Improving Separation of Differentiated Embryonic Stem Cells with a Microfluidic Device

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Improving Separation of Differentiated Embryonic Stem Cells with a Microfluidic Device

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Improving Separation of Differentiated Embryonic Stem Cells with a Microfluidic Device

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Abstract

Differences in cell cytoskeletal stiffness can be utilized to sort differentiated embryonic stem cells into distinct populations through the use of a microfluidic device. An initial microfluidic system was developed and proven by previous researchers to sort cells[1]. Modifications to this microfluidic system were made and aspects have been improved to increase the efficiency of moving large numbers of cells through the device for use in PCR. Preliminary data from the updated microfluidic system shows that vertical integration of cells and increasing cell count along with increasing length of experiment show the greatest promise moving forward. Polymerase Chain Reaction (PCR) is a process to analyze differences in gene expression for genes which produce proteins which may have an effect on cell stiffness. Once total cell throughput is improved to large enough numbers, PCR was then completed in a separate project on the two populations differences in levels of gene expression were compared. The genes to be tested are VIM[2], ACTN1[3], and LMNA[4], along with GapDH as a constant, which previous research suggests produce proteins which may play a role in cell stiffness. These genes would therefore have different levels of expression, as measured by PCR, in cells with different levels of cytoskeletal stiffness. Improving the microfluidic separation system will also allow for future use in research, and for commercial use in the field of artificial organ generation, by collecting larger populations of pure populations of stem cells. A system was developed to generate large quantities of cells for the graduate student advisors' other research endeavors along with other graduate students working on similar projects. Knowing the genes that alter cytoskeletal stiffness will allow for numerous avenues of opportunity, but will greatly change the way populations of cells are isolated and purified.

Introduction

Artificial organ generation requires large numbers of cells that have differentiated to the specific cell type required. Currently, one of the larger setbacks of scaling up artificial organ development is the effort to isolate large, pure populations of stem cells. For the organ to grow as desired, the cells must all be of the same type, which can be characterized by multiple traits, including gene expression and cytoskeletal stiffness. Microfluidics is a growing multidisciplinary field that deals with the flow of fluids of relatively low volume, and is being applied for use in biological research to sort populations of cells and other particles. It has been shown that different cell types exhibit different levels of cell stiffness, which can be used to sort different populations[1]. Our lab has developed a microfluidic chip that sorts cells based upon their relative stiffness and their deformability. Through the application of microfluidic principles, the cells are forced down a corridor with multiple rows of diagonal ridges. Depending on if the cell is above or below the stiffness threshold, it will determine which of the two outlets each cell is drawn to by either hitting the ridge and going over it or going alongside it. The gap between the bottom of the ridge and the bottom of the device is where the soft cells would deform slightly to continue on down the channel, and this gap size changes based on the type of cell and its average size. Previous research with this device has yielded positive results proving the validity of the device in separating cells based on their stiffness[1]. This research also justified the proper flow rates for both the sheath inlets, on either side to direct the cells towards the ridges, and the cell inlet, which is in the center between the two sheath inlets[1]. These experiments were completed with relatively low numbers of cells, and the average population stiffness was only compared using AFM (Atomic Force Microscopy)[1].

This project will later deal with these separated populations of embryonic stem cells and through a process called PCR (Polymerase Chain Reaction), the levels of gene expression can be determined for genes that are thought to control the cell's stiffness. Many different genes code for proteins that have the potential to play a role in cell stiffness, but these genes will have to be tested on separated cell populations to know for certain. The two separated populations will be compared through PCR and through AFM, a process which measures the cell's actual stiffness. These results would be the differences in stiffness, which may be a signal in determining how the stem cell might differentiate. The microfluidic device is a way to sort these cells, but PCR is a

way to guarantee the cell populations are in fact different, in that completely different cell populations will have different levels of gene expression for the genes that control cell stiffness. The data collected from these experiments will go to further prove the validity of the device, and for it to be used in clinical environments for sorting larger populations of stem cells to develop artificial organs efficiently. First though, the total throughput of cells through the device must be increased to a sustainable number of two million cells for PCR, while still maintaining accurate separation.

Background/Literature Review

When an American needs an organ transplant, he or she becomes added to the national transplant list, which ranks each person in need of each specific organ by various factors. Some persons are more likely than others to rank higher on the list, depending on age and lifespan, the reason for losing the organ, and for how long the person can live without the organ. Getting rid of the transplant list entirely sounds like science fiction, but with recent research into the field of artificial organ generation, science fiction is becoming reality. The problem facing most research in the past, was how to effectively isolate a population of stem cells with a precise set of properties, which was nearly impossible in most environments. This problem has led to a halt in other aspects of this field, mainly in how to form the organs from the stem cells provided. Before this research can move forward, a method of extracting a large population of stem cells has to be established. Microfluidic devices have the opportunity to provide significant advances in the field of cellular and molecular biology. Through maintaining the principles of fluid dynamics within small volumes of liquid flowing through equally small environments, multiple cell populations can be separated and specific cell properties can be established. Previous microfluidic devices being produced around the world have been used to separate cells based on their size, density or even their buoyancy, with new types of devices being created and tested continuously[5]. Past research shows that higher populations of cells used in these microfluidic devices do not have significantly higher inertia, which means that there are virtually no ill effects on how quickly a large number of cells can be passed through a device to become separated[6]. This finding is crucial because to gather larger populations of cells, the duration of separation

will be much shorter than previous studies have shown, and will still allow for accurate separation of the two populations.

