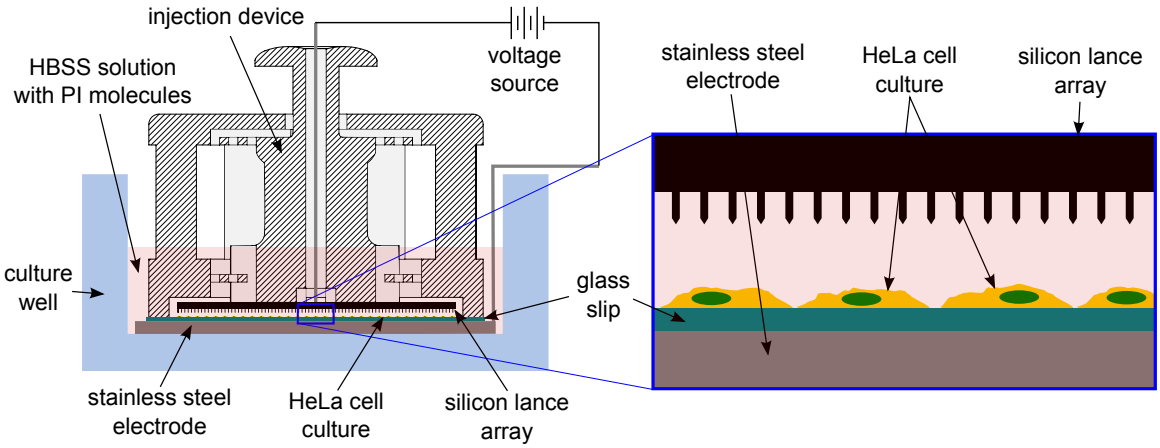

Figure 2.2: Schematic of the setup for the nanoinjection process with a lance array, using the application of an electric field (not to scale).

a single cell culture well is shown in Figure 2.2. The stainless steel plate electrode (for voltage injections), lance array chip, and adhered HeLa cells were completely submersed in HBSS/PI solution for these experiments.

At this step in the nanoinjection process, one of six different tests were performed to determine molecule delivery efficiency and cell death due to different variations of the nanoinjection process.

- PI diffusing through membrane openings created by lances in the nanoinjection process, with no applied voltage

- An application of 1.5 VDC to the lances to accumulate and release PI molecules into cells
- PI entering cells with the assistance of 1.5 VDC accumulation and a 20 ms release pulse alternating between 1.5 and 5 VDC applied to the lances
- An experiment identical to diffusion testing, without addition of PI
- An experiment identical to diffusion testing, but using a bare silicon chip instead of a lance array
- An experiment performed to determine the length of time cell membranes remain permeable following penetration and removal of lances

2.1.1 Diffusion Protocol

A manual force of approximately 30 N was applied to the center fixture of the injection device, moving the lances through cell membranes and into the cytoplasm or nucleus of adhered cells. After holding the force for 5 seconds, the force was released and the injection device removed from the well. The diffusion protocol presented PI nanoinjection data that other variations in the injection process could be measured against.

2.1.2 DC Voltage Protocol

In theory, positively charged PI molecules can be accumulated on lances and released in the presence of an electric field [34]. DC Voltage was added to the diffusion protocol to test for greater PI uptake efficiencies using an electric field. A power supply was used to apply an electric potential across the stainless steel base electrode and the lances. Negative 1.5 VDC was applied to the system for 20 seconds, allowing lances to accumulate positively charged PI molecules. Using a manually applied 30 N force, the lances were lowered into the cell culture, puncturing cell membranes. Immediately following membrane penetration, the 1.5 VDC was switched to positive for 5 seconds, releasing PI molecules from the lances into the cytoplasm or nucleus. The lances were withdrawn from the cells and the entire device was moved to the next injection site. Figure 2.3 illustrates the steps in the nanoinjection process with a lance array chip and DC voltage.

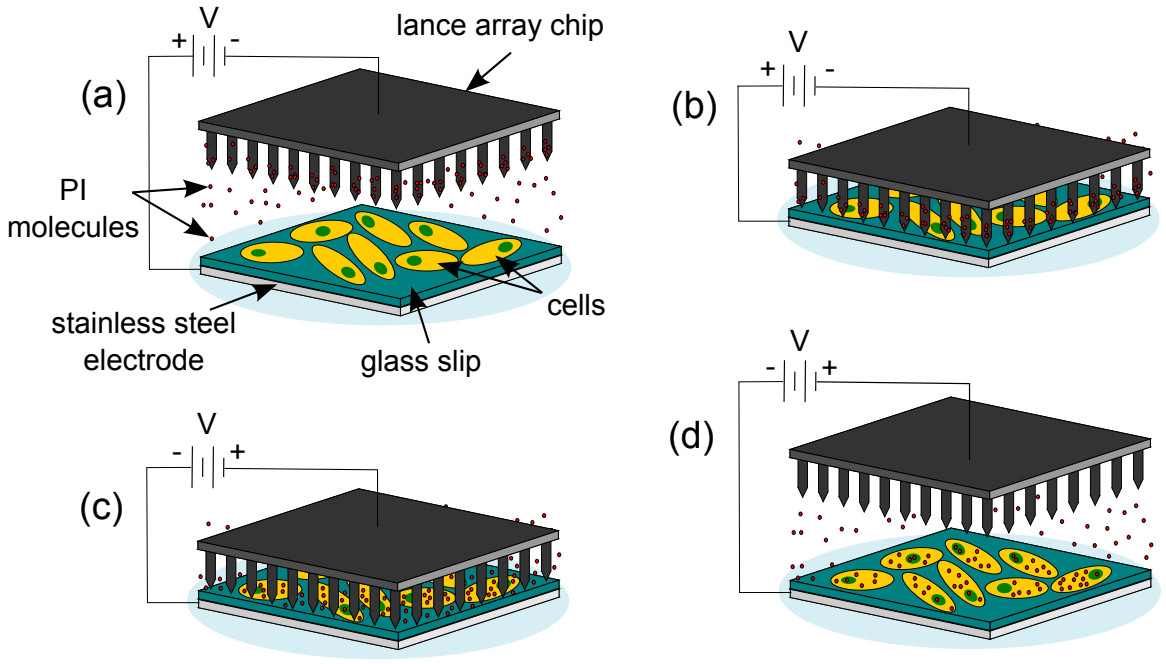

Figure 2.3: Process diagram for the nanoinjection technique using a lance array and voltage (not to scale). The process begins with (a) cultured cells in solution with PI and the lance array, with a negative voltage applied to the lances to accumulate PI molecules. The lances penetrate cell membranes (b), and the voltage is reversed (c), releasing the PI molecules inside the cytoplasm and/or nucleus of cells. The lances are removed from the cells (d), leaving PI molecules inside.

2.1.3 DC Voltage with Pulse Protocol

Negative 1.5 VDC was applied to the nanoinjection system for 20 seconds, allowing lances to accumulate positively charged PI molecules. Lances were pushed into adhered cells with a force of 30 N. Immediately following membrane penetration, a square wave pulse between positive 1.5 and 5 VDC was applied to the electrodes for 20 ms, releasing PI molecules from lances into the cytoplasm or nucleus. Positive 1.5 VDC was applied while removing the injection system from the well. Pulsed voltage was employed to determine if an increased repulsion voltage would increase PI uptake while mitigating cell death. The 2 ms period square wave pulse with 50% duty cycle was applied for 20 ms for a total of 10 pulses per sample, since high voltages for an extended period of time can be fatal to cells [40].

2.1.4 Protocol for Injections Without PI

In order to quantify cell death due to lances puncturing cell membranes, propidium iodide was not added to any samples for these nanoinjections. A manual force of approximately 30 N was applied to the center fixture of the injection device, moving the lances through cell membranes and into the cytoplasm or nucleus of adhered cells. After holding the force for 5 seconds, the force was released and the injection device removed from the well.

2.1.5 Bare Silicon Chip Injection Protocol

To determine the difference in PI uptake between cells punctured with lances and cells strained but not punctured, a bare silicon chip was employed. A manual force of approximately 30 N was applied to the center fixture of the injection device, pressing the bare silicon chip against cell membranes of adhered HeLa cells. After holding the force for 5 seconds, the force was released and the injection device removed from the well.

2.1.6 Cell Membrane Permeability Timing Protocol

To test for cell membrane permeability over time, cell cultures were punctured with the lance array before adding PI to the solution. PI concentration was constant at 0.04 mg/mL for all samples. After cell cultures were injected, PI was added to samples at intervals of 0, 3, 7, or 10 minutes.

2.2 Collection of Cells for Analysis

To make sure different PI concentration samples were not mixed, the lance array chip was rinsed before injecting cells at a new PI concentration. Following the injection of each sample, regardless of previous injection protocol, 5x Trypsin (Sigma) was added to each well and the culture plates were incubated for 5 minutes. After trypsinating the cells, DMEM was added to each well, and the contents of each well were transferred to FACS tubes (BD Biosciences). The tubes were centrifuged at 2000 RPM for 10 minutes. Following centrifugation, supernatants in each tube were removed and the pellet of cells resuspended in the remaining media. 0.25 mL

HBSS was added to each tube, and the experimental samples were then analyzed using a flow cytometer (BD Biosciences).

2.3 Flow Cytometry and Statistical Analysis

Flow cytometry was employed to quantify PI positive cells and cell death. Using flow analysis software (BD FACSDiva, Dako Summit) and control data, live and dead cell populations were determined on a forward scatter and side scatter basis. For all experiments, cell death was determined using gating techniques in the flow software. For each HeLa cell sample, 10,000 events were analyzed. Control data was also used to determine the PI positive population in the PI fluorescence channel.

Confidence intervals were calculated for each data set ($\alpha = 0.05$) and included in bar graphs to visualize variation in statistical analysis. Student's t-tests for small sample sizes ($n<30$) were used to determine statistical significance between population means. When comparing population means, p-values less than 0.05 were considered to show statistically significant difference. Population means with p-values greater than 0.05 were considered statistically the same.

2.4 Results and Discussion

2.4.1 Nanoinjection Efficiency

Figure 2.4 compares averages in PI uptake efficiency for controls, diffusion, 1.5 VDC, and pulsed voltage experiments. T-test results indicate that all differences in PI uptake between population means are statistically significant ($p<0.05$), except for the difference between 1.5 VDC and diffusion at 0.04 mg/mL PI ($p>0.05$). Exact p-values for differences in population means can be viewed in Table 2.1.

Increasing PI concentration resulted in similar trends for all controls, diffusion, 1.5 VDC, and pulsed voltage experiments. Greater concentrations of PI resulted in greater PI uptake into cells. As seen in Figure 2.4, when comparing controls to diffusion tests, it is evident that the nanoinjection method penetrates cell membranes and delivers PI molecules to culture cells. By allowing PI molecules to diffuse into culture cells following the insertion and release of nanoin-

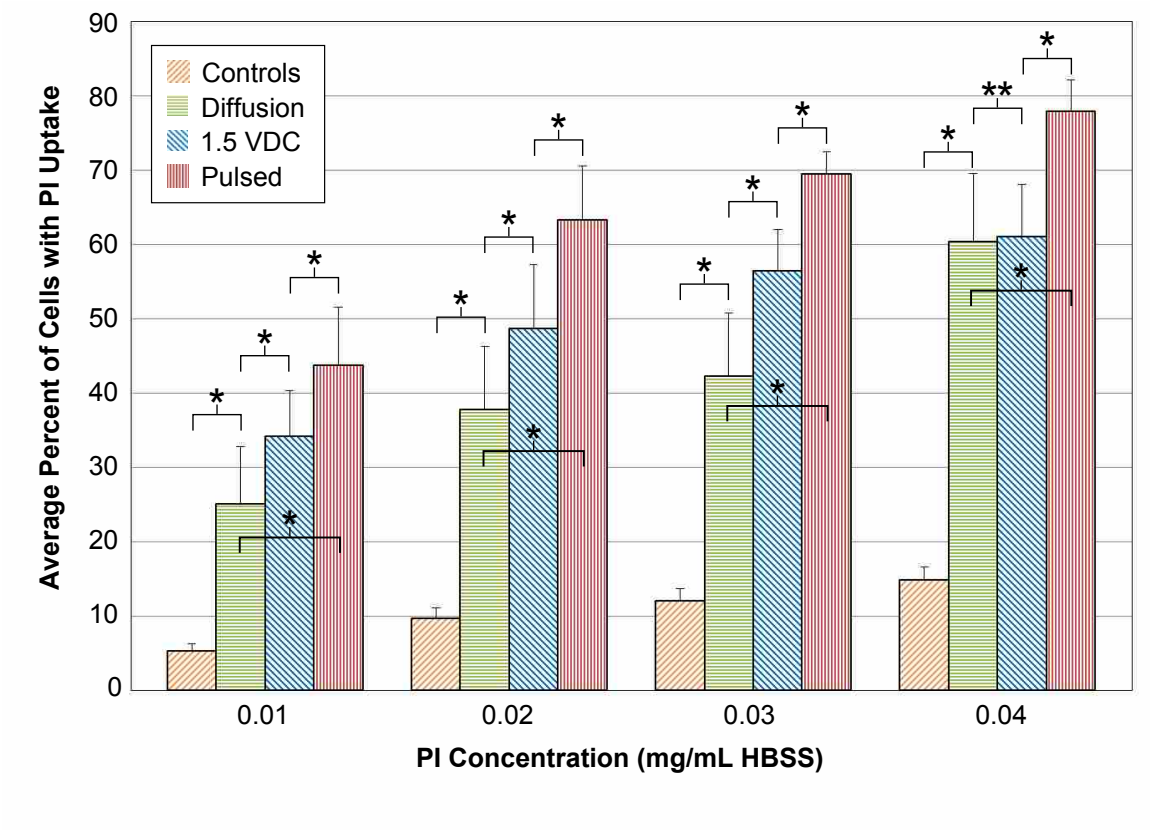

Figure 2.4: Bar graph comparing average PI uptake data for controls, diffusion injections, 1.5 VDC injections, and pulsed voltage injections. The error bars indicate a 95% confidence interval (α = 0.05). *Statistically significant difference in population averages ($p<0.05$) **Population averages are statistically the same ($p>0.05$)

jection lances, approximately 4.1 times more cells take in PI than without nanoinjection. The addition of DC voltage, to accumulate PI molecules and release them into the cellular cytoplasm or nucleus, increases efficiency of the nanoinjection process by an average of 11.4% over diffusion. In the case of the highest PI concentration, statistical evidence shows that the application of voltage is no different than diffusion tests for PI uptake. However, due to the evidence that the other three concentrations are statistically different between diffusion and 1.5 volts, this discrepancy may be a result of the inherent variation in biological data [41]. We can confidently state that the addition of 1.5 DC volts increases average PI uptake when compared with diffusion alone in the nanoinjection process. The pulsed voltage protocol delivers the greatest amount of PI to HeLa cells.

Table 2.1: Table of p-values calculated when comparing the difference in population means for PI uptake.

