Abstract:

Common modern high throughput screening (HTS) efforts involve the plating of cells on the bottom of microtiter plates in a 2-Dimensional (2-D) monolayer. However, rigid 2-D structures are not comparable to *in vivo* 3-D tissue structures in the human body. 3-D bioprinting is the modern alternative to this challenge. This paper describes the pneumatic extrusion-based printing of the HeLa cancer cells directly into 96-well microtiter plates for screening applications. Cells were prepared for printing in a solution of 30% Pluronic F127 dissolved in media. The solution concentration was determined through a dilution experiment that optimized both the print quality and cell viability. Once structures were printed into wells, plates were imaged with a high-content, high-throughput confocal imaging system. The canny edge and flood-fill algorithms used to segment cells performed at a 95.238% accuracy. This shows the potential of using 3-D printed structures in conjunction with confocal microscopy for drug screening. Cell viability remained an issue even after bioink optimizations, and the cells died within 2 days of incubation. A matrigel other than pluronic may be able to maintain cell growth for the necessary weeks during which cells may form an extracellular matrix within the sacrificial gel.

Introduction

This paper demonstrates the feasibility of analyzing 3-Dimensional (3-D) bioprinting with high-throughput screening technology used in drug discovery. The study of bioprinting lies on the frontier of modern biology, and has vast potential, particularly in medicine and healthcare. It is an additive manufacturing method based on the deposition of live tissue in stacked layers [1]. Although it has yet to be fully realized, this technology may eventually have the capability of fabricating synthetic, cell-based human organs for human transplants [1]. An equally important application of 3-D bioprinting lies in the field of pharmaceutical drug discovery. Bioprinting may be specifically useful in high-throughput screening, a process used to search for potentially useful drugs by mass-imaging samples of infected cells. in microtiter plates, each of which may contain up to 9600 wells [1].

The speed of modern high-throughput screening techniques has rapidly improved owing to the advances of automation; robots are able to quickly plate cells into the bottoms wells and subsequently infect them with pathogens or treat them with compounds [2]. However, plating 2-D cell assays may lead to misleading results in drug compound analysis. Moreover, research shows that 3-D cell assays are more effective in modeling *in vivo* drug response in comparison to 2-D assays. For instance, mouse mammary tumor cells cultured as 3-D structures exhibit more drug resistance to certain compounds than those cultured in two dimensions [3]. In addition, in response to the same concentrations of the anti-mitotic drugs doxorubicin and 5-fluorouracil, cancer cells in 3-D arrays were much more resistant to these drugs than those in 2-D monolayers; the drugs took 7 times longer in the multilayered cell culture to take effect [4]. Moreover, results

of drug screening that are determined via 2-D cell-based assays are not necessarily indicative of *in vivo* 3-D cell conditions.

Bioprinters are capable of producing adaptable and precise 3-D cell cultures at similarly high speeds regardless of the microtiter plate size [1]. This adaptability may allow researchers to avoid the cost of purchasing multiple microplate dispensers. Apart from overall adaptability, bioprinting has several advantages over existing 3-D culture methods. One such method is manual seeding, which involves manual pipetting of the extracellular matrix (ECM) [5]. Although cost efficient, manual seeding has major drawbacks. Because of the pressure at the center of a pipetted drop, the cells in each drop are more concentrated at its perimeter than at its center, resulting in an uneven distribution of cells, as demonstrated by Horva´th et al. [5]. In addition, manual printing involves a large degree of human error. In contrast, bioprinting guarantees constant density throughout the matrigel cell structure. Another modern 3-D cell culture is the spheroid [6]. While culturing spheroids is often quick and cost efficient, adaptability still remains an issue. In both forced-floating and natural agitation-based methods, it is impossible to dictate the size of the spheroid. While scaffolds and hanging-drop suspensions are an alternative adaptable measure of developing spheroids, neither of these tactics are suitable for large-scale drug screening due to cost [6].

A more long term application of bioprinting in the field of drug discovery is the bioprinting of cellular tissues/organs to directly observe the effect of drugs on elements of the human body. 3-D bioprinting allows for cells to form extracellular matrices and perform multicellular activities like organs in the human body [7]. By creating such artificial, isolated living systems, scientists may be able to completely avoid the hassle of live animal testing with

specimens such as monkeys and rats: a long and costly process. As an example, HUVEC cells could be printed in a hollow cylindrical shape to mimic the structure of capillaries for hemorrhagic virus drug testing. A study by Truang et al. implements this idea by studying the cell migration differences between cancer and normal cells that were printed in the structure of rat capillaries in response to anti-migratory drugs [8]. This is an important realm of study due to the fact that cancerous blood vessels may not be able to transfer drugs as effectively as normal blood vessels [9]. In general, the applications enumerated above are a result of the adaptability that comes with bioprinting that few other 3-D cell culture methods come with.