The device being used for these experiments was created in our lab, and is used to separate cells based on their deformability, otherwise referred to as their stiffness. The cells are directed towards the center of a long corridor by two sheath streams, and the cells than are sent hurdling towards diagonal walls that have a gap at their bottom[1].This gap size is changed based on the type of cell being separated, due to all cells being different sizes, so the average cell size for that population is used. If a cell is relatively "soft", it will pass through the gap and onwards towards the subsequent walls and gaps until it reaches the so-called "bottom outlet". If a cell is relatively "stiff", it will hit the ridge and go along it, due to the ridge being diagonal, and it will then pass along the top until it reaches the "top outlet". It has been determined though research on this device, that there is a nearly perfect flow rate for accurate cell separation with 25 μL/min[7]. With this flow rate, the viscoelasticity, or time dependent stiffness, has nearly no effect on how each cell reacts to the ridges[7]. If a cell deforms when it hits a ridge, as is expected, it will likely become more stiff for a short period of time, and if this time is longer than the time it takes it to hit the next ridge, it will likely go along the ridge instead of under it. This is known as a low viscoelasticity, which means the cell is not very elastic, and the flow rate must be compensated to allow for that, which is why the rate of flow is so very important, and why it is kept constant while cell concentration is variable based on the experiment being conducted.

Once these cells are separated, a number of cells from each population undergo a process called Atomic Force Microscopy (AFM), where it is determined, among other properties, what the general stiffness of the cell is. AFM essentially uses a cantilever to pop a cell, and calculates the amount of force that was required, which is a great measure of the cells relative stiffness. This process is quite similar to the relatively new optical stretcher tool, which measures a cell's deformability, which could be another way of determining this property in future experimentation[8]. Once the cells stiffness has been determined, Polymerase Chain Reaction (PCR) can be done on the same populations of cells to determine their relative gene expressions to each other. My previous research has shown possible connections of the proteins Lamin A, Actinin, and Vimentin to cell cytoskeleton structure, and the genes which produce these proteins control the overall stiffness. Two different populations of cells separated by the device will

naturally have different levels of expression for a gene which controls cell stiffness, and thus it can be determined if this gene plays a role in this expanding field of research.

The research worked on in this project was to run experiments on different iterations of the microfluidic system setup, which included fine tuning a system to gather the highest throughput as possible while still maintaining accuracy. Once this had been achieved, further research on the genes desired could be analyzed and results produced. Improving throughput on the device will allow it to be used by other research environments for use in artificial organ generation, or for the use of other and new microfluidic devices. Producing a list of genes which actively play a role in cell stiffness will allow for other methods of separating cells based on their stiffness, which has significant ramifications in the field of artificial organ generation.

Methods and Materials

Due to low cell throughput in previous microfluidic system setups, many different factors of the system were altered and tested to see if they significantly improve results. The system is composed of an inlet of cells in a buffer at a specific concentration within a syringe, which initially used to settle out after longer experiments, two sheath buffers both in syringes, the PDMS device on a glass slide, and the collection system.

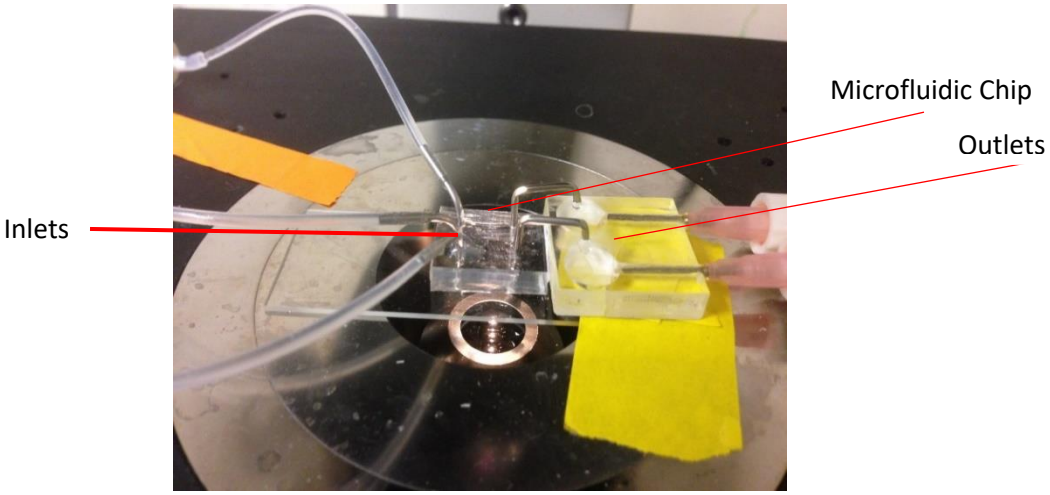

Figure 1. PDMS Microfluidic Device over a Microscope

Microfluidic Separation Device: Cells are directed at subsequent diagonal ridges by sheath inlets, and the cells are directed towards one of two outlets. One outlet for cells which are unable to deform under the ridges, stiff cells (top outlet shown below), and those cells which can deform, soft cells (bottom outlet as shown below).