PI (mg/mL)	**0.01**	**0.02**	**0.03**	**0.04**
Ctrls *vs.* Diff	9.7E-5	8.0E-6	3.0E-6	<1.0E-6
Ctrls *vs.* 1.5 V	<1.0E-6	<1.0E-6	<1.0E-6	<1.0E-6
Ctrls *vs.* Pulse	<1.0E-6	1.0E-6	<1.0E-6	<1.0E-6
Diff *vs.* 1.5 V	0.041	0.043	5.7E-3	0.45
Diff *vs.* Pulse	1.7E-3	1.2E-4	8.0E-6	1.4E-3
1.5 V *vs.* Pulse	0.039	9.4E-3	2.3E-4	2.7E-4

Diffusion protocols delivered PI, on average, to approximately 4 times the number of cells as control data. Constant DC voltage and pulsed voltage protocols resulted in 5x and 6.5x greater PI uptake, respectively, than controls at the same PI concentration.

2.4.2 Nanoinjection Viability

Results of viability calculations are graphed in Figure 2.5 along with confidence intervals (α=0.05) to visualize scatter in the data. The differences in average viability between diffusion testing and all voltage tests are statistically significant ($p<0.05$). There is no statistical difference ($p>0.05$) in average viability of the 1.5 VDC tests and pulsed voltage tests at all PI concentrations. Average viability for controls and diffusion tests were statistically different for each PI concentration, except for 0.03 mg/mL. Specific p-values for differences in average viability between tests are listed in Table 2.2.

The viability results show that diffusion nanoinjections induce greater average cell death than that of controls, but only by 2%-4%. Both experiments performed with voltage caused greater average cell death than diffusion nanoinjections, by 5%-12%. At all PI concentration levels, average viability was higher for 1.5 VDC tests than pulsed voltage tests. However, statistical analysis reveals that there is no statistical difference in average viability between 1.5 VDC and pulsed voltage tests. Therefore, the addition of a 20 ms repulsion pulse does not decrease cell death when compared to 1.5 VDC repulsion tests.

Viability averages between PI concentrations in each experiment were compared for statistical difference. Table 2.3 lists the p-values obtained from the Students t-test. For all average

Table 2.2: Table of p-values calculated when comparing the difference in population means for cell viability.

PI (mg/mL)	**0.01**	**0.02**	**0.03**	**0.04**
Ctrls *vs.* Diff	9.4E-3	0.030	0.056	5.2E-3
Ctrls *vs.* 1.5 V	1.0E-6	3.6E-5	3.0E-6	6.0E-6
Ctrls *vs.* Pulse	5.3E-5	5.7E-4	<1.0E-6	7.2E-5
Diff *vs.* 1.5 V	3.4E-4	3.8E-3	3.1E-4	1.2E-3
Diff *vs.* Pulse	2.3E-4	2.5E-3	1.9E-5	7.6E-4
1.5 V *vs.* Pulse	0.15	0.10	0.37	0.12

viability comparisons in control data and diffusion data, there is no statistical difference between PI concentrations ($p>0.05$). When comparing averages between 0.01 and 0.03 mg/mL PI, as well as 0.01 and 0.04 mg/mL PI for both experiments with voltage, statistical difference is apparent ($p<0.05$). For all other average viability comparisons in voltage experiments, there is no statistical difference between PI concentrations.

The statistical difference in viability in the electrical experiments between concentrations may be explained by a change in the electrochemistry at higher concentrations. For control and diffusion data, PI uptake into cells is approximately linearly dependent on PI concentration. However, PI uptake does not increase linearly with increasing PI concentration in voltage experiments (see Figure 2.4). Instead, there seems to be a decreasing effect of voltage as concentration increases. This data suggests that the application of voltage becomes less effective at accumulating and releasing PI molecules from lances at higher PI concentrations, perhaps due to differences in the electrochemistry of the solution. The decrease in electrical effectiveness may also explain the increase in cell viability as PI concentration increases in voltage experiments. If a smaller effective electric field is being applied to the cells at higher concentrations due to changes in electrochemistry, this would explain both the higher viability and the reduced efficiency effect.

2.4.3 Membrane Puncturing Viability Without Injecting PI

Since PI fluoresces when bound to nucleic acid chains, cells with positive PI expression may have died due to normal passaging procedures, or they may have been successfully injected. An experiment was performed to quantify the difference in cell death occuring as a result of pas-

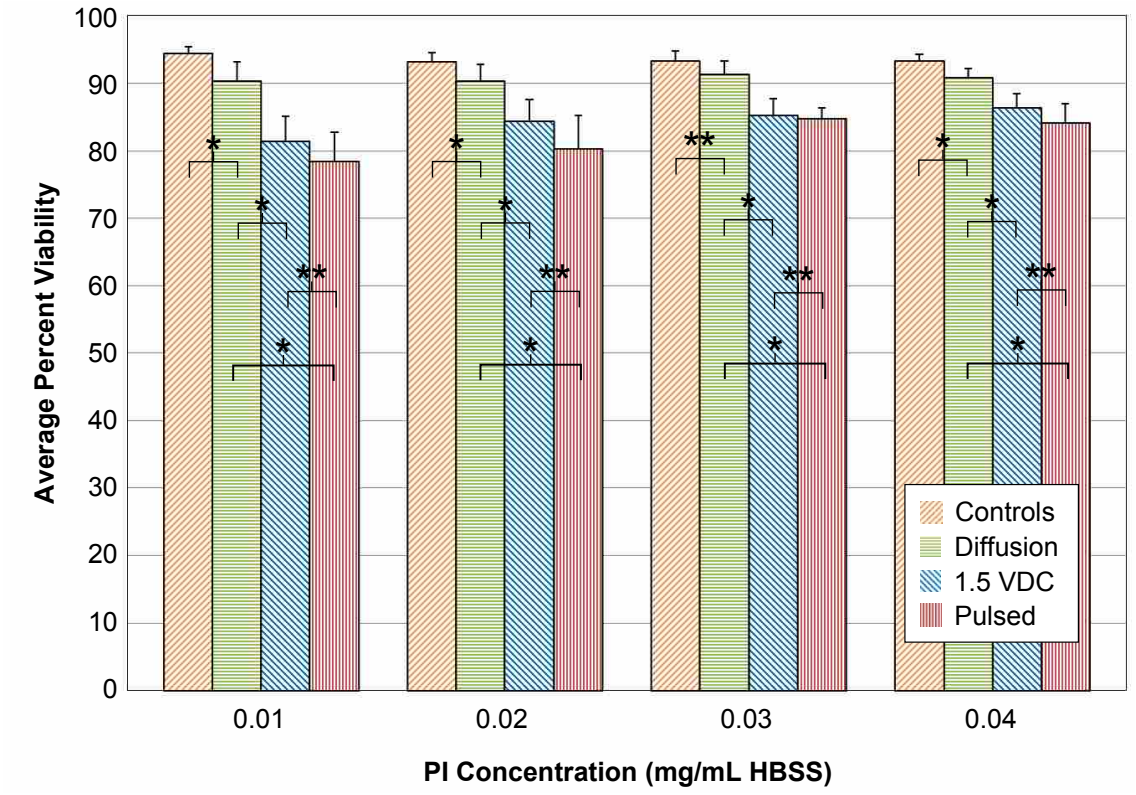

Figure 2.5: Bar graph comparing average viability data for controls, diffusion injections, 1.5 VDC injections, and pulsed voltage injections. The error bars indicate a 95% confidence interval (α = 0.05). *Statistically significant difference in population averages ($p<0.05$) **Population averages are statistically the same ($p>0.05$)

saging and moving cells, and cell death as a result of puncturing membranes with lances. Cell death was determined using gating capabilities of flow cytometry software. Figure 2.6 indicates an average decrease in cell viability of 4.2% due to lances puncturing cell membranes, which agrees with the diffusion vs. controls comparison in Figure 2.5. From the results of a t-test comparing population means, the difference in average viability between the controls and punctured cells is statistically significant ($p<0.001$). We can confidently state that on average the lances induce approximately 4% greater cell death than cells not punctured with lances.

2.4.4 Nanoinjections With Bare Silicon

An experiment was performed to determine if lances were indeed penetrating cell membranes, or if cell membranes were being strained into opening pores for PI delivery. Pieces of a

Table 2.3: Table of p-values calculated when comparing the difference in population means for cell viability, between PI concentrations.

PI (mg/mL)	**Ctrls**	**Diff**	**1.5V**	**Pulse**
0.01 *vs.* 0.02	0.089	0.49	0.13	0.29
0.01 *vs.* 0.03	0.13	0.28	0.050	9.8E-3
0.01 *vs.* 0.04	0.083	0.40	0.015	2.3E-3
0.02 *vs.* 0.03	0.44	0.27	0.34	0.063
0.02 *vs.* 0.04	0.44	0.39	0.16	0.11
0.03 *vs.* 0.04	0.48	0.32	0.26	0.36

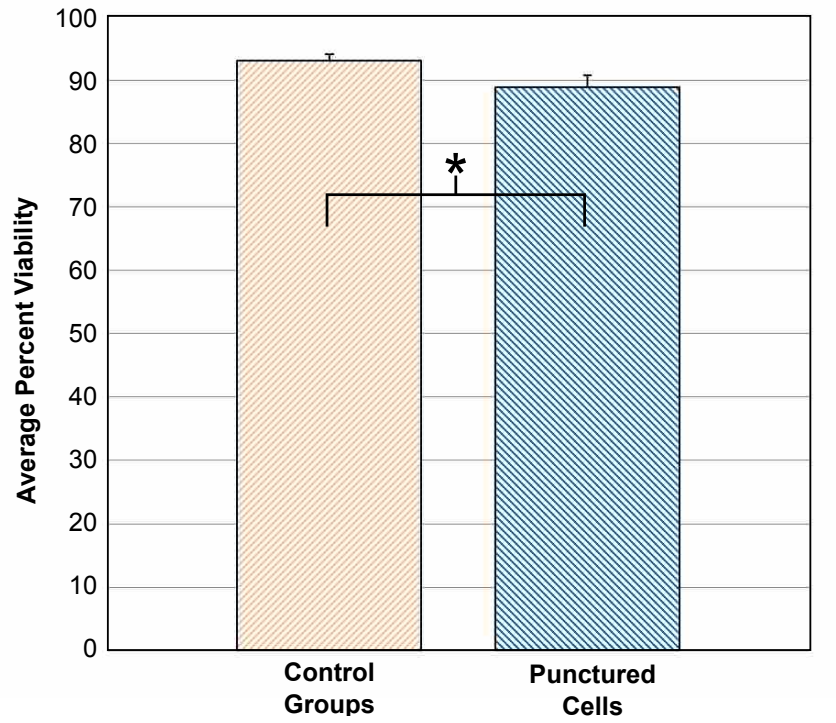

Figure 2.6: Bar graph comparing average viability data for controls and cells punctured with the lance array. The error bars indicate a 95% confidence interval ($\alpha = 0.05$). *Statistically significant difference in population averages ($p<0.05$)

bare silicon wafer, having the same dimensions as the lance array chips (2x2 cm), were used in place of the lance arrays. Figure 2.7 compares experimental data from diffusion lance injections with bare silicon "injections" and control groups with the same PI concentration. Bare silicon delivers more PI than control groups, indicating that there is some cell membrane permeability that

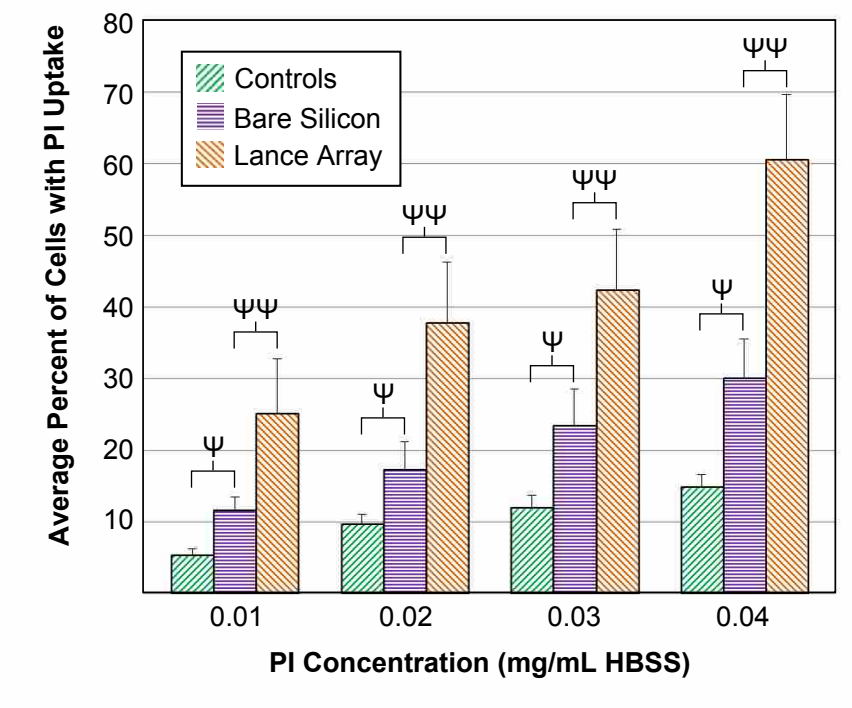

Figure 2.7: Bar graph comparing PI uptake between cells strained with a bare silicon chip, cells punctured with lances, and un-punctured controls. Error bars indicate a 95% confidence interval (α = 0.05). $^{\Psi}$Statistically significant difference in population averages ($p<0.001$) $^{\Psi\Psi}$Statistically significant difference in population averages ($p<0.01$)

occurs, allowing more PI to diffuse into cells. The data indicates statistically significant differences in average PI uptake between bare silicon injections and lance penetration experiments ($p<0.01$). There is also statistically significant difference in average PI uptake between bare silicon "injections" and control data ($p<0.001$). On average, the silicon lance array delivers PI to twice as many cells as a bare silicon chip in similar experiments.

