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DEVELOPING A LOW COST BIOLOGICAL ADDITIVE MANUFACTURING SYSTEM FOR FABRICATING GEL EMBEDDED CELLULAR CONSTRUCTS.

Justin Stewart Minck
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DEVELOPING A LOW COST BIOLOGICAL ADDITIVE MANUFACTURING SYSTEM FOR FABRICATING GEL EMBEDDED CELLULAR CONSTRUCTS

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Justin Stewart Minck
June 2019
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Approved by:

Dr. Nicole Bournias-Vardiabasis, Committee Chair, Biology

Dr. Michael Chao, Committee Member

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ABSTRACT

Organ transplantation has made great progress since the first successful kidney transplant in 1953 and now more than one million tissue transplants are performed in the United States every year (www.organdonor.gov/statistics-stories, 2015). However, the hope and success of organ transplants are often overshadowed by their reputation as being notoriously difficult to procure because of donor-recipient matching and availability. In addition, those that are fortunate enough to receive a transplant are burdened with a lifetime of immunosuppressants. The field of regenerative medicine is currently making exceptional progress toward making it possible for a patient to be their own donor. Cells from a patient can be collected, reprogrammed into stem cells, and then differentiated into specific cell types. This technology combined with recent advances in 3D printing provides a unique opportunity. Cells can now be accurately deposited with computerized precision allowing tissue engineering from the inside out (Gill, 2016). However, more work needs to be done as these techniques have yet to be perfected. Bioprinters can cost hundreds of thousands of dollars, and the bioink they consume costs thousands per liter. The resulting cost in development of protocols required for effective tissue printing can thus be cost-prohibitive, limiting the research to labs which can afford this exorbitant cost and in turn slowing the progress made in the eventual creation of patient derived stem cell engineered organs.
The objective of my research is to develop a simple and low-cost introductory system for biological additive manufacturing (Otherwise known as 3D bioprinting). To create an easily accessible and cost-effective system several design constraints were implemented. First, the system had to use mechanical components that could be purchased “off-the-shelf” from commonly available retailers. Second, any mechanical components involved had to be easily sterilizable, modifiable, and compatible with open-source software. Third, any customized components had to be fabricated using only 3D printing and basic tools (i.e. saw, screwdriver, and wrench). Fourth, the system and any expendable materials should be financially available to underfunded school labs, in addition to being sterilizable, biocompatible, customizable, and biodegradable. Finally, all hardware and expendables had to be simple enough as to be operated by high school science students.
ACKNOWLEDGEMENTS

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Tissue Transplant

The earliest crude examples of tissue transplants utilized xenografts. Tissue, commonly animal hide, would be grafted onto a patient who suffered deep and extensive tissue damage. Later, human cadaver tissue would be used in place of animal hide. This alternative practice is still common, as many transplanted tissues are typically sourced from organ donors. Animal tissues are finding their way back into medicine through xenotransplantation, with the development of immune compatible chimera organs, decellularized animal organ scaffolds, reseeded with patient derived stem cells.

While promising, these methods still have drawbacks. In the case of human organ donor-sourced transplants there remains an insufficient supply of donors to meet the demand for tissues (Figure 1). Although animal derived tissues may provide a solution to the high demand, they also come with their own set of problems which include differences in morphology, greater risk of rejection, and availability (Watson, 2011). Even with chimeras or with decellularization and reseeding methods, patient rejection of the tissue is still a possibility. Both methods would likely fall short of meeting current demand or remaining cost-effective, making their ability to replace or supplement current transplant methods unlikely.
Cell Culture: Then and Now