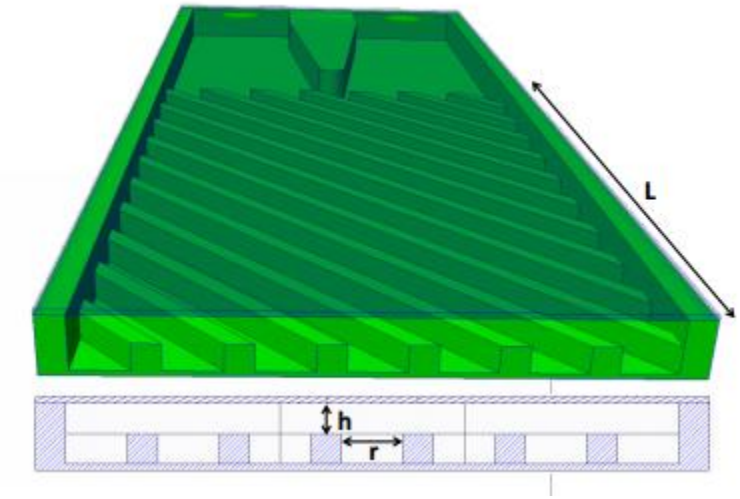

Figure 2. Cross sectional view of rendered device showing ridges and gap size.

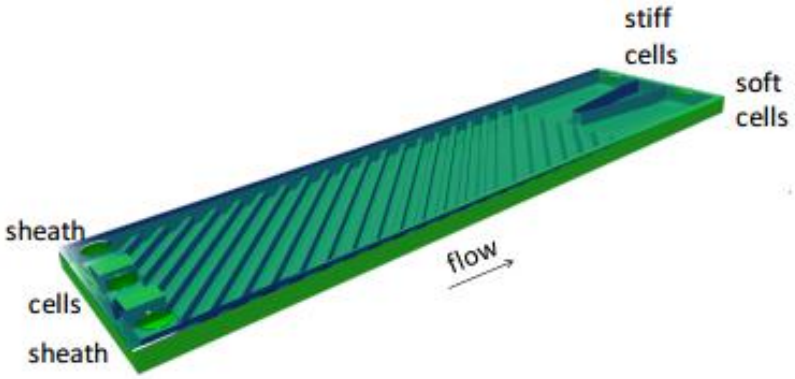

Figure 3. Long render of device showing direction of flow.

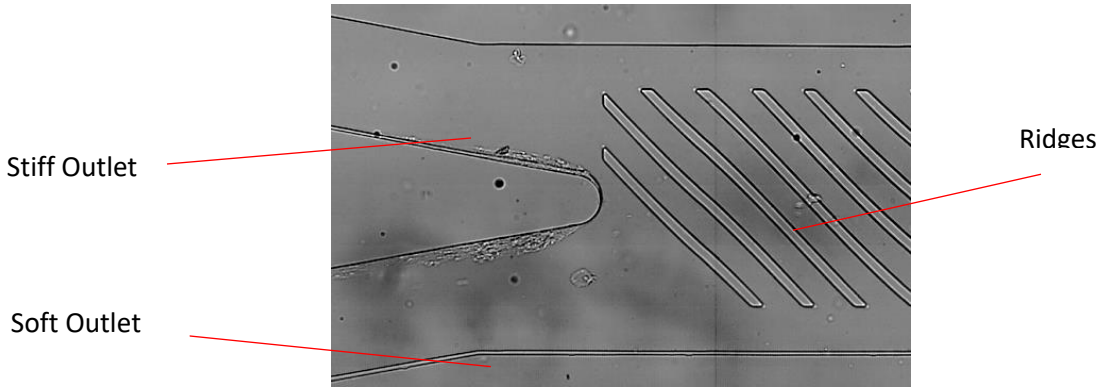

Figure 4. Inside of Microfluidic Separation Device

Cell Count with Hemocytometer: Total cell counts are measured using a hemoctyometer (Figure 7). Then by measuring the total duration of the experiment, the average cell/s rate can be calculated (Figure 8).