2.4.5 Cell Membrane Permeability Following Nanoinjection

PI uptake results from the membrane permeability tests are graphed in Figure 2.8. Adding PI immediately after removing lances from cell membranes gives nearly identical PI uptake results as in the 0.04 PI concentration in Figure 2.4 ($\approx$ 60%). Whether PI is added to the cell culture solution before or immediately after membrane penetration with lances makes no difference in PI uptake. PI uptake decreases in nanoinjected cells the time interval between injections and addition of PI increases. This indicates that cell membrane pores are formed due to nanoinjection, and

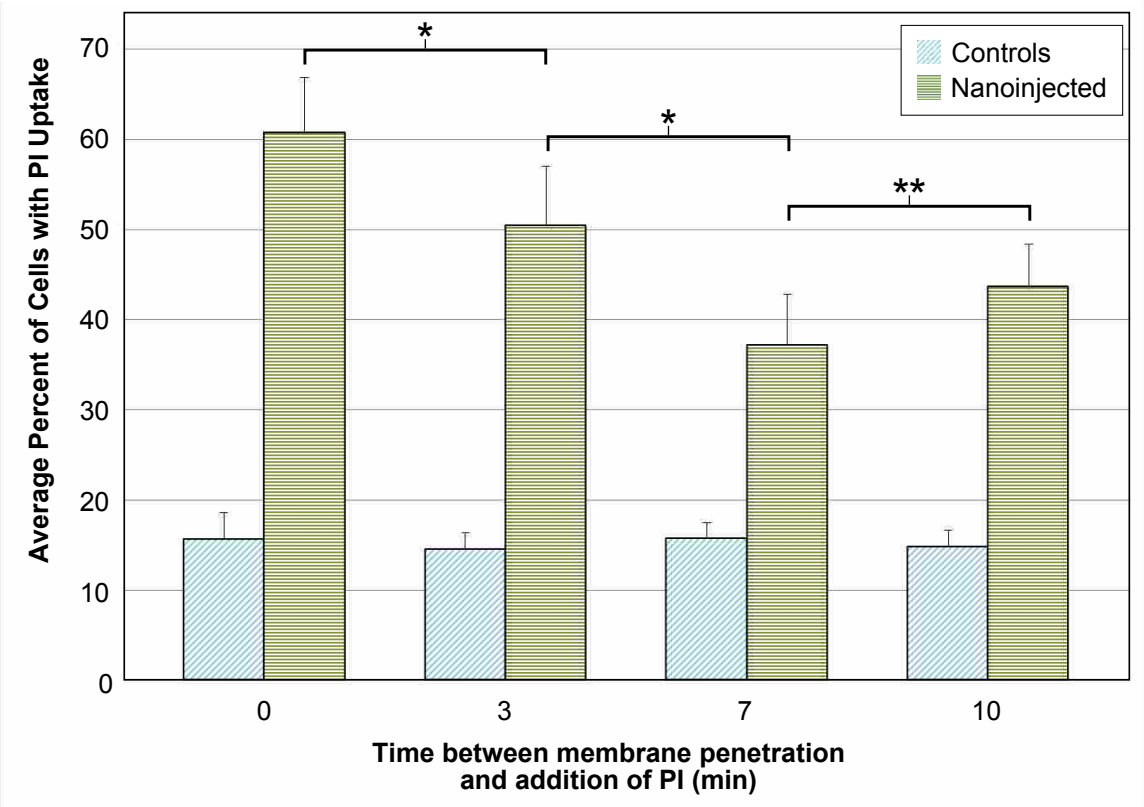

Figure 2.8: Bar graph comparing PI uptake between controls and cells nanoinjected with PI added at several time intervals. Error bars indicate a 95% confidence interval (α = 0.05). *Statistically significant difference in population averages ($p<0.05$) **No statistical difference in population averages ($p>0.05$)

the pores stay open for an additional amount of time following lance penetration and removal. However, the difference between uptake averages in the 7-minute and 10-minute tests are not statistically significant ($p>0.05$). The similarity between the 7-10 minute experiments suggests that the closing of membrane pores becomes more gradual after about 7 minutes. Control data illustrates no difference in PI uptake at any time interval in the experiment.

Figure 2.9 shows cell viability results from the membrane permeability timing experiments. For all time intervals, there is no statistical difference ($p>0.05$) in cell death over time. As discussed in the membrane puncturing section, control viability is an average of 4.2% greater than injected samples. Adding PI at different time intervals after cell membrane penetration makes no difference in cell viability. These results also show that nanoinjection is causing the cell death, and not PI exposure.

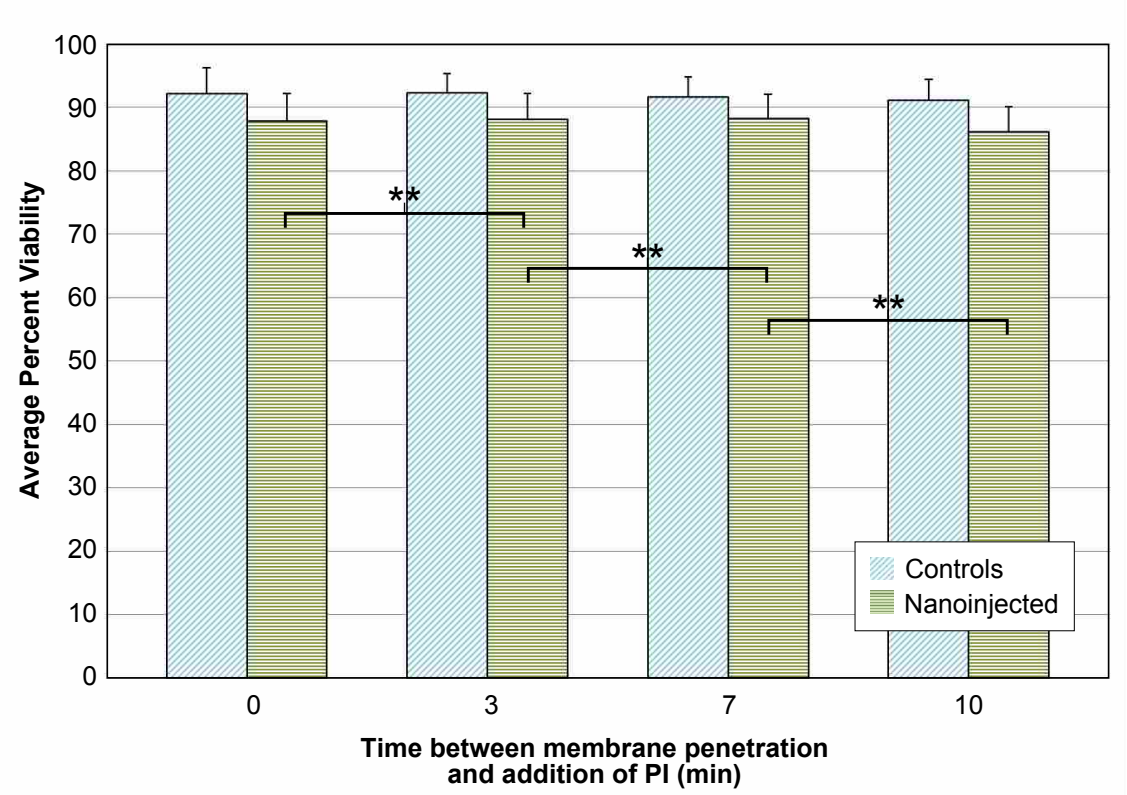

Figure 2.9: Bar graph comparing viability between controls and cells nanoinjected with PI added at several time intervals. Error bars indicate a 95% confidence interval ($\alpha = 0.05$). **No statistically significant difference in population averages ($p>0.05$)

CHAPTER 3. NANOINJECTION OF PCAG-GFP DNA PLASMID INTO HELA CELLS

3.1 pCAG-GFP DNA

To determine the efficiency of nanoinjection as a gene therapy method, DNA plasmids were nanoinjected into HeLa cells. The DNA plasmid used in this is experiment was pCAG-GFP (Addgene, principal investigator Connie Cepko), a 5551 bp plasmid containing the sequence to build green fluorescent protein (GFP). A full description and construction details of pCAG-GFP can be reviewed in [42]. Plasmids were copied using bacteria and purified using common plasmid purification techniques. Some experiments discussed in this chapter were performed using a restriction enzyme digested plasmid (REDP) version of pCAG-GFP. The plasmid was digested to see if nanoinjecting linearized DNA resulted in greater GFP expression in cells.

Figure 3.1 shows a step-by-step illustration of the pCAG-GFP nanoinjection process:

(a) Plasmid DNA in solution around HeLa cells

(b) Positive electrical charge is applied to silicon lances, and negatively charged plasmids accumulate on lances

(c) Lances are pushed into HeLa cells, penetrating cell membranes

(d) Electric charge on the lances is reversed, releasing plasmids inside the cell cytoplasm or nucleus

(e) Lances are removed from the cells with little to no damage to the cells

(f) Plasmids integrated in the nucleus are unzipped to RNA

(g) Cells use the RNA to build green fluorescent protein

(h) Cells expressing GFP are fluorescent under UV light and can be detected with flow cytometry

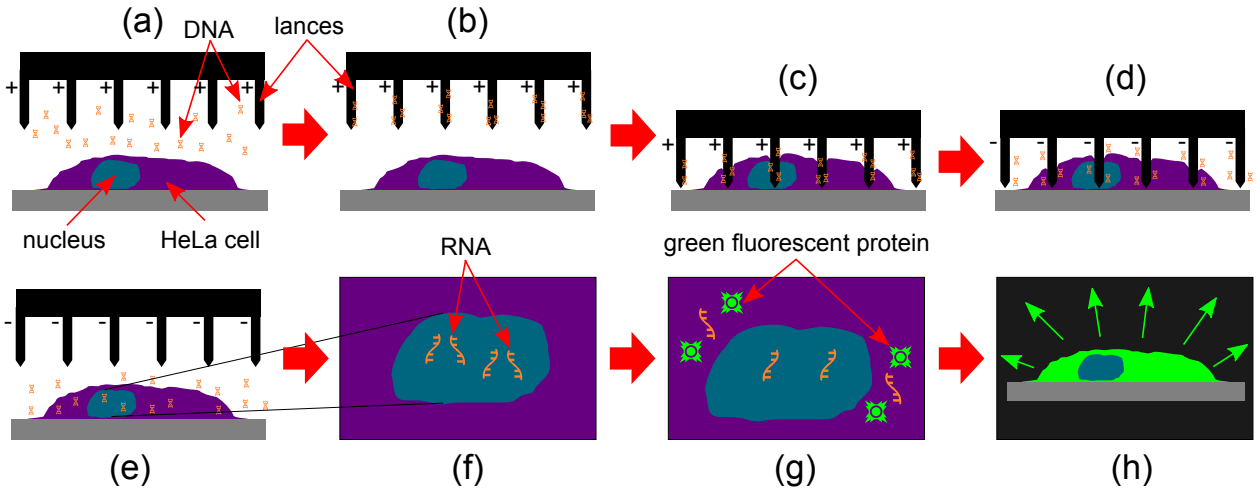

Figure 3.1: Illustration of the pCAG-GFP nanoinjection process.

3.2 Testing Methods

HeLa cells were cultured and prepared in six well plates with the same process outlined in Chapter 2. Two DNA molecule concentrations were used in these experiments: 1 and 3 μg DNA per 1 mL HBSS. A typical GFP injection experiment consisted of six negative controls (one with no DNA and no membrane penetration with lances, and five with DNA added but no membrane penetration), and six injected samples (DNA added and cells punctured with lances). The nanoinjection process was identical to the process for PI injection given in Chapter 2. Six different protocols were developed and tested to determine DNA delivery efficiency due to different DNA concentrations and variations in the nanoinjection process. Table 3.1 shows a brief overview of the 6 protocols.

- Diffusion of full plasmid DNA into cells through membrane openings created by lances, with no applied voltage
- Full plasmid DNA entering cells with the assistance of 1.5 VDC accumulation and release
- Full plasmid DNA entering cells with the assistance of 1.5 VDC accumulation and a 20 ms release pulse alternating between 1.5 and 5 VDC applied to the lances
- Full plasmid DNA entering cells with the assistance of 1.5 VDC accumulation and a 20 ms release pulse alternating between 1.5 and 9 VDC applied to the lances

Table 3.1: Table of variations in DNA concentration and pulse voltage used in the GFP injection experiments.

DNA Type	Injection Type	Pulse Limits (V)	DNA concentration
Full Plasmid	Diffusion	NA	1 μg/mL
Full Plasmid	Constant Voltage	NA	1 μg/mL
RE Digest Plasmid	Diffusion	NA	1 μg/mL
RE Digest Plasmid	Pulsed Voltage	-1.5 to -9	1 μg/mL
Full Plasmid	Pulsed Voltage	-1.5 to -5	3 μg/mL
Full Plasmid	Pulsed Voltage	-1.5 to -9	3 μg/mL

- Diffusion of REDP DNA into cells through membrane openings created by lances, with no applied voltage
- REDP DNA entering cells with the assistance of 1.5 VDC accumulation and a 20 ms release pulse alternating between 1.5 and 9 VDC applied to the lances

3.2.1 Diffusion of Full Plasmid and RE Digested Plasmid DNA

The diffusion protocol presented in Chapter 2 for PI injections was also used for DNA delivery. The concentration of DNA in solution was 1 μg/mL for both diffusion of full plasmid DNA and REDP DNA. Diffusion protocol for DNA was tested to compare DNA results to PI results.

3.2.2 Constant Voltage Protocol for Full Plasmid Nanoinjections

The constant voltage protocol used for this DNA experiment was identical to the constant voltage protocol given in Chapter 2. Taking advantage of the negative charge inherent to DNA molecules [43], accumulation and release voltages applied to lances were +1.5 and -1.5, respectively.

3.2.3 Pulse Protocol for Full Plasmid and RE Digested Plasmid DNA Nanoinjections

In previous studies using a single polysilicon lance to deliver foreign DNA in mouse zygotes, DNA molecules were delivered at much higher rates when voltage was applied to the

lance [17, 18, 44]. Pulsed voltage, identical to the protocol given in Chapter 2 for PI, was added to the diffusion protocol to test for greater DNA expression rates using an electric field. Positive 1.5 VDC was applied to the nanoinjection system for 20 seconds, allowing lances to accumulate negatively charged DNA molecules. Lances were pushed into adhered cells with a force of 30 N. Immediately following membrane penetration, a square wave pulse (between -1.5 and -5 volts or between -1.5 and -9 volts) was applied to the electrodes for 20 ms, releasing DNA molecules from lances and into the cytoplasm or nucleus. Negative 1.5 VDC was applied while removing the injection system from the well. Pulsed voltage was employed to determine if an increased release voltage would increase DNA integration in the cell. The 2 ms period pulse with 50% duty cycle was applied for 20 ms for a total of 10 pulses per sample.

3.3 Incubation and Collection of Cells for Analysis

DMEM was added to each well following the injection process, and the six well plates were placed in the incubator for at least 48 hours. If the plasmid was nanoinjected into the nucleus of a cell, the cell will copy the plasmid when it divides. HeLa cells grow with a doubling time of about 24 hours [45], and the 48 hour incubation gives the cells time to divide and show true GFP expression.

Following the incubation of samples, regardless of previous injection protocol, the media was removed and the cells were rinsed with HBSS. The cells were trypsinated and incubated for 5 minutes. After trypsinating the cells, DMEM was added to each well, and the contents of each well were transferred to FACS tubes (BD Biosciences). The tubes were centrifuged at 2000 RPM for 10 minutes. Following centrifugation, supernatants in each tube were removed and the pellet of cells resuspended in the remaining media. 0.25 mL HBSS was added to each tube, and the experimental samples were then analyzed using a flow cytometer (BD Biosciences).

3.4 Flow Cytometry and Statistical Analysis

A flow cytometer was used to quantify HeLa cells expressing GFP. Using flow analysis software (BD FACSDiva, Dako Summit) and control data, live and dead cell populations were determined using forward scatter and side scatter data. For each HeLa cell sample, 10,000 events

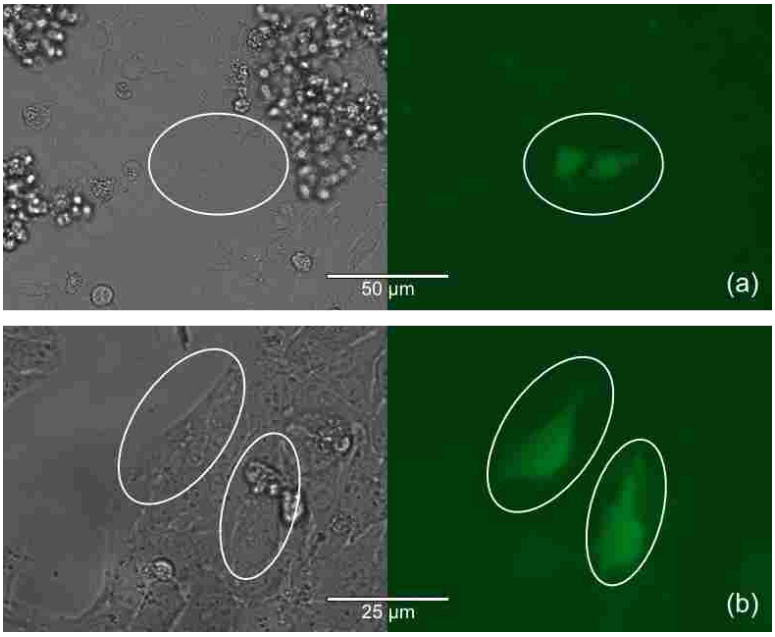

Figure 3.2: Microscope images of two different culture regions containing cells expressing GFP (a,b). The left images were taken using regular light. The right images were taken with fluorescent light.

were analyzed. Control data was also used to determine the GFP positive population in the FITC fluorescence channel.