In 1907 Ross Harrison developed the first protocol for first 3D cell culture and was able to cultivate cells via the Hanging Drop method (Harrison, 1910). In 1916, Peyton Rous and F.S Jones developed a technique for removing adherent cells via trypsin enzyme. In 1948, Earle was able to isolate the first fibroblast cell lines. The first chemically defined media was developed in 1955 by Harry Eagle and is still in wide use today. This project uses a variant of the original Eagle Medium known as Dulbecco’s Modified Eagle’s Medium (DMEM). In 1962, Dr. George Todaro and Dr. Howard Green developed the 3T3, or three-day transfer, mouse fibroblast cell line which is now considered the standard fibroblast line (www.nih3t3.com, 2017). Isolation of the first pluripotent stem cell lines can be credited to Martin Evans and Gail Martin, who in 1981 successfully derived pluripotent stem cells from mouse embryos (MESC). Embryonic stem cell lines

Figure 1: Global Organ Transplant Shortage.
yielded many advancements in the field of medicine however were subject to controversy ultimately research was subsequently restricted in 2001 by the Bush administration. In 2006, Dr. Shinya Yamanaka and his team were able to induce successful pluripotency in mouse cells, creating the first induced pluripotent stem cells (iPSCs). The benefit of iPSCs is that they are easily obtainable terminally differentiated cell samples which can be reprogrammed back into a stem-like state and then differentiated toward a specific lineage. This method when combined with 3D printing certainly has the potential in the clinical setting to produce patient-compatible tissue constructs.

Researchers have clearly made huge advances in cell culture; There remains two major hurdles in the clinical applications of cell culture. The first is the difficulty of culturing large quantities of stem cells suitable for use in treatments. Currently cell sorting and bioreactors seem to be the most promising technologies to overcome this obstacle (Mironov, 2011). The second obstacle is controlled differentiation. Controlled differentiation requires manipulation of the intracellular and extracellular chemical environments over time, as well as the mechanical microenvironments. 3D printing technology has the potential to resolve these issues, as chemical and mechanical gradients can be introduced into the construct’s design. This would emulate the natural environmental signals that normally guide stem cell differentiation in vivo.

Additive Manufacturing (3D Printing)
The earliest roots of modern 3D printing date back to the 1950s with the advent of inkjet printing systems. The first patent for a 3D printing system was filed in 1984 by Charles Hull. The system used a vat of UV sensitive photopolymer with a Z-axis platform; a UV laser activates a photo initiator that in turn crosslinks molecules in polystyrene resin. This occurs layer-by-layer as the Z-axis platform submerges into the vat (www.3dsystems.com/our-story, 2017). The systems were expensive, unreliable, and time-consuming, but could convert a 3D computer model into a physical model overnight. The process was named Stereolithography, or SL[A]. Charles Hull went on to launch one of the first major manufacturers of today’s 3D printers, 3D Systems.

In 1986, Scott Crump developed a method called fused deposition modeling (FDM), using a modified hot glue gun to extrude molten plastic (Horvath, 2014). This system proved to be the forbearer of most 3D printers to come. In FDM, molten plastic is extruded from a heated tip mounted to an XYZ platform, allowing it to create 3D objects. Crump went on to launch Stratasys Systems, the largest 3D printer manufacturer today (Savini, 2015).

In 2005, Adrian Bowyer founded the Replication Rapid Prototyper (RepRap) project. His goal was to develop an open-source 3D printing system capable of self-replication. Project RepRap went on to birth the modern home 3D printing industry, as it allowed for low-cost printers to be built at home. One of its descendants, MakerBot, has become the gold standard of affordable home 3D printers. The RepRap project made use of Arduino modular computers and FDM
printing techniques to make 3D printing an affordable alternative to traditional subtractive manufacturing. Presently, 3D printers are no longer limited to plastics -- they can print glass, metal, sugar, clay, and even cell-infused gels for bioprinting applications (Goldberg, 2017).