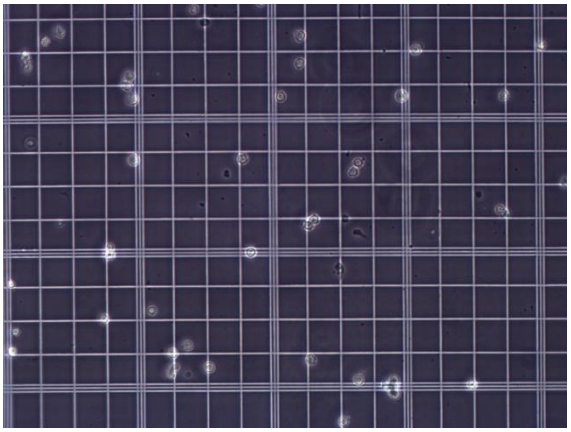

Figure 5. Soft cells in Hemocytometer

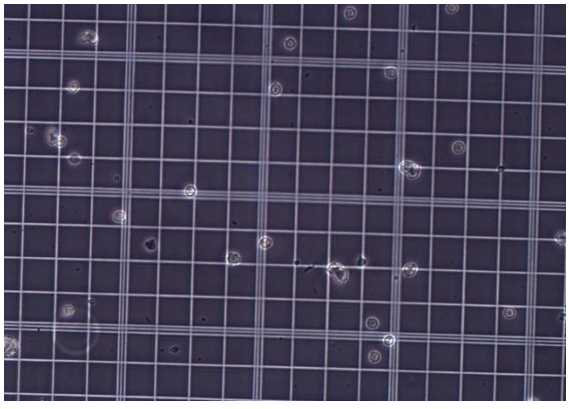

Figure 6. Stiff Cells in Hemocytometer

$$Total\ \#\ Cells = \frac{\#\ of\ Cells}{Square} * \frac{25\ Squares}{1\ Box} * 10{,}000\ dilution\ factor = \frac{\#\ Cells}{mL}$$

$$\#\ Soft\ Cells = \frac{4.95\ Cells}{Square} * \frac{25\ Squares}{Box} * 10{,}000 = \frac{1{,}236{,}000\ Soft\ Cells}{mL} * 1mL = 1{,}236{,}000\ Cells$$

$$\#\ Stiff\ Cells = \frac{6.24\ Cells}{Square} * \frac{25\ Squares}{Box} * 10{,}000 = \frac{1.560{,}000\ Stiff\ Cells}{mL} * 1mL = 1{,}560{,}000\ Cells$$

Figure 7. Formula and sample calculations for counting total cells with a Hemocytometer

$$\frac{Cells}{s} = \frac{Soft\ Cells + Stiff\ Cells}{Duration\ of\ Experiment}$$

$$Average\ \frac{Cells}{s} = \frac{1{,}236{,}000 + 1{,}560{,}000}{7200\ s} = \frac{388.33\ Total\ Cells}{s}$$

Figure 8. Calculation of Cells/s sorted by device

Modifications

Vertical Input Method

This method ensures that when the cells inevitably settle, they settle towards the direction of the device, as opposed to the initial setup which contained the syringe laying horizontal as seen in Figure 9, which most cells would settle to the bottom of, decreasing actual cell concentration to the device over time. The new vertical setup was developed with K'Nex to hold the syringes as shown in Figure 10.

Figure 9. Previous Horizontal Setup

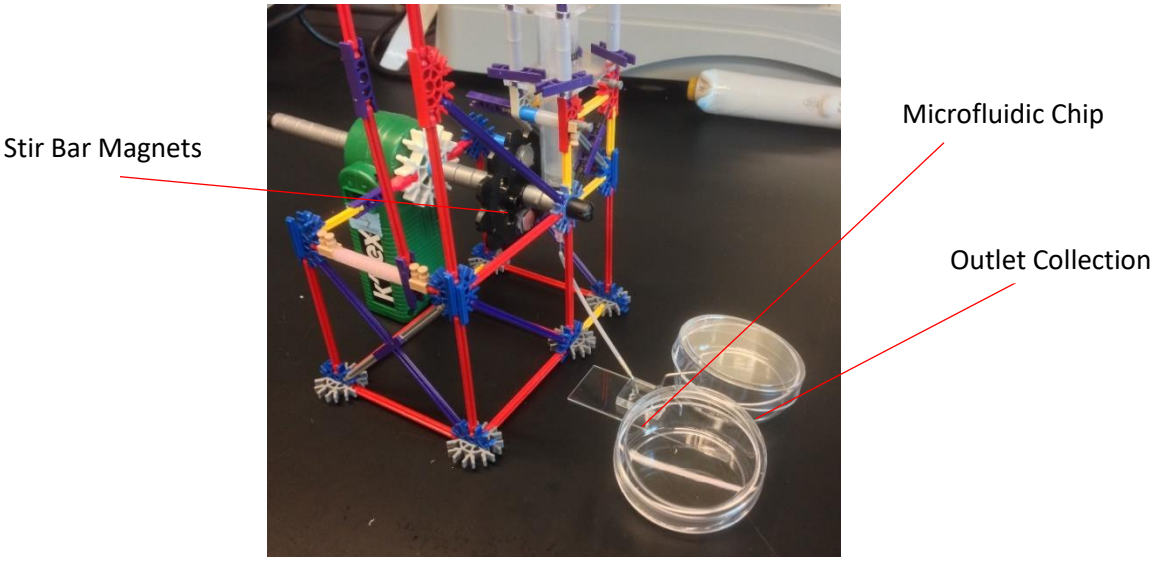

Figure 10. New Vertical Setup

Matched Cell Density Buffer

This process aims to allow for the buffer that the cells are dispersed in to be the same density as the cells, so that they do not settle out over time and so that a constant flow rate can be achieved. Through a process called density centrifugation, the cells can be placed atop levels of buffers of varying densities, then, when placed in a centrifuge, the cells will settle to the density of buffer that they most closely match. The density of the buffer will thus change for each cell type used, and the buffer will still not match every cell, meaning that some cells will still be lost

over time. Due to this and the new vertical input method, it was decided that the buffer should have a density slightly lower than the cells, so that the cells would settle out towards the bottom when given enough time. As seen in Figure 12, the clogging at the cell inlet may have been due to the matched density buffer having microscopic debris that got caught in the inlet, initiating cell shearing.