Despite the advantages of bioprinting, studies that put bioprinting into drug screening practice are lacking. One method of implementation, proposed by Deiss et al. [10], is the Cells-in-Gels-in-Paper (CiGiP) method, in which 200 μm sheets of paper are stacked to form the 3-D cell structure; the sheets are then detachable for analysis in high-throughput screening in 96 well plates. My study proposes the use of confocal microscopy to image structures in a 3-D whole as opposed to imaging the 2-D slices (the strategy used in CiGiP). The goal of my research was to produce a 3-dimensional cell assay that could be easily maintained, imaged, and analyzed with high throughput screening technology.

Methods:

This study was a two-phase proof of concept study. In the first phase, I attempted to optimize the various printing parameters to produce a 3-D cell matrix suitable for high throughput analysis. In the second phase, I attempted to assess the imaging quality to assess the potential of confocal microscopy for analyzing 3-D assays in drug discovery applications.

In this study, I used the *helacyton gartleri* (HeLa) cancer cell line for bioprinting. The cells had undergone 10 cell cycles by the printing stage—roughly 10 days. During these 10 days, the cells were incubated at 37 degrees Celsius with 5% Carbon Dioxide levels in a 20 centimeter petri dish. Minimum essential medium (MEM) of 10% fetal bovine serum and 1% penicillin was used as nutrition to keep the HeLa cells alive during the incubation period. The cells also exhibited red fluorescent protein (RFP) throughout the nucleus that would allow electron excitation in confocal microscopic imaging.

Every two days, I split the cell cultures to reduce the cell population and thus prevent overcrowding and resulting apoptosis activation— ⅔ of the cells were discarded while ⅓ of cells were maintained in order to keep pace with the cell cycle (HeLa mitosis occurs approximately every 24 hours). This also would allow me to add fresh MEM to the petri dish to the cells that I would continue culturing. In order to split the cells, I worked under a sterile hood to avoid any contamination. First, it was necessary to aspirate all the existing MEM in the dish, and add 5 mL of phosphate buffer saline (PBS) to remove the media serum for the subsequent trypsinization. After washing the plate and aspirating the PBS, I added 5 mL of trypsin to the cell plate. I then incubated the plate at 37 degrees Celsius and 5% Carbon Dioxide for 15 minutes, and subsequently examined the cells under a 10x microscope to ensure that all the cells have successfully been removed from the plate. If the trypsin hadn't taken effect, another 5 minutes of incubation time would typically guarantee that the cells had detached. Next, I added 10 mL of fresh MEM + 10%FBS +1%Penicillin solution to the trypsinized plate, and suspended the cells by repeatedly pipetting media in and out onto the plate for approximately 5 iterations; the force of the pipette extrusion ensures that cells are not all lying at the bottom of the plate. Then, I took

5 mL of suspension solution (⅓ of cells, assuming an even distribution of cells after manual suspension), and transferred it to a new plate, subsequently adding 20 mL of extra media to this new plate in order to supply sufficient cell nutrition. The remaining 10 mL of suspension in the other plate were either discarded or distributed to other workers at the laboratory. It was important to keep track of the passage count, to avoid changes in the cells that occur past a certain number of cell cycles; the ATCC, the cell provider, does not recommend over 2 months of cell culture. The HeLa cells I used in my study were passage 7 by the printing stage, approximately 14 days old, and well within the 2 month time limit.

3-D bioprinting requires the use of a sacrificial printing matrigel to encapsulate the cells in a structure until cells form an ECM for self sufficient support. I tested two different types of matrigel to maximize print quality (without infusion of cells). Preparing 4 varying concentrations for both Gelatin and Pluronic F127, I tested print quality qualitatively for each sample. The concentrations for gelatin were 10%, 7.5%, 5%, and 2.5%, and 40%,35%,30%, and 25% for Pluronic F127 (Pluronic requires higher concentrations of material for solidification). I

	1	2	3	4	5	6	7	8	9			
A	40	35	30	25	20	15	10	5	0			
B	40	35	30	25	20	15	10	5	0	Empty		
C	40	35	30	25	20	15	10	5	0			
D												
E												
F	Empty											
G												
H												

initially determined the quality on a yes/no basis—whether or not the matrigel was able to sustain a hollow cylindrical shape without support material. A secondary measure of quality was assessing the consistency in diameter of filament extrusion; gel that extruded messily and inconsistent in size have less structural integrity than gel that extrudes in smooth, constant

diameter. Prints were assessed with both .100 mm and .203 mm inner diameter 1'' long syringe tips.