3D Bioprinting

Bioprinting is at the cutting edge of engineering and biology. The process combines Computer Aided Drafting (CAD), additive manufacturing (3D printing), and tissue culture to produce living, three-dimensional structures. The history of bioprinting is relatively short. In 1999, Dr. Anthony Atala successfully used a 3D printer to print scaffolds. These scaffolds were seeded with living cells, creating an artificially engineered bladder. Later in 2003, Thomas Boland developed the first method for modifying an inkjet printer for cell distribution (Murphy, 2014). In 2009, the company Organovo began development of the NovoGen MMX Bioprinter (Figure 2), one of the first purpose-built bioprinters. The same year, researchers at Organovo successfully integrated vasculature into printed structures. This advancement

![Figure 2: NovoGen MMX.](image_url)
was important as media diffusion limits the thickness and size of bio printed constructs (www.organovo.com/about/history/, 2017).

The issues of controlled cell differentiation and large-scale proliferation are not the only things limiting the clinical applicability of cell culture. Equipment costs are another obstacle because purpose-built bioprinters are exceedingly expensive, often costing in the six-figure range. In 2015, a startup company called BioBots rolled out the BioBot 1, the first purpose-built bioprinter based on technology developed for low-cost desktop 3D printers, but even this system retails for $10,000 (www.biobots.io/, 2017). As of 2018, the bioprinter market has grown extensively as several commercially available bioprinters exist with the cheapest being the direct injection Rebel Series by SE3D for $4,000 (Figure 3) (https://www.se3d.com/,2018).

The objective of this research is to develop a bioprinting process using a common, low-cost, 3D printer modified with 3D printed, and store-purchased components. Any printer modifications made must also be simple enough that a person with no technical experience can perform them. The bioink used in this process will allow for cost-effective bioprinting optimization. By reducing the

Figure 3: SE3D Rebel Mini.
hardware, and overhead costs of this technology I hope to make it available to Biology students at the high school and college level. Providing introductory students meaningful STEM research experience.
CHAPTER TWO

MATERIALS AND METHODS

3D Printer Modification

The printer chosen for this project was the Creator Pro dual-head desktop 3D printer by FlashForge. This printer was chosen for several reasons. The printer is one of the lowest-cost dual head printers on the market. This fulfilled one of the primary goals of the project as the unit cost was under $1,000. The benefit of having a dual head printer instead of a single head printer was that not only could the printer print using two different materials simultaneously without the need to switch syringes, but the printhead carriage and supporting XYZ drive system was larger and more robust. A major office supply retailer carries this product in store, thus fulfilling the goal of being easily obtainable as the machine could be picked up locally without pre-order and added shipping costs. The printer itself was already fully assembled out-of-the-box and is known as a MakerBot clone. MakerBot is currently the most popular model of home desktop 3D printers. MakerBot clones can make use of some of the MakerBot legacy software and hardware options. Overall this compatibility helps the printer meet the third and most important goal of easy modifiability. The Creator Pro was also chosen over lower-cost machines as it was fully enclosed with a steel frame and has a full, removable, external enclosure -- thus making sterilization easy and the steel frame providing more structural integrity (www.flashforge-usa.com/creator-pro, 2017).
The printer is nearly fully assembled out-of-the-box, requiring only the addition of screw-on filament spools that contain the ABS printing plastic. This model printer also comes fully enclosed with a removable plastic shell, which could potentially be modified to act as a sterile cabinet, although for the purposes of this project these outer panels were removed. Removal of the outer panels was relatively straightforward as the printer comes with the necessary wrenches to remove any of the screws. Once the outer panels were removed, the printhead assembly then had to be unscrewed, and the filament extrusion motors as well as thermal sensors had to be unscrewed from the printhead assembly. The extrusion heads, heatsinks, and cooling fans, as well as printhead chassis, were then discarded. The thermal sensors and unneeded wiring were then detached from the harness and conduit tube. The sensors were screwed into an unused socket and the excess wiring was then coiled up and zip-tied under the printer XYZ chassis. The conduit tube was then removed to help mitigate the possibility of sterilization issues. The X-axis touch sensors were removed from their clip-on housings and secured via hot glue to a thin 2 cm x 3 cm ABS card. This card was then slid back into the clip effectively extending the touch sensor beyond the XYZ chassis frame and preventing the bio printhead from contacting the chassis frame. This modification was later reversed after the integration of an extended contact bracket on the PSP2-B and later bio printhead variants. Lastly, the build platform was dropped approximately 5 mm via the lowering wingnuts, which are typically used to level the platform during initial setup. This adjustment allowed
the printhead to have a longer extrusion tip as well as provided room for the petri dish bottom (Figure 4).