Stir Bar

A magnet moves a stir bar inside the syringe with the cells, which are moved around in a general direction to reduce settling and to attain a constant flow rate of cells into the device. This helps to maintain constant single cell suspension, but the velocity of movement within the device may cause more cell to bump into other cells causing clumping, or may cause cells to shear when they hit the stir bar itself. The first type of stir bar was not effective at creating a flow of cells inside the syringe, so a different cross shaped stir bar took its place. The horizontal setup featured a stir plate which could be used to adjust the speed of the stir bar, but this could not be feasibly implemented in the vertical setup, so a new turning series of magnets was implemented. The stir plate can be seen in Figure 9, and the spinning magnets can be seen on the vertical setup on the cog like piece in Figure 10.

Automatic Cell Collection

Automatic cell collection, allows for cells to be feasibly collected over a longer period of time. Two forms of cell collection were tested, the first being a tubing system which had the cells move out of each of the outlets into a faucet that collected into a reservoir. The tubing was connected to the reservoir and to syringes with media on the other end which were being drawn at a constant rate equal to or greater than the rate of flow out of the device. One thing noticed was that the flow rate was still relatively slow in the tubing, meaning cells might have enough time to settle out within the tubing or within the reservoir itself. The second form of automatic cell collection as seen in Figure 10 was to use the same type of faucet, though slightly taller to collect out into two different petri dishes with media inside of them. Prior to the petri dish collection method, small volumes of cells in buffer were collected post separation into .5mL tubes (Figure 11). This seems to be more effective in decreasing the distance the cells must travel, and allows them to immediately reach the media. This method was abandoned when

moving to the single cell PCR process and micro-pipetting was used instead to collect the sorted cells.

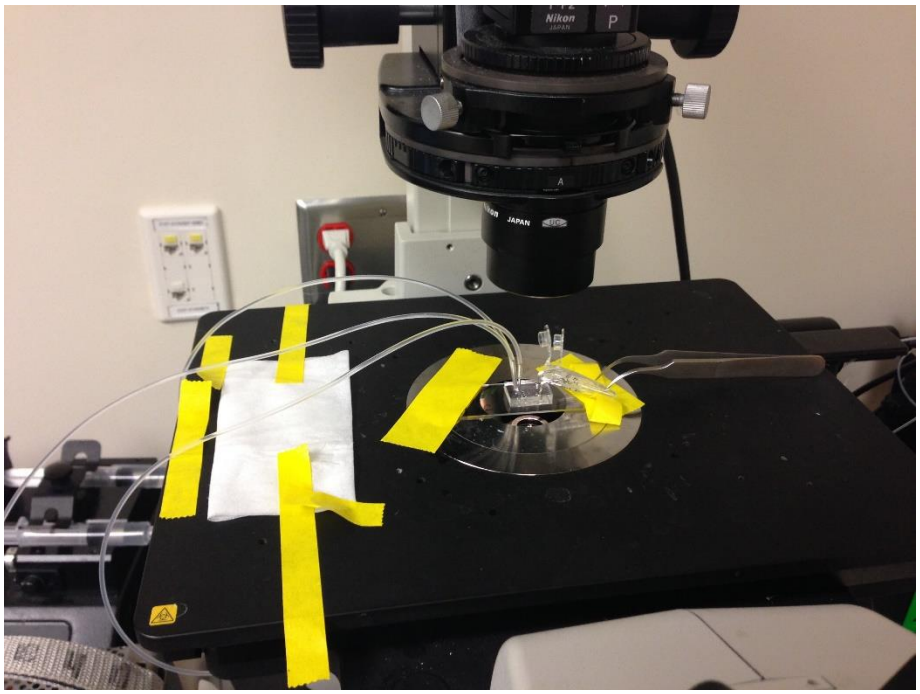

Figure 11. Initial Version of Automatic Cell Collection.

Increasing Cell Concentration

This method is an obvious way to allow for shorter experiments, but may influence cell trajectory, due to cells bumping into each other and in keeping single cell suspension so they can accurately be sorted. Cell concentration was increased from 5 million cells/mL to 12.5 million cells/mL. This method has shown the most promise, especially in conjunction with other methods to maintain constant single cell suspension over longer periods of time. Sheath flow decreases were shown as discussed below to keep the cells flowing per second the same so as to not increase the cells bumping into each other per second.