I also tested the effect of gel concentration on cell viability. It is clearly important that the cells survive in the matrigel for the purpose of drug compound testing, and it is necessary to try to maximize—or at least find a balance between—print quality and cell viability. To do this, I took advantage of the high throughput screening technology available in the laboratory. The experiment consisted of 3 trials of 9 concentration samples. I deposited 300 μL of matrigel solution into 3 rows of 9 wells, as shown in the plate map *Figure 1*. Each column was constant in concentration (3 trials of each concentration) and was 5% less in concentration than the column of wells immediately to its left. I first added gel itself to the wells, cooled to 4 degrees Celsius by storing the plate in an ice bucket under the hood to prevent the Pluronic F127 from solidifying (Pluronic is, for most concentrations, solid at room temperature and liquid below room temperature). I then added the appropriate volume of media to each well (increasing this volume with each column to decrease the gel concentration). I had calculated the volumes of gel and media I needed to add to each plate to achieve the 9 distinct concentrations I needed. The next step was to add a constant population of cells to each of the 27 wells. Unfortunately doing so had the side effect of slightly diluting the concentrations in each well, but only by a fraction of a percent— the overall point of the experiment was to find a general trend, and since each well was diluted by the same amount, the pattern would not be affected. I deposited 1000 cells/10 μL of media-cell solution into each well; I had determined this concentration of cells by using a cell counter. After one day of incubation, the plate was processed through a high-throughput imaging machine, and I used the Opera software in order to analyze the assay.

Next, I used this ideal bioink concentration to begin bioprinting. I used the Biobot 1 pneumatic extrusion bioprinter, along with the Biobot 1.5 software to control and calibrate the printer. In order to create the file for printing, I created a 3mm diameter, 3mm high solid cylindrical .STL file in the open-source Onshape online CAD software. I then used the Slic3r program in conjunction with Repetier Host Version 1.02 in order to create a G-code file. Repetier Host converted the stl file to the G-code, while Slic3r provided Repetier with information regarding the 2-D slices (E.g. thickness of the slices, type of infill, etc.). The G-code is a sequence of positional 3-D coordinates for the stepper motors to follow as they move the print extruders. After calibrating and printing the bioink in a 96 well plate, the plate containing the print-job was incubated for 24 hours at 37 degrees Celsius and 5% Carbon Dioxide. This concluded phase one of the study.

Phase two of this study began with the actual imaging of the structures created in phase one. I repeated the high-throughput screening process used earlier for the concentration testing experiment, producing 86 images 0μm to 1720μm with a step of 20μm. To test the potential utility of these images, I developed a 3-Dimensional cell analysis algorithm.

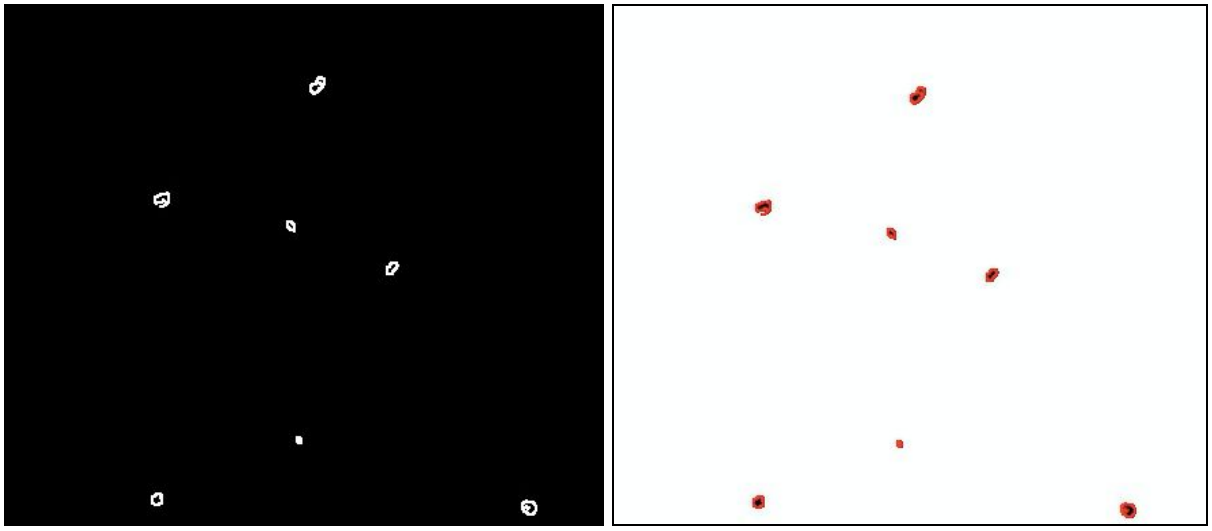
The algorithm was coded in Python 3.5, and used the OpenCV 2 library. The goal of the program was to process sets of 2-D images and determine cell number, size, and position in 3-D. The algorithm utilized the combination of the flood-fill and canny edge detection algorithms in order to segment cells and determine. First, the code converts the image to a grayscale (to avoid having to analyze complicated sets of (R,G,B) values), and subsequently uses the canny edge to determine sharp changes in the gradient of pixel values [10]. The edge detection marks any points with gradients above a certain threshold, as shown in the equations below. In addition,