Confidence intervals were calculated for each data set ($\alpha = 0.05$) and included in bar graphs to visualize variation in statistical analysis. Student's t-tests for small sample sizes ($n<30$) were used to determine statistical significance between population means. When comparing population means, p-values less than 0.05 were considered to show statistically significant difference. Population means with p-values greater than 0.05 were considered statistically the same.

3.5 DNA Expression Results and Discussion

An inverted microscope with fluorescent light capabilities was used to visually identify any cells expressing GFP. Figure 3.2 shows two different regions where expressing cells were located. Very few GFP expressing cells were located with microscopy.

Figure 3.3 compares averages in GFP expression for full plasmid (FP) diffusion, FP 1.5 VDC, FP pulsed voltages, REDP diffusion, and REDP pulsed voltage experiments. T-test results

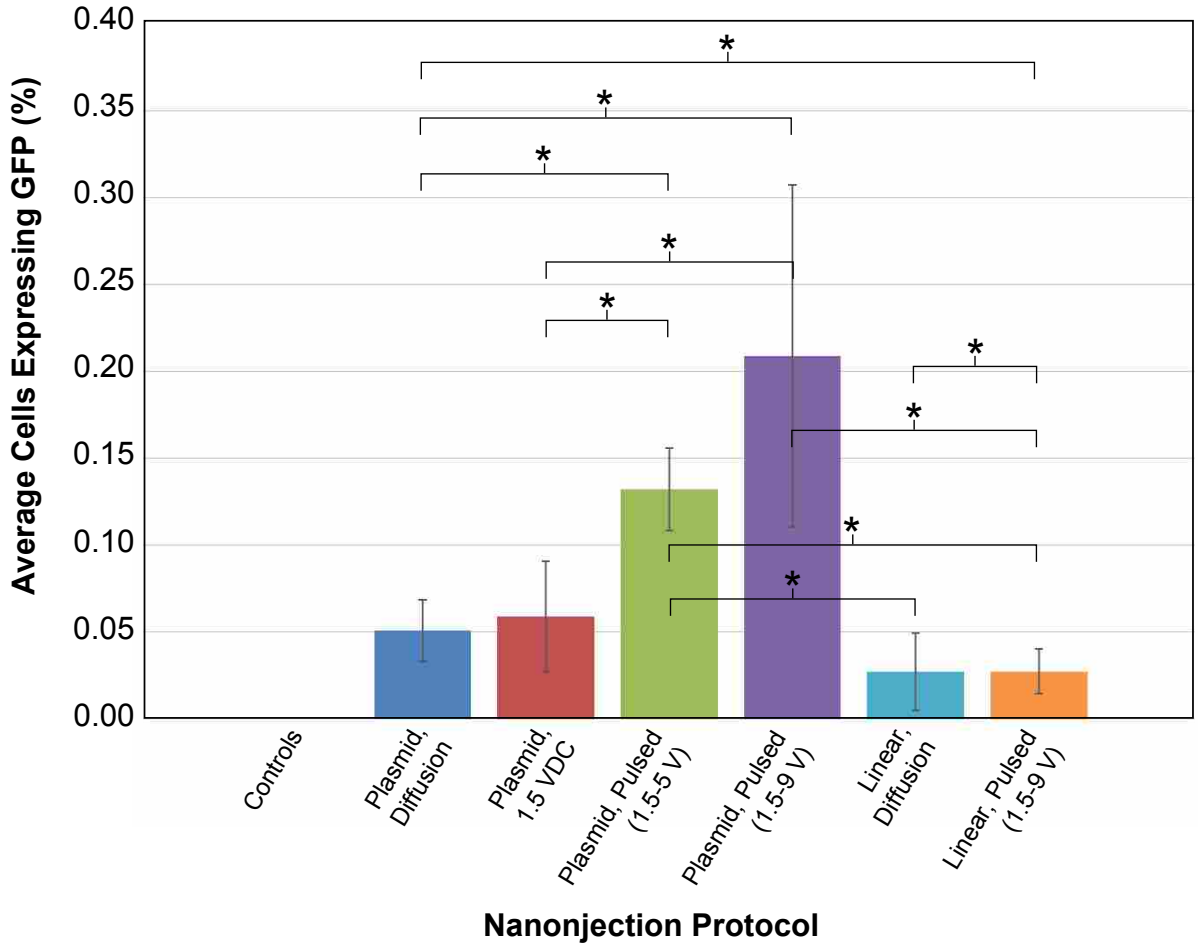

Figure 3.3: Bar graph comparing average GFP expression data for nanoinjection experiments. The error bars indicate a 95% confidence interval (α = 0.05). *Statistically significant difference in population averages ($p<0.05$). All other comparisons of difference in population means were not statistically different.

indicate a variety of statistical significance in average GFP expression between experiments. Exact p-values for differences in population means can be viewed in Table 3.2.

Both expression averages in pulsed experiments with the FP are significantly different from all other experiments, but not different from each other. The pulsed FP experiments were performed with a DNA concentration of 3 μg/mL, which is 3 times greater than all other experiments. Compared to FP diffusion injections at 1 μg DNA per mL solution, the 1.5-5 V and 1.5-9 V FP pulsed injections induced 2.6 and 4.1 times the gene expression, respectively. These results denote

Table 3.2: Table of p-values calculated when comparing the difference in population means for DNA expression.

Comparison			p-value
FP Diffusion	*vs.*	FP 1.5 VDC	0.64
FP Diffusion	*vs.*	FP Pulse (1.5V-5V)	<0.0001
FP Diffusion	*vs.*	FP Pulse (1.5V-9V)	0.0032
FP Diffusion	*vs.*	REDP Diffusion	0.069
FP Diffusion	*vs.*	REDP Pulse (1.5V-9V)	0.021
FP 1.5 VDC	*vs.*	FP Pulse (1.5V-5V)	0.0005
FP 1.5 VDC	*vs.*	FP Pulse (1.5V-9V)	0.0057
FP 1.5 VDC	*vs.*	REDP Diffusion	0.078
FP 1.5 VDC	*vs.*	REDP Pulse (1.5V-9V)	0.059
FP Pulse (1.5V-5V)	*vs.*	FP Pulse (1.5V-9V)	0.13
FP Pulse (1.5V-5V)	*vs.*	REDP Diffusion	<0.0001
FP Pulse (1.5V-5V)	*vs.*	REDP Pulse (1.5V-9V)	<0.0001
FP Pulse (1.5V-9V)	*vs.*	REDP Diffusion	0.001
FP Pulse (1.5V-9V)	*vs.*	REDP Pulse (1.5V-9V)	0.0009
REDP Diffusion	*vs.*	REDP Pulse (1.5V-9V)	0.99

that increasing DNA concentration, adding a DNA accumulation voltage and a release pulse, or combining the two results in significantly greater gene expression.

The two protocol for REDP nanoinjections resulted in lower average gene expression than all experiments performed with FP. However, statistically the REDP expression averages were no different than the FP averages at the same DNA concentration. The equal to lower expression rates indicate that digesting plasmids to make them linear has little effect on gene expression in nanoinjected cells.

PI uptake results presented in Chapter 2 suggest that nanoinjection is an efficient method for delivering foreign molecules into culture cells. However, PI uptake and GFP expression cannot be compared directly. PI molecules fluoresce when bound to any nucleic acids in the cytoplasm or nucleus, so cells with PI anywhere inside the cell membrane will show positive in a flow cytometry test. Cells with successful pCAG-GFP integration will only show positive expression in a flow cytometry test if the plasmid was injected into the nucleus, integrated into the genome of the cell, and then expressed. With a low average expression rate of 0.21% or less, and no solid indication of what direction to take to increase expression, the decision was made to find a way to measure total

DNA count in cells after nanoinjections. The experiments performed to determine DNA count in cells following nanoinjection are discussed in Chapter 4.

3.5.1 Viability

Due the the 48 hour incubation time between nanoinjections and flow cytometry, viability wasn't measured for pCAG-GFP injections. The viability for DNA nanoinjections is assumed to be greater than or equal to viability data gathered from PI injections from Chapter 2, since the DNA concentration used in this experiment is non-toxic to cells [46].

CHAPTER 4. NANOINJECTION OF DNA LABELLED WITH A RADIOACTIVE ISOTOPE INTO HELA CELLS

The experiments performed in Chapter 3 showed low rates of gene expression for pCAG-GFP in HeLa cells. Cells only expressed fluorescent protein when nanoinjections inserted DNA into the nucleus of cells. Another experimental procedure was developed to determine the amount of DNA inserted into the cytoplasm and nucleus of cells during nanoinjection. Radioactive DNA can be quantified with liquid scintillation techniques, allowing discrimination between DNA inserted into cells and DNA left in solution around cells following nanoinjection. This chapter discusses the cellular DNA uptake results obtained from nanoinjecting RE digested DNA labelled with a radioactive isotope.

4.1 Tagging of Restriction Enzyme Digested DNA with ^{32}P

Full plasmid pCAG-GFP DNA was digested at the SpeI and HindIII locations (see Figure 1.1), resulting in two linear DNA segments 2533 bp and 3018 bp long. The two segments of DNA had 5' overhangs, leaving DNA bases available for tagging with free nucleotide triphosphates. For these experiments, dTTP labelled with ^{32}P (Thymidine 5' - Triphosphate [α - ^{32}P]), along with the three other nucleotides (dATP, dCTP, dGTP), were used to fill in the 5' overhangs and blunt the ends of DNA segments. α - ^{32}P is a beta-particle emitting radioactive phosphorous isotope commonly used in microbiological research. Figure 4.1 shows an illustration of the main steps of the DNA tagging procedure (see Appendix A for a full step-by-step protocol for DNA tagging and nanoinjections). Following the tagging step, unincorporated nucleotides were filtered from the DNA solution to eliminate false scintillator readings. Although this filtering step is not 100% efficient, the process by which injected DNA is quantified following nanoinjection gives accurate DNA concentration readings.

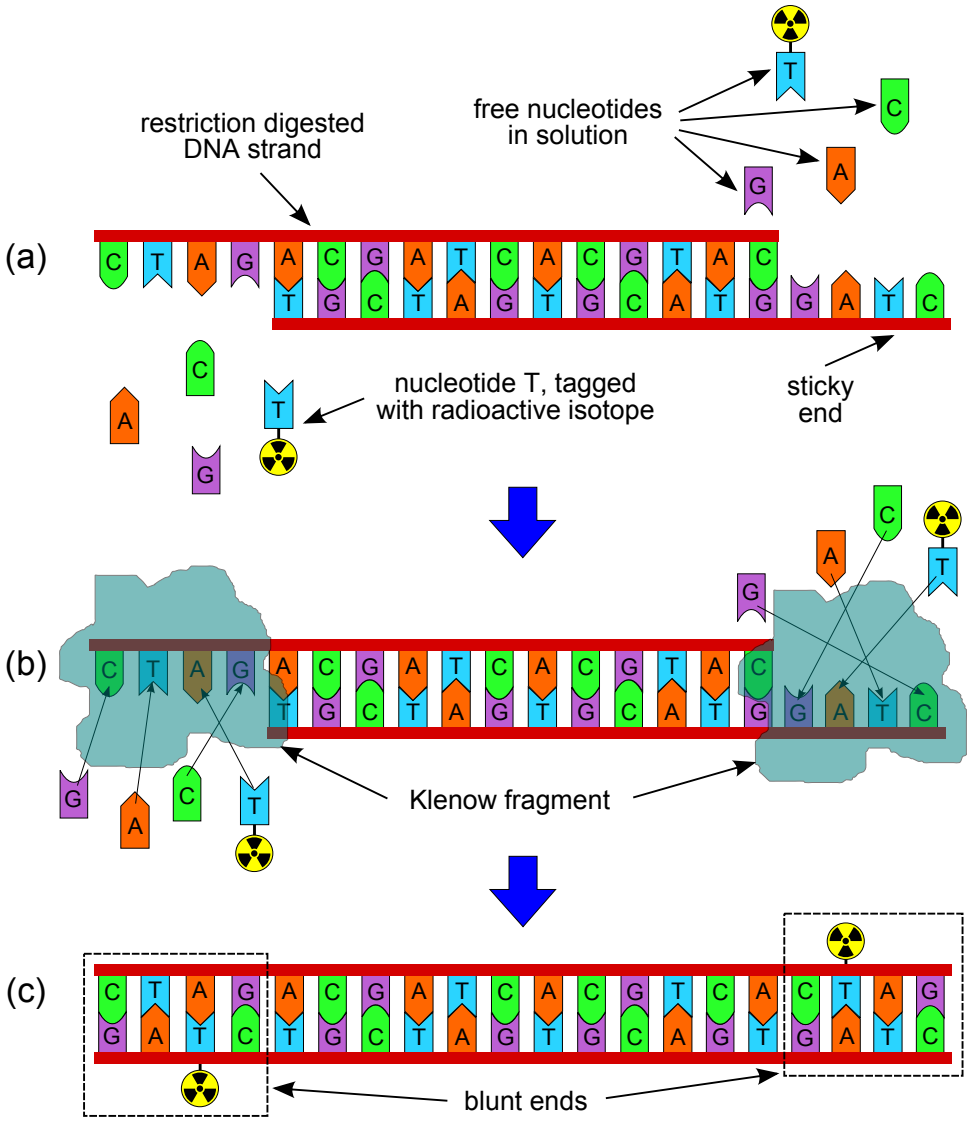

Figure 4.1: Illustration of the DNA tagging procedure. (a) RE digested DNA is placed in solution with free nucleotides (thymidine is purchased with a radioactive ^{32}P tag). (b) Klenow fragment is added to the solution, which binds free nucleotides to DNA overhang sites. (c) The DNA strands are successfully blunted and tagged with radioactive molecules, allowing the DNA strands to be detected with radioactive detection techniques.