Increased Duration

Increasing the length of the experiments is in theory the method which would most dramatically increase numbers of cells collected, but in reality, has harsh effects on the health of the cells, which need to be incubated and should not be left at room temperature for longer than one to two hours. This method may have had an adverse reaction, and ended up killing more

cells than the device was able to effectively sort and separate. The killing of cells caused many problems, either due to the increased duration or not, that caused cells to have more of an inclination to shear and clog the inlets (Figure 12). For these reasons, the total duration of any microfluidic separation involving embryonic stem cells was limited to two hours.

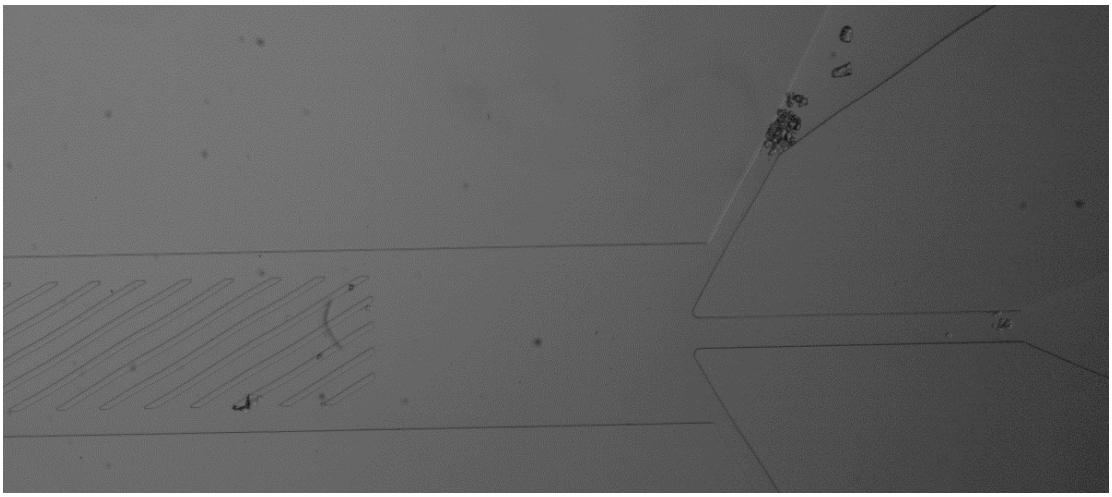

Figure 12. Clogging at the inlet.

Sheath Flow Biasing

Changing the flow rate of the sheath inlets from an even split to 17 μL/min and 23 μL/min allowed for an even split of the pluripotent ESCs from the differentiated ESCs. Extensive manipulation experiments were conducted to measure the best flow rates along with a determined optimal 8 μL/min flow rate for the cell inlet to ensure minimal cell clumping prior to the sorting chamber.

Gap Height

Devices with gap heights, 9.3 μm, 11.5 μm, and 15.6 μm were tested over the course of the project, with 11.5 μm having the best qualitative outcome with significant flow of cells to both outlets with a lack of cell clogging.

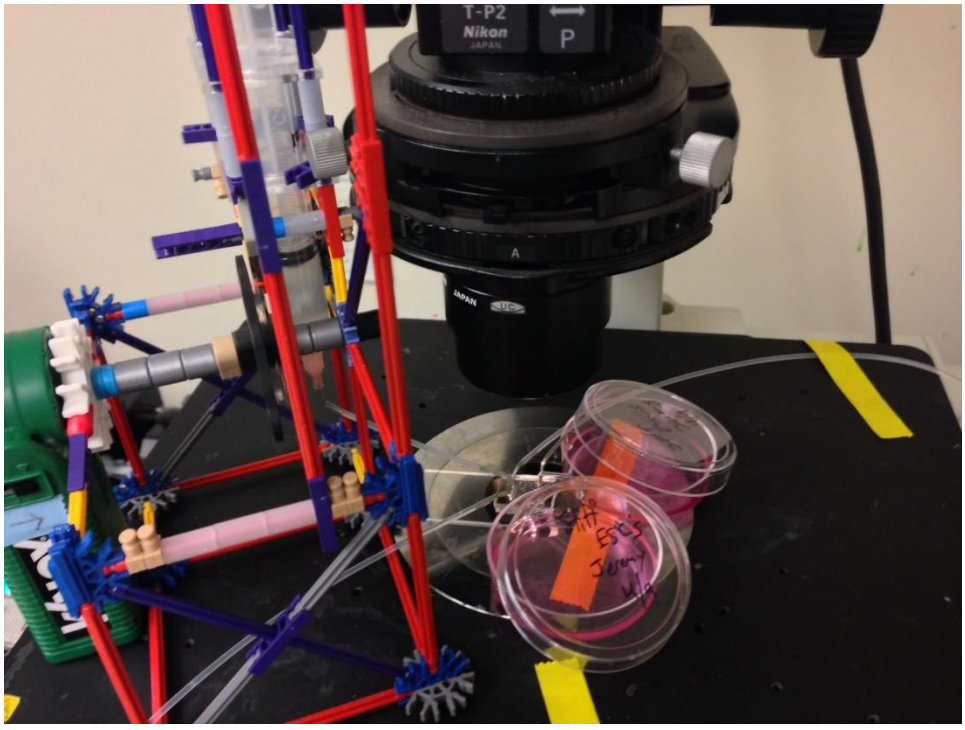

Figure 13. Complete setup on top of microscope stage.