Figure 4: Creator Pro 3D Printer.

Development of 3D Printable Syringe Pump Printhead

Typically, commercially built bioprinters use a pneumatic system to dispense bioink material. Due to the difficulty of developing a low-cost or easily constructed pneumatic delivery system, a screw driven bioink delivery system was given consideration. Screw drives are more precise and reliable than pneumatic systems, hence their heavy use in traditional 3D printing. However, screw drives have less pressure control and as such may cause additional shear forces on the embedded cells (Malda, 2013).
Initial development was relatively straightforward. Measurements were taken of the original printhead assembly, taking careful note of the size limitations, as well as the mounting points and center points for the original extruders. A single-piece 3D printable syringe pump was designed (PSP1). The PSP1 (Figure 5) was a dual-head extruder that utilized two linear actuator five-phase stepper motors. These motors were ordered from www.sparkfun.com. These motors drive a 20 cm threaded rod through the core of the stepper motor with the intention of putting force on the plunger of inserted disposable 5 ml syringes. The PSP1 was designed to occupy the same position as the original 3D printhead, using the same mounting points and hardware. The PSP1 accommodates both 2 mL and 5 mL syringes, with grooves and notches placed to secure firmly the syringe in place at precisely the center point of the original extrusion head. Mounting points were also designed into the PSP1 to allow for the linear actuators to be easily mounted above the syringe plungers.

Issues with the PSP1 were apparent rather quickly. First, the design required the use of support material that did not come with the 3D printer. Second, measurements used
in the design did not consider a notch in the XYZ carriage, which resulted in the syringe pump needing to be trimmed down via a rotary tool by Dremel to fit into the carriage. Third, the force required to compress a gel-loaded syringe was more than the linear actuator could supply. The syringe pump was also heavy even after having been printed using a honeycomb or “low infill” setting plus a large internal cavity. The syringe fitting was also unsatisfactory as it was difficult to load and unload syringes without risking tip contamination.

The PSP2

The PSP2 was designed in response to these challenges. The PSP2 was significantly reduced in material and designed with flat printing surfaces in mind, thus removing the need for printing of support material. The PSP2 (Figure 7) also

Figure 6: Flashprint Software Adjustments.
better considered the profile of the original printhead, allowing a snug fit into the
XYZ carriage while using the original attachment points. Instead of a direct
compression system, a screw drive system was used, similar to how
conventional syringe pumps operate. A 10 mm, metric-threaded steel rod was
purchased from Home Depot along with 10 mm hex nuts. The five-phase stepper
motors from the original 3D printhead were salvaged and mounted via designed
attachment points to the bio printhead. Steel couplings also purchased from
Home Depot were used to join the shaft of
the stepper motor to the threaded rod. 3D
printed compression plates were designed to
slip over the hex nut and threaded rod and
ride on a pair of 10 cm ¼-inch aluminum
rods. These rods were also purchased from
Home Depot and hacksawed to size just as
the threaded rod was. The PSP2 was then
able to be secured into the XYZ carriage,
and the stepper motors could be
reconnected to the 3D printer control board.
The syringe ports were made to fit only 5 mL
syringes, with a larger lower opening still
centered where the original extruder was but
able to be easily loaded and unloaded.
without risking contamination. Subsequent testing of the PSP2 revealed two major design issues. One, the printhead was top-heavy and prone to print tip vibration during the printing process. This instability resulted in substantial loss of print quality. Only rough test-prints could be created as the printhead vibration caused bioink layer deposition inconsistency and subsequent layer delamination. Two, the printhead extrusion mechanism, or a combination of both the printhead extrusion tip and the syringe pump compression mechanism, suffered decreased print resolution. Stepper motors driving the syringe pump had difficulty driving the threaded syringe compression rod as bioink had to be of firm gelatinous viscosity to achieve usable print quality. The printhead extrusion tip itself also needed to be of high gauge in order to maintain print resolution, although the 26-gauge resolution of the original plastic extrusion printer hardware could not be matched as driving the bioink through this gauge was not possible at the required viscosity. These issues were addressed in variants of the PSP2 printhead design.