$$M(n,n) = \sqrt{g_m^2(m,n) + g_n^2(m,n)}$$

before the actual gradient calculation process, canny edge uses a Gaussian filter to reduce the

$$M_T(m,n) = \begin{cases} M(m,n) & \text{if } M(m,n) > T \\ 0 & \text{otherwise} \end{cases}$$

noise of the image and subsequently avoid any "false edges" or unnecessary edges that aren't part of the main edges of objects the algorithm is looking for. Ultimately the canny edge process produces a binary black and white image (where the black is background and whites are edges as shown in the first panel of *Figure 2*).



In the next step, the white edges are colored red, and a recursive flood-fill algorithm is executed in the background, coloring all black pixels in the background white. This leaves only the cells themselves colored black. The program then iterates through the pixel array of this entire image searching for black pixels, and flood filling any areas where they are detected. In doing so, the program flood-fills individual cells, and at each flood-fill location, the program is able to measure the area and number of cells.

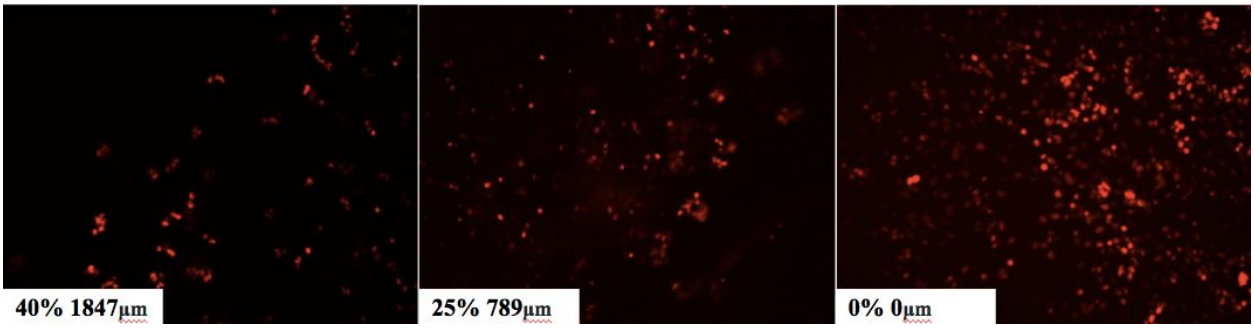
The position is not so easily found, due to the fact that images are slices of a 3-D structure. The program must be able to determine the z position of each cell. To accomplish

this, the algorithm crops sections of image slices above and below the image being analyzed. It then repeats the canny edge and flood-fill algorithms on these cropped images, and determines the area of the cell slices. If the area of the current cell slice being analyzed is greater than the area of cell slice above and below it, the program determines that this slice is the z position of the cell, and marks the position. This process negates the possibility of overcounting cells for each slice of a cell. The only scenario in which this algorithm would fail is if a cell happened to have two local maxima in cell width (if the cell was not shaped as a paraboloid).