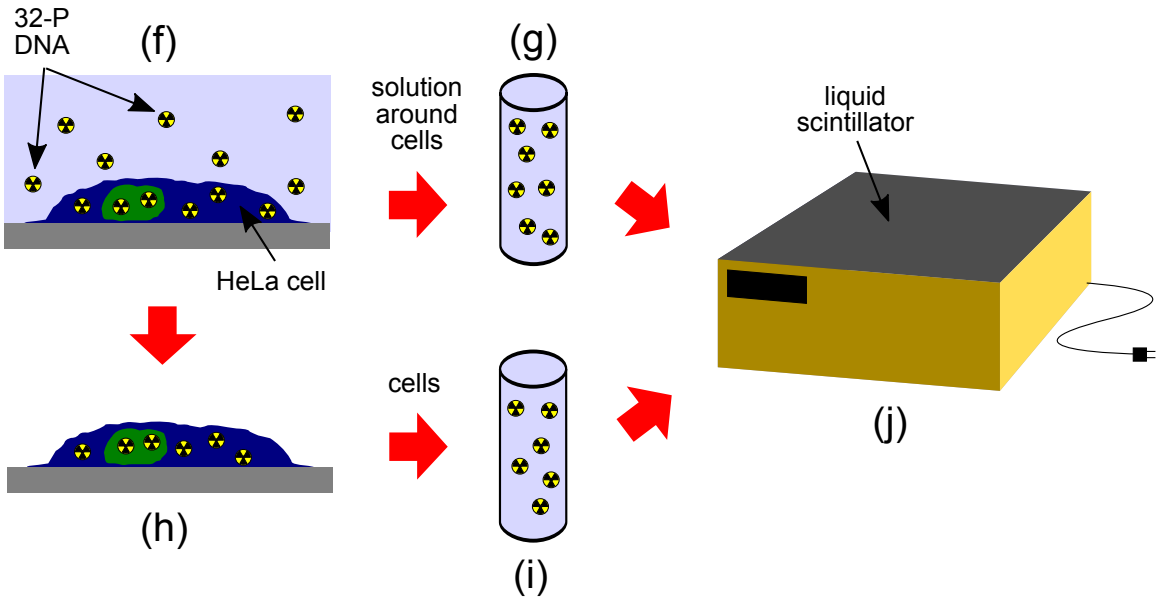

Figure 4.2: Radioactive DNA nanoinjection procedure (steps (a)-(e) are not shown here since they are identical to those in Figure 3.1). (f) Sample well with ^{32}P DNA previously nanoinjected into cells following pulsed nanoinjection protocol. (g) Solution around cells is transferred to a scintillation vial. (h) Cells remaining are trypsinated and transferred to a (i) separate scintillation vial. (j) Samples are placed in a liquid scintillation counter to determine cpm of each sample.

4.2 Experimental Procedure

HeLa cells were cultured and prepared for testing using the same protocol outlined in Chapter 2. The total number of cells per well was determined with a hemocytometer. Results from Chapters 2 and 3 showed that nanoinjection with accumulation voltage and a short release pulse was most effective at delivering molecules into cells. Three protocols similar to those found in Chapters 2 and 3 were used for the radioactive DNA nanoinjection experiments:

- Diffusion of DNA molecules into cells following cell membrane penetration with lances
- DNA accumulation at +1.5 V, penetration of cell membranes with lances, and -1.5 V release voltage
- Accumulation voltage at +1.5 V, penetration of cell membranes with lances, 20 ms release pulse between -1.5 V and -9 V, and a constant release voltage of -1.5 V

A typical radioactive nanoinjection experiment consisted of a control with no DNA nor injection, a control with DNA but no injection, and four samples with DNA and injection. Figure 4.2

depicts the steps in the nanoinjection process that are specific to injections with radioactive DNA. Following the delivery of DNA into cells, cultures were left to incubate for 15 minutes at room temperature, allowing pores in cell membranes to close [47]. For each sample, the solution around cells was transferred to a scintillation vial. Each sample was then rinsed with 1 mL HBSS, and the rinse was transferred to the respective scintillation tube. At this point in the experiment, any DNA not transferred into cells during nanoinjection was in scintillation vials. Following the rinse step, the cells were trypsinated and transferred to new scintillation vials, along with a 1 mL HBSS rinse per well. Two scintillation vials contained the results from one sample: a vial with the DNA not injected into cells, and a vial containing cells. Separating the rinse from the cells allowed us to discriminate bewteen DNA that entered cells during nanoinjection and DNA that was left in solution following nanoinjection. 3 mL of scintillation cocktail (Ecoscint A, National Diagnostics) was added to each vial prior to liquid scintillation.

4.3 Specific Activity and Statistical Analysis

DNA in rinse vials and cell vials were quantified with a liquid scintillation analyzer (Tri-Carb B2910TR, PerkinElmer). The contents of each vial were counted three times, for one minute each time. Each vial count was reported in counts per minute (cpm). When counting ^{32}P with liquid scintillation, cpm is nearly 100% efficient when compared to actual disintegrations per minute (dpm) of beta particles. To calculate the average number of DNA molecules per culture cell (DNA_{cell}), Equation 4.1 was used

$$DNA_{cell} = \frac{(N_A)(S_{CPM})}{2(S_A)(C_F)(N_{cells})} \tag{4.1}$$

where N_A is the Avogadro constant (6.02x10^{23}molecules/mol), S_{CPM} is the cpm from the liquid scintillator for the injected sample (cpm), S_A is the specific activity of the ^{32}P on the day the injections were performed (Ci/mol), C_F is a conversion factor (2.22x10^{12} Ci/cpm), and N_{cells} is the number of cells in the sample.

Confidence intervals were calculated for each data set ($\alpha = 0.05$) and included in bar graphs to visualize variation in statistical analysis. Student's t-tests for small sample sizes (n$<$30) were used to determine statistical significance between sample means. When comparing sample means,

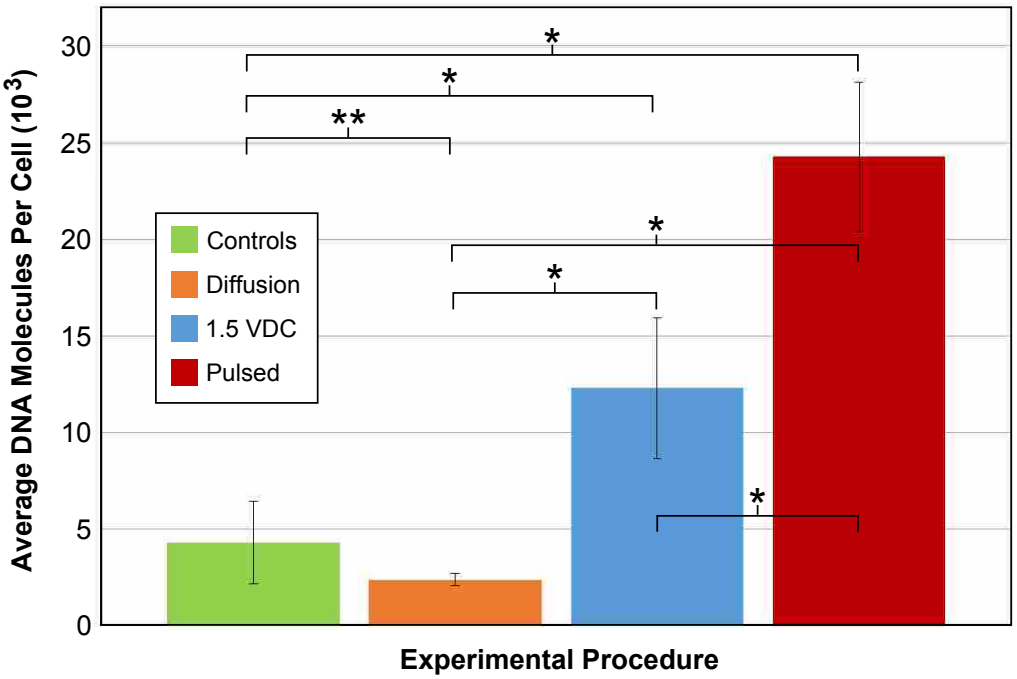

Figure 4.3: Bar graph comparing average DNA molecules per cell for controls, diffusion, 1.5 VDC, and pulsed injections. The error bars indicate a 95% confidence interval ($\alpha = 0.05$). *Statistically significant difference in population averages ($p<0.05$) **Population averages are statistically the same ($p>0.05$)

p-values less than 0.05 were considered to show statistically significant difference. Sample means with p-values greater than 0.05 were considered statistically the same.

4.4 Results and Discussion

4.4.1 DNA Molecules Per Cell

The purpose of the experiment discussed in this chapter was to develop an accurate procedure to count the number of DNA molecules delivered to each cell during the nanoinjection process. Figure 4.3 compares averages in DNA molecules delivered per cell for all radioactive DNA nanoinjections. T-test results indicate that differences between sample means are statistically significant ($p<0.05$), except for the difference between control averages and diffusion averages ($p>0.05$). Calculated p-values for all sample mean comparisons can be viewed in Table 4.1.

Table 4.1: Table of p-values calculated when comparing the difference in population means for DNA molecules per cell.

		Comparison	**P-Value**
Controls	*vs.*	Diffusion	0.12
Controls	*vs.*	Constant 1.5 VDC	2.2E-3
Controls	*vs.*	Pulsed (1.5V-5V)	<1.0E-6
Diffusion	*vs.*	Constant 1.5 VDC	3.6E-4
Diffusion	*vs.*	Pulsed (1.5V-5V)	<1.0E-6
Constant 1.5 VDC	*vs.*	Pulsed (1.5V-5V)	3.0E-4

The DNA per cell averages represented by Figure 4.3 follow trends observed in the Chapter 2 experiments: diffusion experiments showed the lowest PI delivery efficiency, 1.5 VDC experiments resulted in a greater amount of PI molecules delivered than diffusion, and the pulsed voltage tests delivered the greatest amount of PI to the cell. Subtracting average control data from the constant voltage and pulse voltage data, average DNA per cell was $8.0\text{x}10^3$ and $20.0\text{x}10^3$, respectively.

This data suggests that DNA molecules behave similarly to PI molecules in the nanoinjection process. However, in radioactive DNA experiments, average diffusion data was not significantly different than control averages. Since controls and diffusion averages are not statistically different, we can conclude that DNA does not diffuse through cell membrane pores opened by lance arrays in the nanoinjection process. DNA molecules must be accumulated to and released from lances via electric charge in order to be delivered into the cytoplasm or nucleus of cells. The results from single lance nanoinjection experiments described in [18] also show that voltage is needed to effectively deliver DNA into cells.

It is important to note that controls in this experiment were non-zero, compared with controls from the GFP expression experiments in Chapter 3, which are zero. For GFP to be expressed by the cell, the plasmid must first enter the nucleus of the cell. DNA outside a cell will not passively diffuse into the nucleus or even across the cell membrane. DNA molecules must be given a transportation mechanism to enter the cell, therefore GFP expression controls will always be zero. Since the controls in radioactive DNA experiments were non-zero, it is hypothesized that there is a small amount of DNA that clings to the outside of cells even after the rinsing step.

Table 4.2: Table showing average percent of total DNA delivered into cells following the nanoinjection process.

Sample Type	Diffusion	1.5 VDC	Pulsed
Average DNA Injected/Total DNA (%)	0.95	2.77	2.88

4.4.2 DNA Delivered to Cells vs. Total DNA

We also wanted to determine the amount of DNA delivered into cells by nanoinjection, compared to the total DNA added to the well prior to injection. The DNA concentration in each well for all the experiments in this chapter was 0.3 μg/mL. Table 4.2 lists the average percent of total DNA delivered into cells by nanoinjection. As determined in the previous section, diffusion delivered the least amount of DNA to cells, while constant voltage and pulsed voltage delivered greater amounts of DNA to cells. These results indicate that only a small amount of total DNA added to each well is being transported across cell membranes via nanoinjection: just under 3%. Future nanoinjections of this nature should include variation in DNA concentration in order to determine the effect (if any) that initial DNA concentration has on final DNA transportation across cell membranes.

4.4.3 Verification of DNA Calculations

To verify Equation 4.1 used to calculate DNA molecules per cell, we calculated DNA molecules per well using initial DNA concentration. One base pair of DNA weighs approximately 650 Daltons (New England Biolabs, www.neb.com). Using Equation 4.2, we calculated the number of DNA molecules per well (DNA_{well}):

$$DNA_{well} = \frac{W_{DNA}}{(W_{molecule})(C_D)} \tag{4.2}$$

where W_{DNA} is the weight of DNA added to the well (μg), $W_{molecule}$ is the average weight of one DNA molecule (Daltons), and C_D is the conversion factor between Daltons and μg (1.66x10^{-18} D/μg). The average DNA molecule count per well calculated from the scintillation results and Equation 4.1 is 1.5x10^{11}. Using Equation 4.2, we found an average DNA molecule per well count of 2.0x10^{11}. The two results are on the same order of magnitude, verifying Equation 4.1. Since

the DNA tagging and purification procedure is not 100% efficient, it is more likely that a lower concentration of DNA was added to each well than the 0.6 μg assumed previously. If the DNA tagging and purification procedure leaves only 75% of DNA available for nanoinjections, which is more likely, then the total DNA molecules per well (using Equation 4.2) would be $1.5\text{x}10^{11}$. Whether the tagging and purification process is fully efficient or not, our calculations are accurate enough to give proper measurements of DNA molecules per cell.

CHAPTER 5. DESIGN AND TESTING OF AN AUTOMATED NANOINJECTION SYSTEM TO REDUCE VARIABILITY

One of the challenges inherent to experimental testing with culture cells is variability between test samples [41]. Multi-cell nanoinjections in previous experiments have been performed by several different researchers in the same lab. Since each user applies a different force to press lances into the cell culture, greater variability is added to the process. This chapter describes an automatic nanoinjection system developed to reduce variability in the nanoinjection process. The purpose of the automatic nanoinjection system is threefold:

1. Deliver the same injection force for each experiment, reducing variability caused by different users
2. Automate the injection process to puncture cells with lances and release lances from cells
3. Easily control any injection movement and voltage input by programming and the push of a button

5.1 Mechanical Device Design

The mechanical device used for the experiments discussed in this chapter was an extension of the device described in Chapter 1 and [38]. Additional structural material was designed on the top of the mechanism for stepper motor attachment. A cup shaped structure to house a spring was designed on the surface of the fixture where force is applied. A similar structure was designed to connect the linear actuator with the spring. Figure 5.1 shows a photo and an illustration of the automatic injector mechanism.