Results

After testing many different modifications and alterations to the system setup over various experiments culminating in the setup above (Figure 13), the most obvious calculation to make was the average total cells per second, which is the rate most fitting to tell how many cells can be collected over a given time with each setup. Each week, different modifications were either added or removed, based upon the previous week's results. For the first four experiments, the throughput was not counted, but qualitatively compared to previous experiments, so that subsequent experiments could be developed with the information retrieved. The results from seven different preliminary experiments were compiled into Figure 14. Once these results were gathered, the focus stopped being primarily on gathering quantitative results, but on keeping the cells alive for the transfer to PCR and AFM.

Focus was still maintained on gathering the cells effectively and repeatedly over multiple experiments and in having roughly equivalent cells number to each outlet, leading to the flow rate optimization process. The next step was to determine if biasing would allow for better

separation between the pluripotent stem cells and the differentiated ones. This hypothesis was tested on each population independently, by subjecting the populations to the same biased flow rates and then comparing the numbers of cells to each outlet. The data was then compared (Table 1), and by comparing the flow rates to the day of differentiation, the best flow rate was chosen to get a large ratio of one cell type to another in one of the outlets, which turned out to be the no biasing, a 1 µL/min for both the bottom and top sheath inlets and 0.5 µL/min for the cell inlet. Most of the pluripotent cells went to the bottom in this scenario, while the highest number of differentiated cells went to the middle outlet when comparing this to the other flow rates.

Results of Experiments Testing System Modifications							
Date	Stir Bar	Buffer	Vertical Integration	Auto Cell Collection	Increased Duration	Increasing Cell Concentration	Average Total Cells/s
9/14/2014	No	No	No	No	No	Yes to 10 Million cells/mL	-
10/11/2014	Yes	No	No	No	No	No	-
10/28/2014	Yes	Yes	No	No	Yes	No	-
2/12/2015	Yes	Yes	No	No	No	No	-
2/19/2015	Yes	Yes	No	Yes	Yes	No	2.9
3/5/2015	Yes	Yes	Yes	No	Yes	Yes to 2.5 million cells/mL	29.8
4/2/2015	Yes	Yes	Yes	Yes	No	Yes to 5 million cells/mL	48.1
4/9/2015	Yes	Yes	Yes	Yes	No	Yes to 8 million cells/mL	561.1
4/16/2015	Yes	Yes	Yes	Yes	No	Yes to 8 million cells/mL	388.3

Figure 14. Results of Experiments Testing System Modifications

Top Outlet (%)	Middle Outlet (%)	Bottom Outlet (%)	Bottom Flow Rate (	Day of Differentiation
1	1	97	0.6	0
1	28	71	0.6	5
1	7	91	0.8	0
4	41	55	0.8	5
2	7	91	1	0
0	50	50	1	5

14	62	24	1.2	0
10	84	7	1.2	5
24	71	5	1.5	0
17	79	3	1.5	5

Table 1. Flow Rate Biasing Optimization Data Table

The conclusion of my role in the project saw an introduction of a new device with three outlets to further characterize the sorted cell populations. Due to the various viscoelastic effects on the cells in the new device, as determined by the faculty advisor and the graduate student advisor, the flow rate had to be significantly increased with a strong increase in the initial cell concentration. Through repeated experiments over the course of the semester, it was first determined that the slower flow rates would allow for better separation of cell populations, due to allowing the viscoelastic properties to take hold, so the 2.5 μL/min total flow rate was chosen and with a distinct lack of cells being collected, that number was increased to 5 μL/min. The total flow rate was compared to the percentage of cells that went to the middle outlet, which is shown in the graph (Figure 15) that a lower flow rate allows the cells to move from the bottom outlet to the middle outlet. One such experiment is pictured below, where most cells were going to the bottom third outlet, decreasing the flow rate was shown to increase the number of cells to the middle outlet, thus increasing the effectiveness of separation (Figure 12). Biasing was thus found to not have a factor on the new device as the three outlets were able to move cells effectively along with the 11.5 μm gap size and the culmination of all aspects of the final system. Both sheath inlet flow rates were set to 2 μL/min and the cell inlet flow rate was set to 1 μL/min. This change saw a decrease of 2.5 times the initial flow rate used after the cell/s optimization in Figure 11, which led to the increase of the cell concentration to 2.5 times the initial concentration of 5×10^6 to 10×10^6.