PSP2-A attempted to correct for this printhead instability by reinforcing the lower portion of the printhead with additional material laterally along the printhead interfaces to resist X-axis movement forces as well as by introducing a forward strut to resist Y-axis movement. These modifications combined with the replacement of the standard-thread compression drive rods with fine metric-thread drive rods resulted in negligible improvement in print quality as testing revealed the guide rods and rocking of the printhead mount itself were causing
much of the printhead instability. Software settings were adjusted in attempt to compensate for the printhead instability by reducing the printhead travel speed by 90% (Figure 6). However, this adjustment still yielded unsuitable test prints as well as further worsening the issue with bioink extrusion quality as the gel would become too viscous for extrusion during the printing process, resulting in unstable bioink flow and frequent tip blockages.

The PSP2-B (Figure 8) was redeveloped in attempt to further mitigate the previously mentioned issues. The most notable modification to the printhead was the use of zigzag supports in place of the solid 15% infill honeycomb lower printhead attachment. This change was done to reduce overall material weight without sacrificing structural integrity. The decreased weight would presumably reduce the strain of the print sled and XY bearings.

Additionally, a 4 mm extension of the sled mounting surface of the PSP2-B provides greater support, as earlier printhead variants had a notable wobble in the Y direction, which could presumably be mitigated using an

Figure 8: PSP1, PSP2, PSP2-A, PSP2-B.
additional supporting lip, thus allowing the printhead to be better supported on the XY shelf. Beyond these stability enhancements, the PSP2-B was given an L-bracket protrusion on its lower-right surface and a reduced mid-section cross plate. These modifications allowed the PSP2-B to make contact with the X-axis positional switch without the need for additional modification of the 3D printer’s existing X-axis switch as was required in earlier printhead variants. The mid-section cross plate reduction was an attempt to minimize the overall printhead weight as well as to minimize any accidental contact contamination during syringe loading. Ultimately the reduced weight and stability enhancements did not prove to be sufficient to stabilize the printhead nor to provide notable improvement in the print quality.

The PSP3

The PSP3 (Figure 9) was designed to eliminate the top-heavy instability of previous printhead designs. The direct injection method was abandoned after over a year of development ultimately because it was not feasible with the current hardware. Keeping this method would have required significant modification of the 3D printer XY chassis yet still would likely have problems with vibration in the XY axis print sled bearings. Additional likely problems might include flexing in the steel guide rods as a result of the bio printhead’s large stepper motors’ being mounted to the top of the syringe pump assembly, resulting in instability and poor print quality. Even with software modifications
Involving 90% reduced printhead travel speeds, test prints of simple circles were of insufficient resolution (Figure 6).

In the end, the PSP3 was designed with two components: a print chassis--mounted syringe pump and an XY sled--printhead syringe mount. The two components were based on earlier variants of the PSP2 designs, divided into two halves with the syringe pump mechanism being heavily conserved in the redesign and the stepper motor mounting plate being extended with additional mounting holes added to secure this component to the chassis. The second

**Figure 9**: Printed Syringe Pump 3.
component was created using the PSP2’s print chassis mounting plate and L-bracket X-axis contact. Additional modifications were included to provide luer lock connections for printhead tip stability as syringes were no longer directly mounted to the printhead, thus necessitating greater reinforcement to maintain tip stability.