Many automated systems require precise movements. Stepper motors are commonly used to apply very precise movements in robotic applications [48]. To apply precise linear nanoinjection movements to the automated system, a linear actuator stepper motor (Anaheim Automation) was

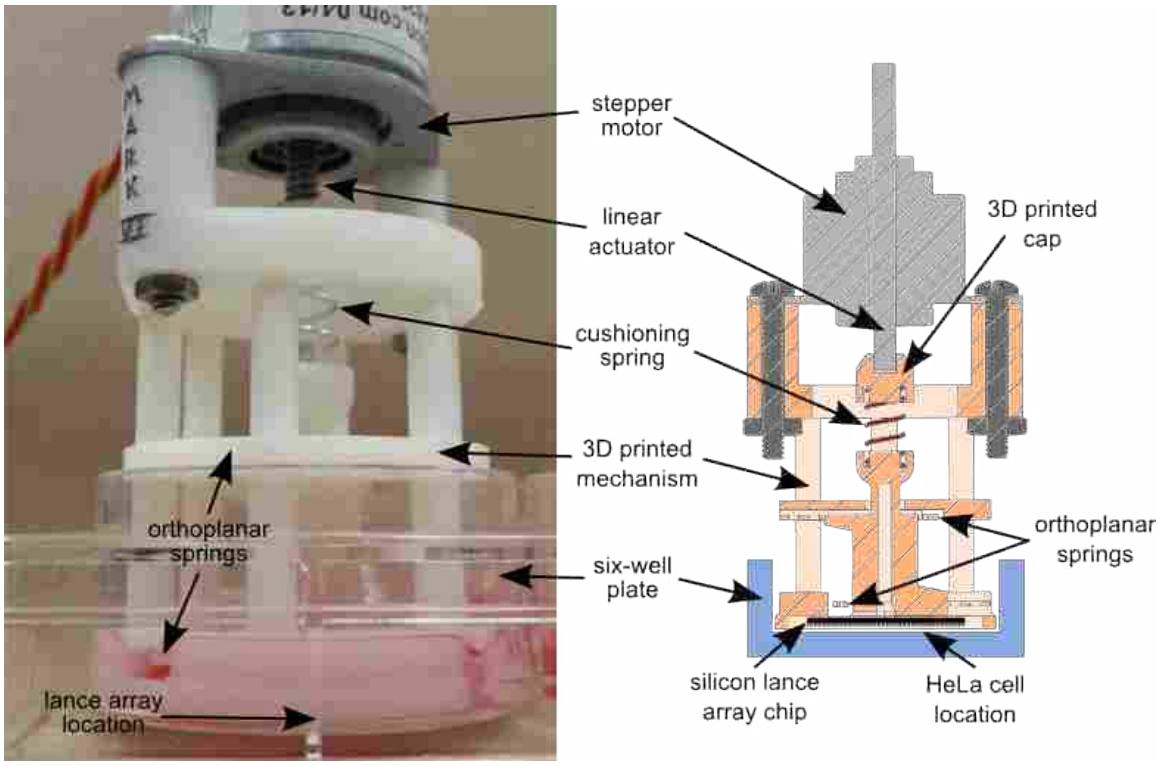

Figure 5.1: Photo image and illustration of the injection mechanism and stepper motor in a culture well, as in the nanoinjection process.

fixed to the top of the 3D printed structure. A small 3D printed cap was attached to the linear actuator, and a steel spring connected the actuator to the mechanical device. The purpose of the spring was to create a cushioning effect when an injection force was applied. Cushioning was desired to avoid possible impulse damage from the linear actuator impacting the silicon chip or well base too quickly. The force applied by the stepper motor could be controlled precisely with the addition of the cushioning spring. Before any actuation of the device, the distance from lance tips to the base of the cell culture was approximately 1 mm. The stepper motor delivered 0.04 mm linear distance for each step, allowing for very precise vertical distance control of the lance array.

5.2 Control Box Design

The automated system was housed inside a simple project box (see Figure 5.2), and provided a way to switch between three different electrical inputs from outside sources. Each of the three input ports were designed to take either coaxial or banana jack wiring. The two different

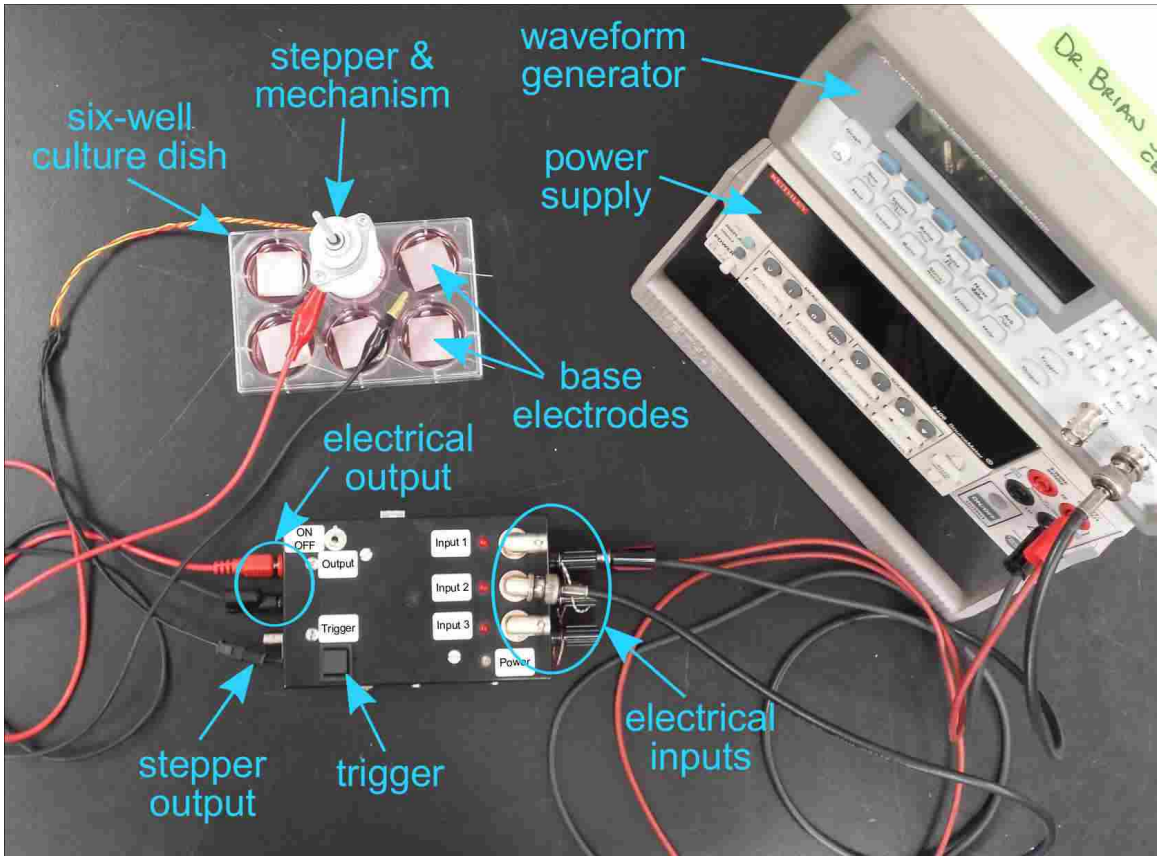

Figure 5.2: Image of a typical setup for automated nanoinjections.

input types allowed for the use of either a common power supply or a waveform generator, since constant DC voltage or pulsed voltage was desired in certain experiments. There was one electrical output pair, which could be wired to the silicon lance array (top electrode) and the stainless steel plate (base electrode). One of the three inputs could be routed via mechanical relays to the output at any given time. The relays were controlled with a microcontroller (Arduino Duemanilove). LED's were wired to the microcontroller to provide visualization of the activated electrical input.

The stepper motor was driven with a quadruple half-h driver (L293DN), and powered and controlled by the microcontroller. The number of steps the stepper motor traveled and the speed at which the stepper traveled was programmed into the microcontroller. A common push-button switch mounted on top of the project box was the means by which the user activated the system. Figure 5.3 shows the full wiring diagram of the switchbox.

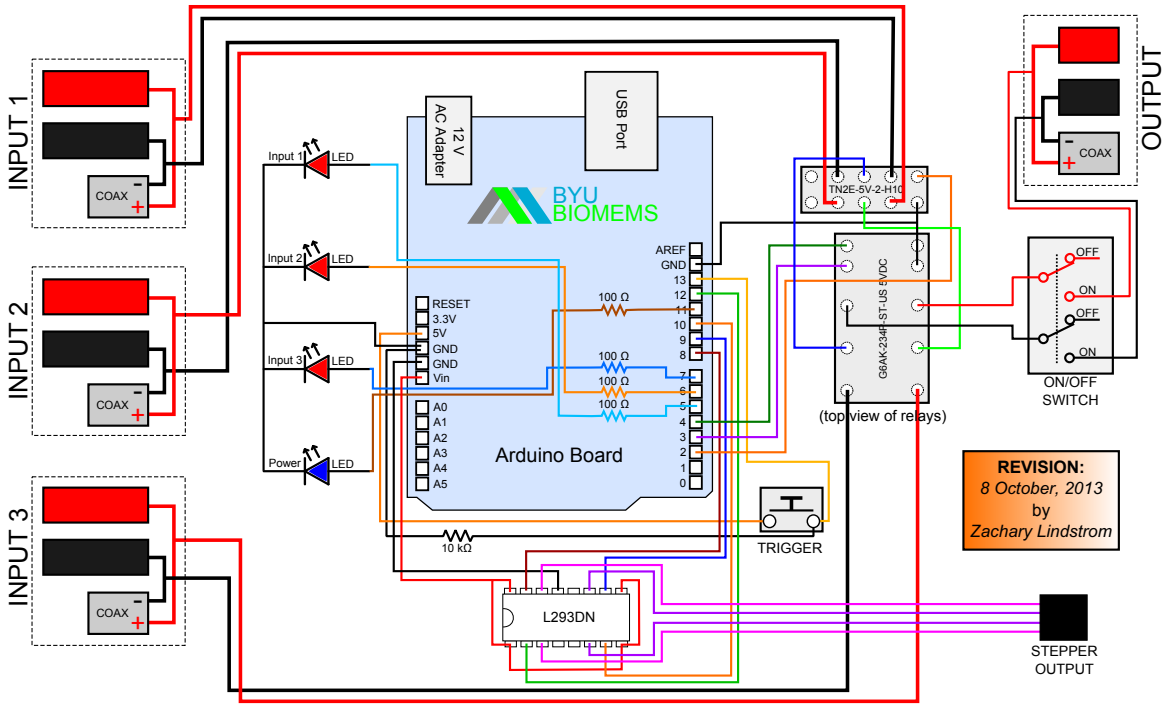

Figure 5.3: Wiring diagram for the automated nanoinjection system control box.

5.2.1 Example Nanoinjection Program

The full setup of the automated nanoinjection system can be seen in Figure 5.2. A typical nanoinjection process using DNA is described in this section as an example of how the system can function.

1. The system is set up and the injection device placed in a dish containing culture cells.
2. Constant +1.5 VDC is routed to input 1.
3. A waveform generator is attached to input 2, supplying a 2 ms period square wave pulse with 50% duty cycle between -1.5 and -5 VDC.
4. Constant -1.5 VDC is routed to input 3.
5. The output is switched to ON (+1.5 VDC), and negatively charge DNA molecules accumulate on lances for 20 s.
6. The trigger button is depressed, which activates the program on the microcontroller:

(a) The stepper turns a predefined number of steps, pushing the lance array into the cell culture, penetrating cell membranes.

(b) Input 1 (+1.5 VDC) is switched off and Input 2 (square wave pulse) is activated for 20 ms, releasing DNA molecules from lances into cells.

(c) Input 3 (-1.5 VDC) is switched on for 10 s, allowing time for the user to remove the mechanism from the well and place in the next well.

(d) Input 1 (+1.5 VDC) is switched back on in preparation for the next injection site.

7. The process is repeated for all culture samples.

The full C++ Arduino sketch can be reviewed in Appendix B. The experiments performed for this chapter did not use voltage as a mechanism to deliver PI molecules into cells. This chapter compares data from diffusion nanoinjections with a manual force (Chapter 2) to diffusion injections with the automated system. In diffusion nanoinjections, PI molecules diffuse into the cell via cellular membrane pores opened by the lance array.

5.3 Experimental Testing

5.3.1 Cell Preparation

In preparation for the experiment, HeLa 229 cells were cultured as mentioned in Chapters 2-4. Four six-well culture plates were prepared for each experiment.

After 24 hours of incubation, the media was removed from the plates and the cells were rinsed with HBSS. 1 mL HBSS and 0.04 mg of PI were added to twenty-three of the twenty-four wells, leaving one well as a negative control (no PI or nanoinjection). Eleven wells were used as positive controls (PI in the solution, but without injection). The remaining twelve wells were used for injection with the automated injection device and lance array.

5.3.2 Device Preparation and Force Calibration

The automatic injection device was prepared by manual calibration and programming the microcontroller. Calibration was accomplished by manually adjusting the threaded rod of the

stepper motor to bring the device base to its level, equilibrium position. The equilibirum position of the device is considered to be the position that results in zero tension or compression in the ortho-planar springs. Visually, when ortho-planar springs on the 3D printed mechanism were level, the device was calibrated. After the actuating section of the device was level, the microcontroller program was coded to the desired number of linearly actuated stepper motor steps. Finally, the lance array was attached to the actuating base of the mechanism using double sided carbon tape.

The force (F) exerted by the lances during injection was approximated using Equation 5.1:

$$F = k(nd_s - d_{lc}) - d_{lc}(k_{op}) \tag{5.1}$$

where k is the steel cushioning spring constant, n is the number of steps taken by the motor, d_s is the linear distance traveled by one stepper step, d_{lc} is the distance between lance tips and cells in the pre-actuated position, and k_{op} is the combined ortho-planar spring constant [38]. Four different numbers of steps were selected for testing: 50, 100, 150, and 200 steps. These steps corresponded to 2.8, 8.8, 14.7, and 20.7 N, respectively.

5.3.3 Nanoinjection

After the cells and the automated device were prepared, the automated mechanism was placed in a well of culture cells. The mechanism was manually held firmly to the well bottom. The trigger button was then pressed by the user, and the linear actuator of the stepper motor pressed the lances into the cells. After 5 seconds at the programmed step extension, the lances were retracted by the stepper to the initial position above the cells. After nanoinjection in a well, any fluid remaining on the device was released into the injected well.

After injections, 5x Trypsin (Sigma) was added to each well and the six-well plates were incubated for 5 minutes to release any cells adhered to the well base. 1 mL of DMEM was added to each well to deactivate Trypsin, and the contents of each well were transferred to FACS tubes (BD Biosciences). Samples were placed in a centrifuge at 2000 RPM for 10 minutes. Supernatants were then removed and the cell pellet was resuspended in the remaining fluid. 250 μL of HBSS was added to the tubes, and the tubes were analyzed with a flow cytometer.

5.3.4 Flow Cytometry and Statistical Analysis

Flow cytometry was utilized to count PI positive cells and cell death in these experiments. Live and dead cells were determined with forward and side scatter using flow analysis software (BD FACSDiva, Dako Summit). For each sample, 10,000 events were recorded and analyzed. The negative control sample was used to approximate live and dead cell populations, as well as PI positive cells (see Chapter 2).

After gathering flow data for 24 samples per force level, the data was averaged and standard deviations calculated. For uncertainty in graphs, confidence intervals were used where $\alpha = 0.05$. A Student's t-test was used to compare differences in population averages. Bar graphs of the data were created to identify potential trends in the data following force testing.

5.4 Results and Discussion

5.4.1 PI Uptake Into Cells

The automated nanoinjection experiments described in this chapter were performed to determine average cellular PI uptake and viability, compared to the results in Chapter 2. Figure 5.4 shows average PI uptake at four different forces for controls and nanoinjected samples. The results show a varying distribution of PI uptake, but above an average of 51% at all injection forces. However, at injection forces greater than and equal to 8.8 N, average PI uptake of the injected cells appears uniform around 60%. This is the value obtained in Chapter 2 with a user applied force at the same PI concentration. Therefore the automated nanoinjection system does not deliver PI to more cells than the manual injection method.

From statistical analysis we see there is no statistically significant difference in PI uptake at automated injection forces 8.8 N or greater (see Table 5.1 for exact p-values from the t-test). Therefore, average PI uptake in injected cells is independent of injection force at 8.8 N or greater. Further testing could be performed to determine the exact force at which PI uptake becomes independent of injection force, but is beyond the scope of the objectives outlined in this thesis.