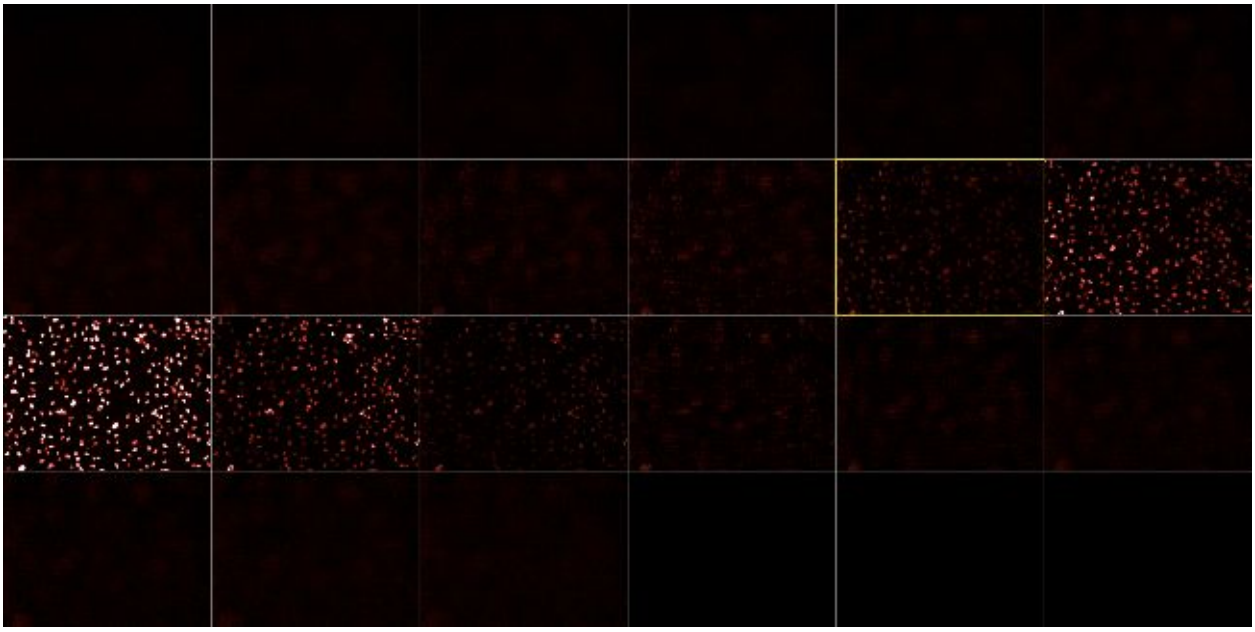
Results

The optimization of print quality required systematic trial and error. Given the infinite permutations of various parameters for printing, it was difficult to determine the best set of parameters. The clearest result was that pluronic print quality is superior to that of gelatin. This is because pluronic has a more suitable viscoelasticity for extrusion printing than does gelatin. Whereas pluronic tends to undergo extrusion at a relatively constant diameter, gelatin does so at various diameters often clogging the printer nozzle or forming clumps before being extruded in large masses. In addition, gelatin from 5-10% concentration in PBS solution could not be extruded at all out of syringes with inner diameters of 0.3 mm or less. Owing to these limitations, I determined that pluronic gel was the only bioink feasible to be printed in the small holes of 96-well plates, in which great precision is necessary.

Tuning the exact concentration of Pluronic was a more difficult task. As expected, an increase in Pluronic concentration resulted in an overall decrease in cell viability. *Figure 3*



shows the cell viability at 3 different concentrations (of the 9 total tested). The images shown were selected because they showed the clearest images of cells at all depths in the local depth



range of 0 to 2000 μm. As depicted by *Figure 4*, which is an array of images of a single-concentration sample taken at various depths of the solution in the well, the image on the third row and first column are the depth at which the cells primarily dropped to within the solution.

Two hypotheses can be inferred from these images. First, a higher concentration of pluronic allows for less cell diffusion through the solution; the cells fell to a depth of 1847 μm above the bottom of the well for the 40% solution, while falling straight to the bottom of the well for the 0% solution. Second, a higher concentration of pluronic is likely to result in lower cell viability. In order to test this second hypothesis, which was of importance to the study, I plotted the number of viable cells in order to further examine the correlation using all 27 wells.