This configuration puts the bulk of the printhead’s weight (i.e. syringe pump mechanism and stepper motors) onto the printer chassis, making use of mounting points formerly used by cable guides in the printer’s factory hardware setup. Screws were also repurposed to secure the syringe pump to the chassis. The printhead sled was designed to make use of the luer lock groove profile to provide additional support for the printhead itself while utilizing the PSP2-B’s XY sled mounting profile and a modified X-axis contact switch--extension concept.

Initially the extruded bioink from the syringe pump was carried through prepackaged sterile disposable IV extensions. These were hoped to minimize the cost and steps of sterilization required for bioprinting. However, the high gauge tubing and integrated valves used to prevent embolism created a bottleneck in the bioink injector flow. This bottleneck impeded bioink flow to the point where bioink of printable viscosity could not be extruded with the existing stepper motors. Ultimately, traditional, inexpensive polyvinyl ⅛-inch tubing combined with inexpensive, sterilizable, barbed, acrylic, male and female luer lock connectors provided a more stable bioink flow at the cost of additional sterilization steps.
The printhead component, while being highly conserved from the PSP2 models, was modified to have a barrel extension with V groove slots matching the luer lock connectors on the surgical tubing. This change allows a 3D printed V clip to be placed in the slot for improved syringe tip stability. Moreover, this design offers locking in the surgical tubing with a barbed acrylic luer lock, thus decreasing the likelihood of connection failure from positional strain on the tubing. Ultimately test prints with the PSP3 demonstrated the stability required for higher resolution bio printed constructs to be generated.

Initial Testing of PSP2 and PSP3

Initial tests as with the PSP2s focused on printing 1 cm rings (Figure 6), chosen for their ready ability to create simple, structurally sound shapes, in addition to their proclivity in cell culture tests for ready media diffusion into cultured cells. 120 mm polystyrene petri dishes were used to provide a removable, sterile build surface. The petri dish lid was affixed to the printer build platform with the application of a few drops of bioink as the capillary action between the build surface and petri dish secured the lid in place. The build surface was then manually adjusted on Z-axes to compensate for the thickness of the petri dish. With several rings printing successfully, a more complex final test print was chosen. The complex shape of a human ear was selected as its
thin structures would still provide media
diffusion while at the same time
demonstrating the bioprinter’s resolution
potential and outlining a shape which
envisions the system’s biomedical
possibilities.

As when doing the ring prints, the
PSP3’s reduced load on the print chassis
sled, without the weight of the stepper
motors, was able to move at a rapid pace with no notable speed wobble. Due to
this improved printhead stability, the print speed (i.e. bioink deposition) did not
require adjustment from machine factory settings. This rate allowed for small
complex structures such as the human ear (Figure 10) to be fabricated in bioink,
in 40-50 minutes. A few additional adjustments were required for the larger
printed construct of the ear, primarily the 120 mm polystyrene petri dish lid that
served as the build surface. This lid had to be scored repeatedly in a hashed
pattern (Figure 10) with a sterile spatula to provide additional friction for the
larger, more complex structures to adhere as not scoring it would cause sections
of the initial print to detach, producing overall construct deformity.

Additional user interaction was required during test prints as the print
platform required occasional leveling adjustments every 3 mm or so in the
vertical axis to compensate for increased bioink deposition when compared to

Figure 10: Human Ear Construct.
the printer’s original plastic deposition. This issue was discovered early on. As the print increased in height, vertical adjustments were required to maintain resolution and to prevent the printhead from contacting and disturbing the previously deposited bioink layers. Manual adjustment was required as the bioink deposition flattening, and temporal curing profile vary with the viscosity of the bioink.