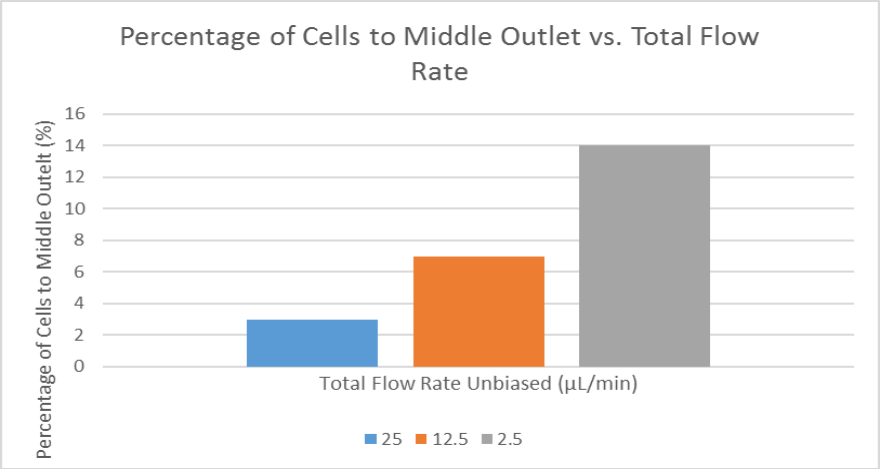

Figure 15. Percentage of Pluripotent Cells to Middle Outlet Vs Total Unbiased Flow Rate.

Discussion

Each experiment tested a different method, as seen in Figure 9, leading to numerous experiments being conducted week after week to determine the optimal separation and collection system. The final determination separating each factor from the others was based on qualitative observations during experimentation as well as quantitatively measured cells/s based off a MATLAB program that the graduate student advisor developed using the Phantom® video capturing software as well as the collected cell concentration when possible. Not every experiment ended in collection as was the purpose of the project, to collect cells effectively, so the MATLAB program was used extensively to track our progress with the experimentation system and setup changes happening so frequently.

It was initially qualitatively noted that the stir bar and the density buffer both had positive impacts on the average cell throughput, and that by introducing vertical integration, increased cell concentration, and increased duration, higher numbers of cells could be collected. Current experiments have been shorter in duration, but have higher cell concentrations, to determine the cutoff point at which increased cell concentration increases clumping between cells. Experiment duration was finalized at a maximum cap of two hours, due to the importance of maintaining healthy cells and incubating them as soon as possible. The desired flow rate to achieve the number of cells for PCR for an experiment of two hours is around 280 cells per second.

Conclusion

This thesis is meant as a focus on the primary goals of my research and the end conclusions gained from the experiments I conducted. The first semester was focused on PCR, while it was determined that the low numbers of cells in the microfluidic separation process were not enough to be able to attain any valuable findings. The rest of the time I spent on the project was focused on this singular goal, with the final outcomes being discussed here. It was determined that a team needed to be assembled as the tasks of these experiments needed extra manpower, which significantly increased the speed of the results obtained. Each experiment tested a different method, as seen in Figure 9, leading to a large timeline to obtain all of the results needed, along with simultaneously growing the two populations of cells in-vitro. The final microfluidic system was an optimized K'Nex setup that integrated the syringe vertically to allow for cell settling towards the device with a stir bar and magnet system to keep the cells moving within the syringe. A buffer that matched the density of the cells was what the cells were kept in as well as used for the sheath buffer, and the cell concentration was increased 2.5 times with an inverse decrease in the flow rate overall. The system allowed for assured cell collection assuming the cells were only out of incubation for a total of two hours.

The desired flow rate to achieve the number of cells for PCR was determined, which allowed for smooth experiments lasting the full two hours that the cells could be absent from an incubator safely. The changes to viscoelasticity during differentiation were minimal, but the device parameters were also tuned to optimize viscoelastic-dependent cell separation as seen with the gap height and the flow rate optimization. At the conclusion of my project, the team was able to gather the necessary populations of cells for their use in single cell PCR as well as AFM experiments. Single cell PCR was utilized for certain experimentation not related to my current project with a shorter duration for the actual experiment as a result, leading to a lessening of complications that my project ran into.

The final months of the project were spent with my absence having conducted an internship out of state. My graduate student advisor, Tom Bongiorno, was able to conduct the final experiments and utilize the results gained, along with his other research projects, to successfully graduate with his doctorate. My project was constantly evolving and changing, but the opportunity to optimize the microfluidic system will hopefully have beneficial results moving

forward. As my project was formed from a previous graduate student in the Sulchek Lab, this new system formed around the microfluidic chip will hopefully be utilized and further perfected.

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Addendum

Work Plan for LMC 4702
(May 2015)

Low Target

- Flow cells through microfluidic device without clogging
- Collect PCR data from at least a million cells on two known genes.
- Get some growth from cells post separation
- Collect enough data to compose a paper which will be submitted to a journal in subsequent semester

Ideal Target

- Flow large populations of cells through device without clogging into two populations
- Collect PCR data on gene expression from two known genes and two new genes on separated populations of cells
- Grow cells post separation
- Submit a paper to a well-known journal

High Target

- Flow millions of cells without clogging into two separated populations.
- Collect PCR data on a wide range of genes, to gather valuable data on multiple genes which may play a role in cell stiffness
- Grow cells post separation with the ability to passage and collect data on the new population of cells
- Submit a paper to PNAS or to Nature

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