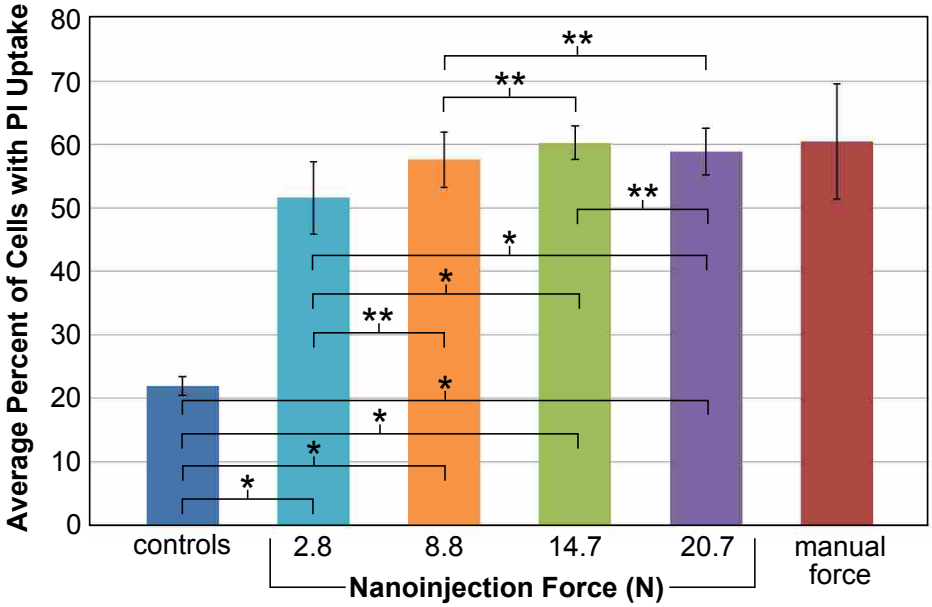

Figure 5.4: Bar graph comparing average PI uptake data for controls, automated nanoinjections, and manual force nanoinjections (Chapter 2). The error bars indicate a 95% confidence interval (α = 0.05). *Statistically significant difference in population averages (p<0.05) **Population averages are statistically the same (p>0.05)

Table 5.1: Table of p-values calculated when comparing the difference in population means for automated nanoinjections.

Comparison			**PI Uptake**	**Viability**
Ctrls	*vs.*	2.8 N	<1.0E-6	<1.0E-6
Ctrls	*vs.*	8.8 N	<1.0E-6	7.6E-4
Ctrls	*vs.*	14.7 N	<1.0E-6	<1.0E-6
Ctrls	*vs.*	20.7 N	<1.0E-6	1.2E-4
2.8 N	*vs.*	8.8 N	0.11	0.26
2.8 N	*vs.*	14.7 N	9.3E-3	2.6E-3
2.8 N	*vs.*	20.7 N	0.039	0.37
8.8 N	*vs.*	14.7 N	0.32	0.34
8.8 N	*vs.*	20.7 N	0.66	0.092
14.7 N	*vs.*	20.7 N	0.56	2.0E-4

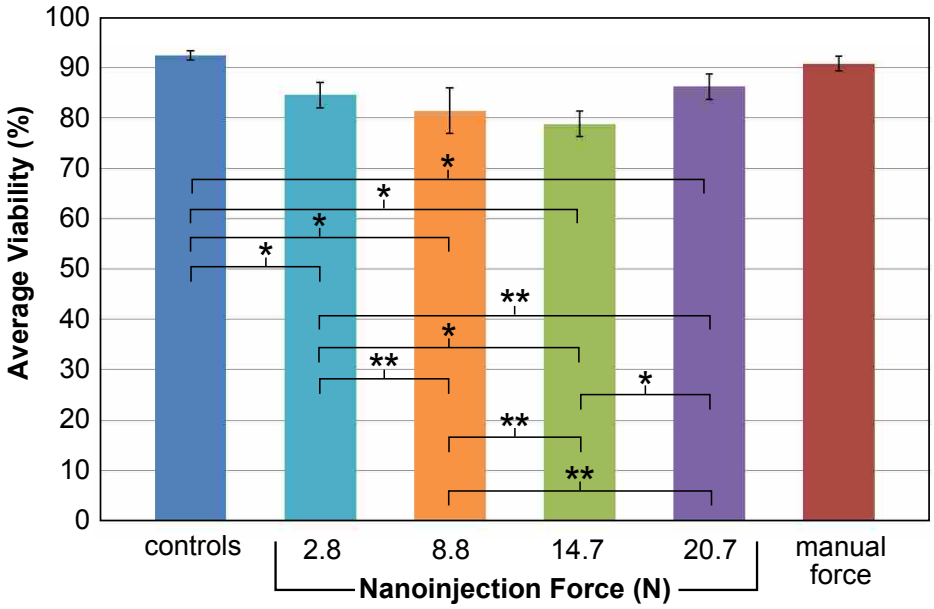

Figure 5.5: Bar graph comparing average viability data for controls and automated nanoinjections, and manual force nanoinjections (Chapter 2). The error bars indicate a 95% confidence interval (α = 0.05). *Statistically significant difference in population averages ($p<0.05$) **Population averages are statistically the same ($p>0.05$)

5.4.2 Data Variability Study

The standard deviation of average PI uptake for the injected samples at 8.8, 14.7, and 20.7 N is 7.8%, 6.6%, and 9.1%, respectively. Standard deviation calculated from PI studies with a manual injection force from several different users is 18.0% (Chapter 2). The ratio of automated injection standard deviation to that of previous experiments is 0.43, 0.37, and 0.51. On average, this amounts to reduced variability in PI experiments by 56%. The automated nanoinjection system delivers the same amount of PI to cells, while eliminating over half of the variability in test data.

5.4.3 Cell Viability

Figure 5.5 shows average viability of controls and injected cell populations. Average viability in nanoinjected samples is around 80% or more, which is an acceptable rate of cell death for nanoinjection.

CHAPTER 6. CONCLUSION

6.1 From Nanoinjection to Gene Expression

Particle delivery methods for mammalian cells are numerous, with each one having a unique set of advantages and disadvantages. To optimize any gene therapy method, it is vitally important to understand the efficiencies inherent to each step a foreign molecule must take in the gene delivery process. The nanoinjection process has the promise of high delivery efficiencies while maintaining very low cell death. This thesis answers many questions about fundamental aspects of the multi-cell nanoinjection process through testing on HeLa 229 culture cells. The experiments performed for this thesis have defined the relationship between extracellular foreign molecule concentration, the variables that influence nanoinjection of those molecules into cells, the quantity of those molecules transported past the cell membrane, and even gene expression.

6.2 Propidium Iodide

Propidium iodide, a fluorescent dye, was used to tag nanoinjected cells and quantify nanoinjection efficiency and cell death. Different concentrations of PI were used to determine the affect of PI concentration on uptake into cells. DC voltage, along with the addition of a short square wave pulse, was used in some experiments to assist in PI delivery. Delivery efficiencies for these experiments ranged from 25%-78%, depending upon PI concentration and application of voltage. Diffusion alone has previously produced efficiencies as high as 98% at much higher PI concentrations [21]. PI delivery efficiency increased with increasing PI concentration. The addition of an electric field also increased PI delivery into cells, as did adding a square wave release pulse. Further studies determined that by straining cell membranes with a bare silicon chip, PI was delivered to half as many cells as diffusion tests with a lance array.

Cell death was also quantified for the lance array nanoinjection process. Depending upon PI concentration and application of voltage, cell viability ranged from 78%-91%. Nanoinjections without voltage induced 2%-4% cell death, while injections with a voltage applied induced 5%-12% cell death. Experimental data suggests that higher PI concentrations reduce the effectiveness of voltage in delivery efficiency, which may cause higher viability at higher PI concentrations. Further testing of the lance array without the addition of PI presented an average decrease in cell viability of 4.2%. HeLa cell membranes stay porous for several minutes following penetration and removal of lances. Most membrane pores close quickly within 7 minutes of nanoinjection, after which the pores close at a slower rate. Nanoinjections with PI resulted in valuable preliminary data that assisted in the development of gene delivery protocols.

6.3 Plasmid DNA

Plasmid DNA containing the gene to create green fluorescent protein was also nanoinjected into culture cells. GFP plasmids are simple yet visually effective genes commonly employed in gene therapy research. HeLa cells were nanoinjected with both full and linearized DNA plasmids. Experimental protocols nearly identical to those used in PI naninjections were performed. In order for a cell to express GFP, the plasmid must be delivered into the nucleus and integrated into the genome of the cell. The highest average gene expression rate acquired in these experiments was 0.21%. Higher DNA concentrations resulted in statistically greater gene expression than lower DNA concentrations. Linearizing DNA plasmids had no affect on gene expression in injected samples. The low gene expression from this experiment led to the protocols developed and discussed in Chapter 4.

6.4 Radioactively Tagged DNA

Since the GFP plasmid protocols only resulted in data pertaining to gene integration and expression, further protocols were developed to quantify the amount of DNA delivered into the cytoplasm or nucleus of the cells. DNA plasmids were linearized using restriction enzyme digestion procedures. The ends of these DNA strands had unpaired base pairs available for attaching free nucleotides. Thymidine labelled with the radioactive isotope ^{32}P was attached to the free ends of

linearized DNA strands. After nanoinjecting the DNA into HeLa cells using the same techniques outlined in previous experiments, DNA delivered to cells was quantified with liquid scintillation. Constant voltage and pulsed voltage experiments resulted in average DNA molecules per cell of $8.0x10^3$ and $20.0x10^3$, respectively. Further calculations revealed that up to 2.9% of total DNA molecules in solution before injections was delivered into cells.

6.5 Automated Nanoinjections

An automated nanoinjection system was designed and tested to reduce user variability in the nanoinjection process. A programmable microcontroller and mechanical relays output one of three electrical inputs to the lance array and base electrode, allowing for several variations in electric field protocols. The microcontroller also controls a motor driver, which in turn determines the force applied by the lance array to the cell culture. A simple push-button switch allows the user to activate the entire nanoinjection process with the push of a button. The automated nanoinjection system has proven to reduce variability in average number of cells exhibiting PI fluorescence by 56%, while maintaining high viability rates.

6.6 Future Direction

There are several experiments that could be performed to understand the reason behind the low rates of gene expression found in Chapter 3. One possible explanation for low gene expression is poor DNA plasmid integrity. If DNA plasmids are damaged beyond repair before being delivered to the nucleus of cells, they will never integrate into the genome of the cell. To verify the integrity of DNA, plasmids could be run through an electrophoresis gel, or could be transfected using well known transfection reagents. It is also possible that DNA is being damaged beyond repair during the accumulation and release phases of voltage protocols. During voltage application to the lances, the electric field is much higher immediately surrounding lances than elsewhere in the solution. DNA accumulated on lances may be damaged by the higher electric field, resulting in low expression rates even if delivered to the nucleus. Ramped voltage could be used to release DNA molecules from the immediate vicinity of lances with a low initial voltage, avoiding DNA damage. The higher voltage of the ramp would still move the DNA further from lances into cells.

Another reason for low gene expression could be caused by improper timing in the cell cycle when nanoinjections are performed. HeLa cells divide approximately every 24 hours. During nanoinjection, lances only penetrate cell membranes once. It is possible that gene expression is limited by a low percent of cells being in the proper life cycle phase to integrate and express foreign DNA. An experiment could be developed where each cell culture sample is nanoinjected at 3-4 hour intervals over the course of 24 hours to ensure DNA delivery at the proper phase in the life cycle of each cell.

Other future work can be done with radioactive DNA nanoinjections. Radioactive DNA testing was performed with set DNA size and concentration for all experiments (Chapter 4). Out of the total DNA added to the solution prior to nanoinjection, just under 3% entered cells during the nanoinjection process. It is hypothesized that decreasing the DNA concentration would not affect the number of DNA molecules delivered to cells. Future work should include decreasing DNA concentration, and varying DNA molecule size. By decreasing DNA size it is hypothesized that greater amounts of DNA could be delivered into cells. Theoretically, since foreign DNA is very minimally toxic to cells [49], increased DNA concentration may also increase DNA delivery efficiency with no negative side effects.

Since gene expression only occurs if foreign DNA is delivered to the nucleus, further work should be done to determine the number of DNA molecules delivered to the nucleus during nanoinjection. Radioactive nanoinjection protocols similar to those found in Chapter 4 could be used, employing enucleation techniques to quantify DNA in the nucleus. Since gene expression from the results in Chapter 3 was very low, estimating the number of foreign DNA molecules delivered to the nucleus is key to optimizing nanoinjection protocols for maximum gene expression.

Ultimately, nanoinjection should be tested on a cells taken immediately from the body. Immortal cell lines, cultured in labs for decades such as the HeLa cells tested in this thesis, may not behave like fresh cultures from the body.

6.7 Final Remarks

Several nanoinjection protocols were designed and tested in this thesis. Although high DNA counts were found in cells following nanoinjection, further experiments should be performed to determine the cause of low gene expression. In conclusion, high delivery efficiencies obtained

with PI and DNA, as well as high viabilities maintained in these experiments, prove that nanoinjection using a silicon lance array is a promising physical method for gene delivery in a culture of cells.