Gelatin Bioink Development

Several different options were considered for the formulation of a bioink. One commercially available bioink distributed by BioBots is a proprietary blend based primarily on Poly (lactic-co-glycolic acid), or PLGA. Unfortunately, this synthetic, biocompatible and biodegradable polymer is also $200 per gram (www.biobots.io/, 2017). Other, more conventional tissue engineering materials such as Thermo Fisher's Geltrex or Corning's Matrigel require high concentrations for 3D cell culture and average around $50 per mL. Ultimately a gelatin-based bioink was selected as the most cost-effective candidate that was both highly biocompatible and mechanically suitable for use as a bioink. Gelatin when combined with the enzyme microbial transglutaminase MTGase creates a hydrogel that congeals at 37 °C. The resulting bioink costs less than $1 per mL (Zhao, 2016).
The gelatin first used was Gibco type B bovine gelatin as bovine gelatin is commonly available, and is the lowest cost. A 2% gelatin dissolved in 10% phosphate buffered solution was created. This mixture was then autoclaved for 30 minutes at 121 °C and allowed to cool to room temperature. MTGase (MooGloo RM) was purchased from Amazon.com. Several different concentrations were tested, including 5 mg/mL, 15 mg/mL, 30 mg/mL, and 60 mg/mL. These concentrations were then dissolved into the gelatin solution and incubated at 23 °C, 37 °C, and 60 °C. Concentrations of 15 mg/mL and 30 mg/mL were selected since lower concentrations produced gels with low viscosities. 60 mg/mL concentrations appeared to have similar properties to the 15 mg/mL and 30 mg/mL concentrations. Both refrigerated and 37 °C incubations produced gels, but 60 °C incubation appeared to denature the enzyme (Table 1).

**Table 1: Bovine Based Bioink Concentration Optimization.**

<table>
<thead>
<tr>
<th>Temp</th>
<th>MTG 5 mg/mL</th>
<th>MTG 15 mg/mL</th>
<th>MTG 30 mg/mL</th>
<th>MTG 60 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>23°C</td>
<td>Unstable</td>
<td>Unstable</td>
<td>Unstable</td>
<td>Unstable</td>
</tr>
<tr>
<td>37°C</td>
<td>Unstable</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
</tr>
<tr>
<td>60°C</td>
<td>Denatured</td>
<td>Denatured</td>
<td>Denatured</td>
<td>Denatured</td>
</tr>
</tbody>
</table>

30 minutes at 121 °C and allowed to cool to room temperature. MTGase (MooGloo RM) was purchased from Amazon.com. Several different concentrations were tested, including 5 mg/mL, 15 mg/mL, 30 mg/mL, and 60 mg/mL. These concentrations were then dissolved into the gelatin solution and incubated at 23 °C, 37 °C, and 60 °C. Concentrations of 15 mg/mL and 30 mg/mL were selected since lower concentrations produced gels with low viscosities. 60 mg/mL concentrations appeared to have similar properties to the 15 mg/mL and 30 mg/mL concentrations. Both refrigerated and 37 °C incubations produced gels, but 60 °C incubation appeared to denature the enzyme (Table 1).
Successful gels were then soaked in PBS over 72 hours to confirm they did not dissolve (Figure 11). Unfortunately, when the experiment was repeated with gels being submerged in DMEM culture media, the gel dissolved. Both integration into uncongealed gel, as well as submersion of completed gel, showed the same results. After further literature review, type B gelatin was found to have been processed using a different technique which resulted in chemical differences making it unsuitable for cell culture.

Corning type A porcine gelatin was then selected. Concentration optimization was repeated with the porcine gelatin with similar results. Ultimately 15 mg/mL and 30 mg/mL MTGase and 2% porcine type A 10% PBS solutions were shown to survive submersion in DMEM for 72 hours under 37 °C. Even after gel solution autoclave and careful sterile practices with the enzyme, bacterial contamination was quickly and consistently found present (Figure 12).