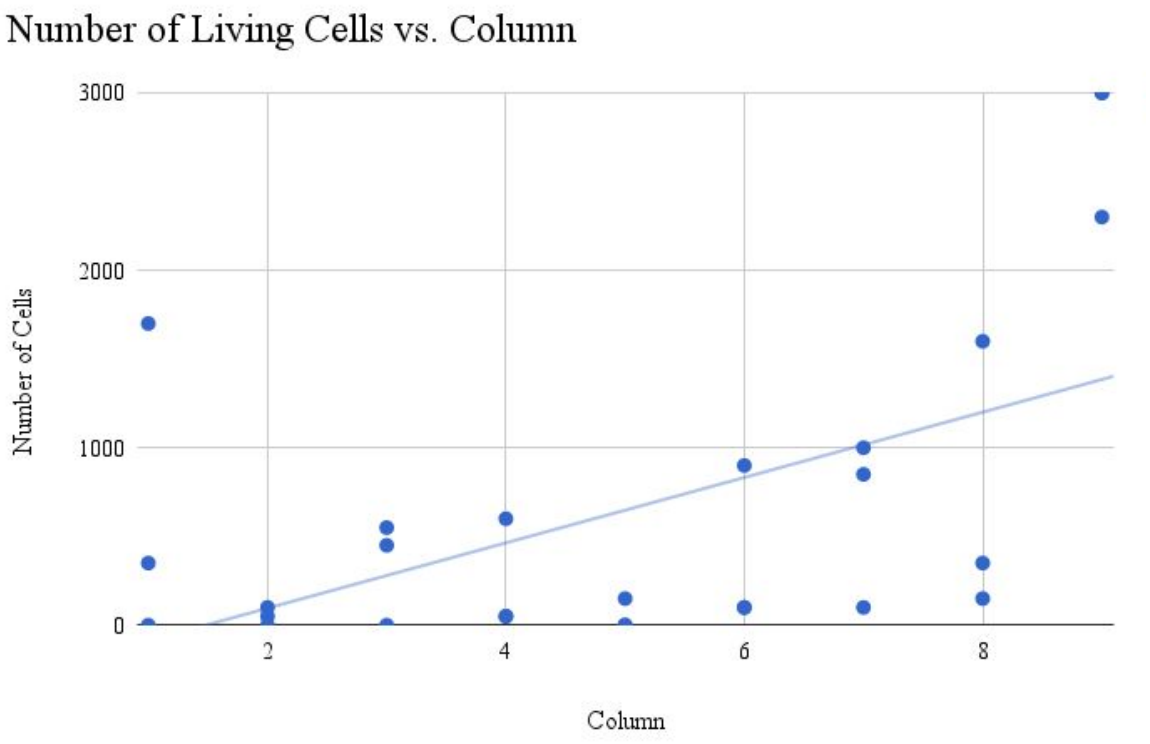
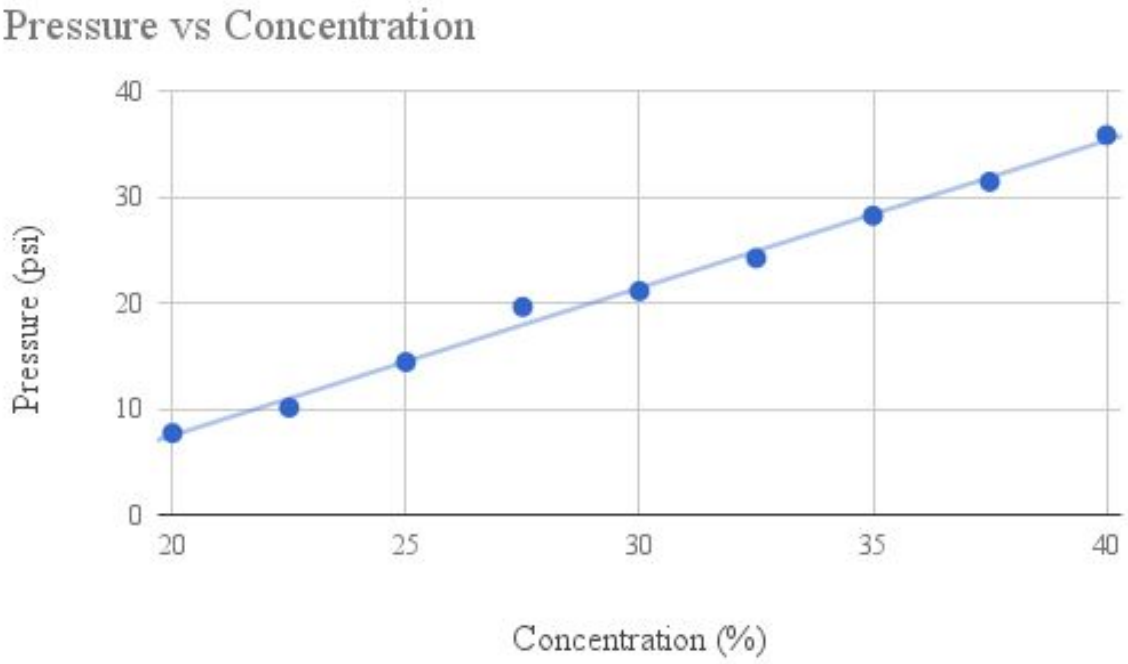


Figure 5 shows a low correlation (R-squared = 0.302) between the matrigel concentration (which increases in tandem with the column number) and cell viability (the number of cells). It is noteworthy that cell viability dropped off heavily for concentrations higher than 5%.