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APPENDIX A. STEP-BY-STEP PROTOCOLS

A.1 HeLa Cell Passaging Protocol

A.1.1 Materials

- Sterile Dulbeccos Modified Eagle Medium (DMEM)(with 10% FBS and gentamicin)
- 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 Passage and Plate Preparation Protocol

A.2.1 Materials

- Sterile Dulbeccos Modified Eagle Medium (DMEM)(with 10% FBS and gentamicin)
- 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 μL size) set at 150 μL, 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 μL 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 Nanoinjection Protocol for Propidium Iodide

A.3.1 Materials (for four 6-well plates or 24 wells)

- Cells that have been cultured in 6-well plates for $\approx$ 24 hours
- Suspension and lance array chip
- (x4) 10 mL serological pipettes
- (x4) 5 mL serological pipettes
- (x24) FACS tubes
- Pipette-aid
- Non-sterile Hanks Balanced Saline Solution (HBSS or Hanks)
- Non-sterile Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + gentamicin)
- Propidium iodide solution

- 12 mL of 5x Trypsin
- Micropipette (green, 2-200 μL size) and a pipette tip
- Micropipette (blue, 100-1000 μL size) and a pipette tip
- Waste beaker
- If using voltage in the process:
  - Power supply
  - 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.3.2 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 Hanks ($\approx$ 8 mL per plate) and discard
4. 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)
5. Using thegreen micropipette, add desired amount of PI solution to each well (to keep PI concentrations uniform between controls and injected samples, remember to double the PI added to wells with stainless electrodes, since they have double the Hanks)
6. If not using voltage in the process:
   (a) Lower injection device and attached chip into well
   (b) Press center fixture of injection device into cells, penetrating the cell membranes
   (c) Continue holding lance array in cells for 5 seconds
7. If using voltage in the process:
   (a) Set desired settings on power supplies (PI has a positive charge)
   (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 wait 20 seconds to attract PI to lances
   (f) Press center fixture of injection device into cells, penetrating the cell membranes
   (g) Immediately push the switch button on the Arduino controller to activate pulsed input
   (h) Continue holding lance array in cells for 5 seconds
   (i) Flip black box toggle switch to "off"
   (j) Remove black alligator clip from wire

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 6-8 for each well to be injected

A.3.3 Flow Cytometry Preparation

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
5. Transfer contents of each well to their respective FACS tubes (use a new 5 mL pipette each time you move to a new plate with a new PI concentration)
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 μL Hanks to each tube using the blue micropipette
10. Take tubes up to RIC facility for flow cytometry analysis (take a CD to put the data on)

$\Rightarrow$ If you need to wait longer than 5-10 minutes to take your samples to flow, put them in the fridge or on ice (WIDB room 794)

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

A.4 Nanoinjection Protocol for DNA

A.4.1 Materials (for two 6-well plates or 12 wells)

- Cells that have been cultured in 6-well plates for $\approx$ 24 hours
- Mark IV injection device and lance array chip
- (x3) 10 mL serological pipettes
- (x2) 25 mL serological pipettes
- Pipette-aid
- Sterile Hanks Balanced Saline Solution (HBSS)
- Sterile Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + gentamicin)
- Tube of DNA in solution
- Micropipette (red, 2-20 μL size) and a pipette tip
- If using voltage in the process:
  - Waste beaker
  - Power supply
  - 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.4.2 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 HBSS ($\approx$ 8 mL per plate) and discard
6. Using a 25 mL pipette, add 1 mL HBSS to each well (if using stainless steel plates for a base electrode, you need to add 2 mL HBSS to cover the cells adequately)
7. Using the micropipette, add desired amount of DNA solution to each well (remember to double the concentration for stainless steel electrode wells)
8. If not using voltage:
   (a) Lower injection device and attached chip into well
   (b) Press center fixture of injection device into cells, penetrating the cell membranes
   (c) Continue holding lance array in cells for 5 seconds
9. If using voltage in the process:
   (a) Set desired settings on power supplies (DNA is negatively charged)
   (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 wait 20 seconds to attract DNA to lances
   (f) Press center fixture of injection device into cells, penetrating the cell membranes
   (g) Immediately push the switch button on the Arduino controller to activate pulsed input
   (h) Continue holding lance array in cells for 5 seconds
   (i) Flip black box toggle switch to "off"
   (j) 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 8-10 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.4.3 Flow Cytometry Preparation

- 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 μL size) and a pipette tip
- (x12) FACS tubes
- Pipette-aid
- Non-sterile Hanks Balanced Saline Solution (HBSS or Hanks)
- Non-sterile Dulbeccos Modified Eagle Medium (DMEM)(10% fetal bovine serum + gentamicin)
- 6 mL of 5x Trypsin
- Waste beaker

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
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 μL Hanks to each tube using the micropipette
14. Take tubes up to RIC facility for flow cytometry analysis (take a CD to put the data on)

$\Rightarrow$ If you need to wait longer than 5-10 minutes to take your samples to flow, put them in the fridge or on ice (WIDT room 794)

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

A.5 ^{32}P DNA Tagging and Purification Procedure

A.5.1 Tagging DNA with ^{32}P dTTP

Materials Needed

- RE digested plasmid

- NE buffer 2 (10x)
- dATP (100x)
- dCTP (100x)
- dGTP (100x)
- ^{32}P dTTP (radioactive thymidine)
- Klenow fragment
- EDTA
- DEPC H_2O (DNase and RNase free distilled water)

Procedure*

Chemical	**100 μL Mixture (μL)**
NE buffer	10
dATP	1.0
dCTP	1.0
dGTP	1.0
^{32}P dTTP	12.5
Klenow	1.5
DNA solution	40.0-70.0†
DEPC H_2O	33.0-3.0†
EDTA	2.0

† *Sum of DNA solution and DEPC must be 73*

1. Thaw DNA, NE buffer, Klenow, and nucleotides
2. Turn on the hot plate on the radioactive bench (set switch to HIGH and high dial to zero to get 75°C) to give adequate time for it to heat
3. Use table above to determine amounts of each chemical needed
4. Add NEBuffer to the tube containing DNA solution
5. Add each dATP, dCTP, and dGTP
6. Add ^{32}P dTTP
7. Add DEPC H_2O
8. Add Klenow
9. Incubate at room temperature (25°C) for 15 minutes
10. Stop Klenow reaction by adding EDTA
11. Heat for 20 minutes at 75°C

**Adapted from https://www.neb.com/protocols/2014/01/13/protocol-for-blunting-ends-by-3-overhang-removal-and-fill-in-of-3-recessed-5-overhang-ends-using*

A.5.2 Separation of Free ^{32}P dTTP Molecules and ^{32}P-Tagged DNA

Materials Needed

- NucAway kit (elution tube, collection tube, and column)
- DEPC H_2O (DNase and RNase free distilled water)
- ^{32}P-tagged DNA from section 1

Procedure**

1. Tap the column to settle the dry gel into the bottom of the spin column
2. Hydrate the column with 650 μL of DEPC H_2O
3. Cap, vortex, tap out air bubbles, and hydrate at room temperature 5-15 min (once rehydrated, columns can be stored at 4°C for up to 3 days)
4. Place the spin column in a 2 mL collection tube and spin the column at 750 x g for 2 min to remove excess interstitial fluid, keeping track of the orientation of the column in the rotor (just use the small centrifuge with a purple lid)
5. Discard the collection tube and immediately apply the sample from section 1 to the center of the gel bed at the top of the column.

⇒ ! Do not disturb the gel surface or contact the sides of the column with the pipette tip or reaction mixture!

6. Place the spin column in the 1.5 mL elution tube and place in the rotor, maintaining orientation
7. Spin the spin column in the tube at 750 x g for 2 min.

⇒ Your sample will be in the elution tube

8. Discard the spin column in the ^{32}P solid waste bin
9. Place a small piece of masking tape on the lid of the solid waste bin. On the tape, write your initials, the date, and the amount of μCi discarded into the bin
10. Place tube of tagged DNA in shielded box in refrigerator until use
11. Update the lab disposal log with the proper dry disposal amount, and perform a radioactive survey of the work area and materials

**Adapted from http://tools.lifetechnologies.com/content/sfs/manuals/4386459B.pdf

A.6 Radioactive ^{32}P DNA Nanoinjection Protocol

WIDB 655

A.6.1 Cell Passaging and Plating *(30-45 min)*

Materials Needed

- Flask of cells to be passaged
- (1x) six-well plate with glass slips (and electrodes if with voltage)
- Micropipette and (1x) 200 μL tip
- Pipet-aid
- (2x) 25 mL pipette
- (2x) 10 mL pipette
- (3x) 5 mL pipette
- (1x) 1 mL pipette
- (1x) 15 mL conical tube
- Waste beaker
- Sterile DMEM, Hanks (HBSS), & 5x Trypsin

Cell Passaging

1. UV non-liquid materials for at least 15 minutes and use proper sterile techniques to avoid cell contamination
2. Using a 25 mL pipette, remove old media from the flask and discard into waste beaker
3. Using a 10 mL pipette, add 10 mL Hanks solution to the flask, then rinse flask bottom and discard the Hanks into waste beaker
4. Using the 5 mL pipette, add 2.5 mL Trypsin to the flask
5. Make sure the Trypsin entirely covers the cells on the flask bottom, and place flask in incubator for 5 minutes
6. Remove flask from incubator and swirl until all cells are no longer adhered (if needed, hit flask against countertop to further break up cells)
7. Place flask back in hood (spray arms, hands, and flask with ethanol first)
8. Using a 10 mL pipette add 7.5 mL DMEM to the flask
9. Pipette the solution up and down several times to thoroughly mix the contents and break up any cell clumps, and make sure to rinse any remaining cells from the bottom of the flask
10. Pull all the cell solution back into the 10 mL pipette
11. 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)
12. Add 2 mL cell solution to the 15 mL tube
13. Discard remaining cell solution into waste beaker
14. Using a 25 mL pipette, add enough DMEM to the flask to bring the total amount of solution to 20-25 mL
15. Using a 5 mL pipette, add 2 mL DMEM to the 15 mL tube
16. Cap 15 mL tube and invert several times to mix cell solution
17. Using the micropipette, place 150 μL cell solution from the 15 mL tube into each well-bottom

18. Using a 5mL pipettte, dilute the cell solution in the tube in half by adding 3 mL DMEM (this tube will be used for hemocytometry counting in Section 2)
19. Using the 1 mL pipette, spread the cell solution in each well into a circle roughly 2 cm in diameter
20. Place flask and 6-well plate into incubator, being sure to loosen the lid to allow CO_2 flow
21. Incubate plated cells for 4-5 HOURS before nanoinjecting to allow cells to adhere to glass

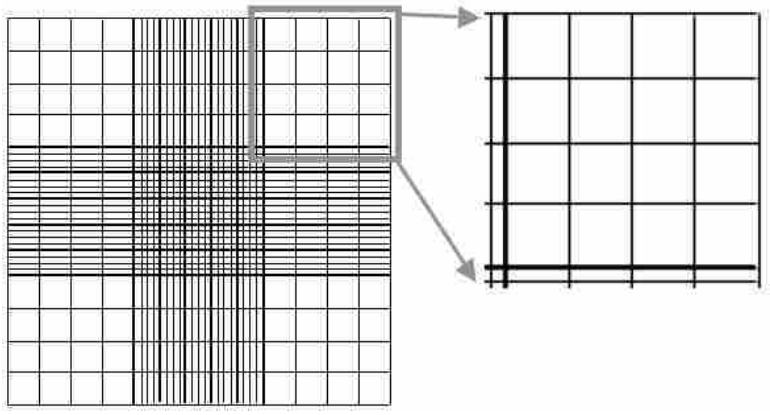
A.6.2 Cell Counting on Hemocytometer *(10-20 min)*

Materials Needed

- 15 mL tube of cell solution from Section 1
- Hemocytometer
- Hand counter/clicker
- Microscope
- 25x25 mm glass cover slip
- Micropipette and (1x) 20 μL tip
- Kimwipes

Cell Counting

1. Wipe away any visible debris on shiny hemocytometer grids with a Kimwipe
2. Place the glass cover slip on the hemocytometer in the center
3. Using the micropipette, pipette 14 μL of cell solution into each of the grooves at the edge of the hemocytometer (capillary action will pull the cell solution up beneath the glass and spread it uniformly)
4. Place the hemocytometer under the microscope and set the microscope to either 20x or 40x
5. Count the cells (keep track with the hand counter) in each of the 4x4-grid corners, and find the average of the four corner counts
6. Multiply the average by 2 (to account for the 1/2 dilution from Section 1) and then multiply by 0.15 (to account for the amount of cell solution in each well)
7. Multiply by 10^4, and the final number is the average number of cells per well



8. When finished, dispose of the glass slide, rinse the hemocytometer, and dry the hemocytometer with a Kimwipe

WIDB 742

A.6.3 Nanoinjection Protocol *(30-45 min)*

Materials Needed

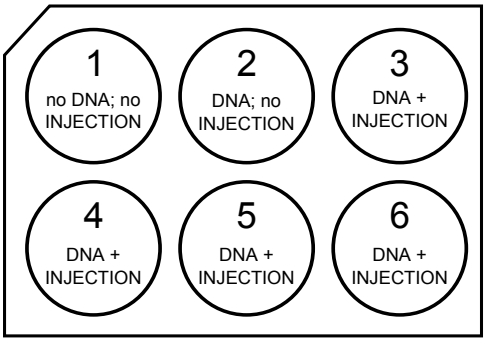
- A 6-well plate of cells that have been incubated for 4-5 hours
- Mark IV inejction device and lance array chip
- Carbon tape, scissors/knife
- Micropipette (2-20 μL) and (1x) 20 μL tip
- (1x) 10 mL serological pipette
- Hanks solution
- Radioactively tagged DNA solution

$\Rightarrow$ Power supply, function generator, black box Arduino switch
$\Rightarrow$ Coaxial cable (connect from function generator output to input 2 of black box)
$\Rightarrow$ Red and black cables (connect from power supply to input 1 of black box)
$\Rightarrow$ Red/black alligator clips (connect to output of black box)

Nanoinjection Protocol

1. Using the 10 mL pipette, add 2 mL Hanks to each well
2. Using the micropipette, add 7 μL DNA solution to wells 2-6 (see figure below for typical plate setup)
3. Refer to previous protocols for the nanoinjection process for diffusion, constant voltage, or pulsed voltage
4. Nanoinject wells 3-6 only (see figure below for typical plate setup)

5. Rinse off injection mechanism and chip thoroughly in sink, air dry on towel, and store in shielded box
6. Wait 15 minutes before transferring solution to scintillation vials



A.6.4 Transfer to Scintillation Vials *(15-30 min)*

Materials Needed

- (x12) 6 mL scintillation vials
- (4x) 5 mL serological pipettes
- (1x) 10 mL serological pipettes
- Non-sterile Hanks (HBSS) and Trypsin

Rinsing Protocol

1. Wait 15 minutes after nanoinjections before transferring cells to scintillation vials
2. Label scintillation vials R1-R6 and L1-L6
3. Using a 5 mL pipette, transfer supernatant from each well to their respective vials (R1-R6)
4. Using a 5 mL pipette, add 1 mL Hanks to each well
5. Swirl the plate by hand to rinse cells
6. Using the same 5 mL pipette, transfer supernatant from each well to their respective vials (R1-R6)

$\Rightarrow$ Be sure to discard pipettes in radioactive waste bin

Transfer Remaining Cells

1. Using a 10 mL pipette, add 1.5 mL Hanks to each well
2. Using the same 10 mL pipette, add 0.5 mL Trypsin to each well
3. Allow Trypsin to detach cells for at least 15 minutes
4. Using a 5 mL pipette, transfer contents of each well to their respective vials (L1-L6)

⇒ You will probably need to pipette up and down a few times to make sure the cells are detached and in solution

5. Using a 5 mL pipette, add 1 mL Hanks to each well
6. Swirl the plate by hand to rinse cells
7. Using the same 5 mL pipette, transfer contents of each well to their respective vials (L1-L6)

⇒ Be sure to discard pipettes in radioactive waste bin

BNSN E240

IMPORTANT! Carry samples to and from BNSN E240 in radiation shielded box!

A.6.5 Scintillation Counting *(45-60 min)*

Materials Needed

- Ecoscint A scintillation cocktail
- Scintillator and rack

Using the Scintillator

1. Add 3 mL Ecoscint A to each sample vial
2. Load samples into larger vials in scintillator rack
3. Find flag 2 (corresponds with the ^{32}P assay in the software) and attach it to the scintillator rack
4. Load the rack into the scintillator
5. Leave samples in the dark for 5-10 minutes
6. Open Quanta Smart on the laptop, and click the start button in the window to start counting the samples
7. When counting is finished, transfer vials back to shielded box

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A.6.6 Disposal and Clean Up *(15-20 min)*

1. Dump vial contents down the drain, rinse inside of vials several times, and recap
2. Place empty vials in radioactive waste bin
3. Update the lab disposal log with the proper sink disposal amount, and perform a radioactive survey of the work area and materials

APPENDIX B. ARDUINO SKETCH CODE FOR PULSED NANOINJECTIONS

```
// ************************************************//
// // // // Nanoinjection 10V Pulse Program // // // //
// ************************************************//

#include <Stepper.h>

// Input delay times
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 = 50; // 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)
  {
   // 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);

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

    // extra delay for removing everything
     delay(input3duration);

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

    // Turn on input 1
     digitalWrite(input_1_LED_pin, HIGH);
     digitalWrite(relay_1_pin, HIGH);
  }

  previousBtnState = buttonState;
}
```