This correlation presented a cost-benefit analysis problem. A sufficiently high concentration is necessary to achieve a suitable print quality (any pluronic concentration lower than 20% would not produce a cylindrical structure that could maintain its shape for over 24 hours). However, excessively high concentrations would result in a high rate of cell death. As a result, I was constrained to use the lowest possible concentration of 20% in order to ensure a degree of HeLa cell viability. In sum, I determined that a pluronic F127 gel of 20% concentration in PBS solution was the optimal bioink for this solution.

Other printing parameters that I needed to address were the pressure and temperature settings of the Biobot 1. From qualitative observations, a high pressure resulted in large globs of

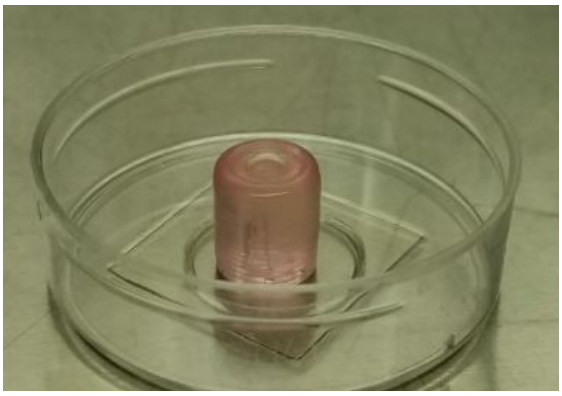
gel extruded at varying diameters, whereas low pressure settings would fail to create a constant



stream of bioink extrusion. As the gel concentration increased, the pressure required for the ideal print quality increased (*Figure 6*). Although this was a qualitative assessment, there was a strong correlation between concentration and the pressure parameter (R-squared = 0.944). Temperature did not contribute significantly to print quality. As long as the temperature was not excessively high or low, and was anywhere from 23°C to 28°C, the pluronic maintained the optimal viscoelastic state. It was also necessary to test various G-code file structures to create a structurally sound model. The ideal printing parameters for the Slic3r program were as follows: layer height, 0.3 mm; fill density, 20%; infill and perimeter speed, 10 mm/s; travel speed, 130 mm/s; and number of solid layers on the top/bottom of the printbed, 3.

By optimizing these parameters, I was able to produce a high-quality cylindrical print in a petri dish (*Figure 7*). Using the 96-well plate mode on the Biobot 1 was a success, and I was

able to print 96 copies of the cylindrical structure into the well plate for image analysis in three hours and seventeen minutes. Although this is a long time for cell assay preparation, the print-time could be drastically decreased by printing a shorter cylinder (the cylinders printed in



the 96-well plates had a height of 7mm). Because confocal screening images layers at micron-level precision, 7 mm would be unnecessary for most screening applications.

The image processing algorithm described above was initially faulty. The canny edge algorithm often failed to create a closed border, and as a result, the flood-fill algorithm filled in the cells in conjunction with the background. In a sample of 10 randomly selected slices, the algorithm identified 33 of 42 cells (78.6% accuracy). To address this problem, I implemented a step that dilated the white edges of the canny edge. The rationale for this step was that by thickening the edges, many of the previously open cells would close. For the same sample of 42 cells, this improvement increased the position-identification accuracy to 40 out of 42 cells (95.2% accuracy). When the x and y positions were correctly determined, the algorithm to calculate the z position was 100% accurate (there were no abnormal, non-paraboloid shaped cells as expected).

Discussion

This proof-of-concept study is significant because, although there are studies that have described 3-D cell culture preparation methods (e.g., spheroid shaped cultures described in the Introduction) for high-throughput screening, studies that examine the feasibility of using extrusion-based bioprinting are lacking. Other papers, including the research by Fang and Eglen [12] and Ventola [1], describe the general applications of 3-D bioprinting in the pharmaceutical drug discovery industry, without describing in detail the necessary steps and parameters required to quickly and precisely prepare 3-D assays for screening. Optimization of parameters is a huge barrier that must be overcome for bioprinting, and this study outlines exactly how cell assays should be prepared.

The sub-experiment that tested the effect of Pluronic concentration on cell viability did not show a strong correlation, it did show a noticeable drop off in cell viability for concentrations higher than 5%. Given that 20% is the minimum concentration required for printing, these results show that Pluronic may not be the optimal bioink to be used as the supporting matrix.

Another point of inquiry in this study was whether or not the confocal microscope imaging would be able to clearly image cells in the matrigel matrix. The clarity of images is extremely important for drug image analysis, since many segmentation algorithms fail to produce accurate data analysis if images are blurry. This study showed that matrigel infused with cells could be imaged relatively clearly. This addresses the concerns raised about imaging 3-D structures in previous papers. For instance, the CiGiP method described by Deiss et al. [10] was developed to avoid labor-intensive, low-throughput micro-dissections required to isolate slices of 3-D structures for 2-D analysis: hence, this method implements a stack of easy-to-disassemble papers impregnated with cells. However the use of confocal microscopy for

high-throughput imaging avoids the hassle of disassembling any 3-D structures whatsoever, and slices of printed cells can be easily imaged with high quality. Because speed and simplicity with which printed structures can be analyzed are vital for screening, the manually laborious task of imaging slice by slice could severely hinder the progress of drug discovery.

There is no doubt that a degree of accuracy and precision is lost when imaging a 3-D assay in its entirety as opposed to imaging its individual slices. However the results show that the accuracy lost is well worth the time saved by using high-throughput confocal imaging. From a qualitative standpoint for example, it is worth noting that a 3-D model of a subsection of the matrix could be created using the velocity program successfully (*Figure 7*). This 3-D modeling capability shows the feasibility of analyzing the effect of drug behavior on a 3-dimensional, high-throughput scale. The high accuracy rate of the image processing program (95.2%) also shows that it can readily measure characteristics such as cell morphology, which are necessary for drug/toxicity assessments.

Conclusions

In this study, I successfully engineered a 3-D cylindrical structure that could be easily printed into 96-well plates, and showed that the images produced by confocal microscopy in a high-throughput screening device were of sufficient quality for computer analysis. However there were a few drawbacks to my design.

First, the cell viability was a major problem in this study; I was not able to meet the goal of producing a maintainable model. After two days of incubation, all cells in the matrigel structure had died. One workaround for this problem would be to simply prepare the Pluronic

solution with cells after infecting the cells with a virus during the drug screening process. This way, there is enough time for the viruses to either succeed or fail in infecting cells in the presence of the inhibitor compounds being tested. Still, ideally most drug screeners would like to have a sustainable 3-D model that could be analyzed over a period of weeks. For successful application in drug discovery, this viability issue must be addressed.

In addition the concentration vs. cell viability mini-experiment answered questions posed by a recent study conducted in 2016 on Pluronic tissue engineering by Gioffredi et al [13]. Gioffredi and her team were unable to deduce the long term effects of Pluronic concentrations on cell viability because their Pluronic 3-D constructs could not maintain a solid state of matter when incubated with media. My experiment shows a moderately clear trend that higher concentrations indeed reduce cell viability. While the concentrations prepared in my experiment were not actually printed, both my work and Gioffredi et al.'s work suggest that the bioprinting process has no effect on viability, so this variable can be excluded.

The second drawback of this study is the fact that I used HeLa cells. These cancer cell lines cannot be equated to normal human cells. For instance, HeLa cells undergo apoptosis under polo-like kinase 1 depletion, while normal cells survive this phenotype [14]. The fact that HeLa cells were used for this study also draws more concern to the viability of cells printed in the matrigel; if cancer cells have difficulty surviving, it isn't likely that normal cells will survive longer. To improve upon this study, a bioink other than pluronic should be tested to optimize cell viability and print quality.

If possible, I would have liked to continue my study at the institute by infecting cells, and creating a separate segmentation algorithm that was able to identify both viral and cell channels,

and produce information concerning cell viability. I also would have repeated a concentration experiment with gelatin, to see if cells could survive more readily in gelatin than in pluronic. These findings establish a solid foundation for identifying the ideal bioink--a key question in the field of drug discovery. Once this is accomplished, it will be possible to address various other questions. What is the effect of the matrigel matrix on cellular growth? Will cells eventually be able to develop an extracellular matrix and maintain structure independent of the sacrificial gel? Can hydrogels have an effect on a viral strain's ability to infect cells? When solutions to these issues are found, bioprinting may greatly expedite the process for finding truly effective drug compounds.

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