Autoclaving the completed bioink was impossible as earlier experiments and literature showed that MTGase would denature around 60 °C (Chen, 2014). A second supply of MooGloo was purchased with the same result. The enzyme was suggested to be dissolved into PBS and purified by filter sterilization. Unfortunately, MTGase proved to be too difficult to filter sterilize directly, as it
was too thick. Ultraviolet sterilization using a UV crosslinker at exposures of 10, 20, and 30 minutes was tested, but the contamination persisted. Several pre-filters were used from 40 μm, 2 μm, 0.45 μm, to 0.22 μm, which resulted in a significant loss of the initial solution. The MTGase solution was then centrifuged as a pre-filtration step. Centrifuge speeds of 5,000 RPM for 5, 10, and 15 minutes were tested, however, this did not result in a filterable solution. A centrifuge speed of 9,500 RPM for 20 minutes resulted in an MTGase solution that could be effectively filter sterilized with a 0.22 μm syringe filter.

Later bioink cost optimizations found that 3,000 RPM centrifugation for 60 minutes provided a supernatant that was filter sterilizable at speeds that could be obtained using conventional low-cost desktop centrifuges. In addition to cost optimizing MTGase filter sterilization, porcine gelatin sterilization was also cost optimized as 60 °C for 60 minutes of hot plate gel dissolution combined with filter sterilization, proving to be as effective as the previous autoclaving protocol and ultimately eliminating the need for laboratory autoclaving altogether. This finding further highlights the financial feasibility of this bioink for under-funded and or under-equipped laboratory facilities. The centrifuged MTGase solution and porcine gelatin solution were filter sterilized in hood.

The final developed bioink loading protocol required immediate filter sterilization of the gelatin into a 12 mL sterile conical tube. Any delay increased gel viscosity inhibiting filtration. Next the MTGase supernatant was syringe filter sterilized directly into the filtered bioink. The bioink was then agitated via pipette
to intermix, and 9 mL of bioink was then transferred into 1 mL of cell-laden DMEM. The result was then agitated via pipette, and then 10 mL of the resulting cell-laden bioink was pipetted into a sterile 10 mL syringe. The syringe was then lure-locked to the sterile syringe tip and ⅛-inch tubing assembly and was compressed to remove any remaining inline DMEM used previously to flush the print tip assembly. The bioink was left to congeal to a viscosity where syringe air bubbles could not migrate when the syringe was inverted.

In the following phase, cells were cultured and transferred into the 15 mg/mL and 30 mg/mL concentration gels to investigate visually any possible cytotoxic effects of the enzyme. The gels were created and transferred into Corning culturing flasks with NIH3T3 cells. After six days there was no observed change in cell morphology in either of the two concentration groups (Nguyen, 2017). This experiment was repeated with cells that were freshly seeded into the flasks. The cells again showed no differences in morphology. Additionally, there were no observed differences in either cell adhesion or time to confluency. The 3T3 cells adhered to both the surface of the gel fragments as well as the culture flask (Figure 13). Next, 15 mg/mL and 30 mg/mL bioinks were produced. Bioinks
were allowed to incubate for 3 hours prior to cell introduction. Cells were
detached from flasks by using 1 mL 1% trypsin for 30 seconds and then
resuspended with the addition of 4 mL of DMEM. Cells were taken from two 80%
confluent flasks. Cells were then put into a 15 mL conical tube and spun down to
form a pellet. The media was siphoned off, and the cells were resuspended in 1
mL of DMEM. The cell suspension was then added to the bioink, pipetted into a
Corning six-well plate and incubated for 30 minutes for 30 mg/mL and 60 minutes
for 15 mg/mL 4 mL DMEM was then pipetted dropwise onto the gels, which were
then incubated at 37 °C for six days. Cell survival and proliferation were then
observed. Cells exhibited a healthy morphology within the bioink. There was no
observable difference between the 15 mg/mL and 30 mg/mL concentration
bioinks. Upper layers of the bioink were found to be subject to fungal
contamination. The experiment was repeated a second time with the same
results. A third repeat of the experiment was conducted; however, flasks were
used in place of plates; fungal contamination was not observed. Ultimately, 30
mg/mL MTGase with 2% porcine gelatin in 10% PBS was selected as the best
candidate bioink (Figure 15).

Syringe Loading and Tip Optimization
Commercial bioprinters use an extrusion tip of around 20 gauge. The higher the gauge, the finer the tip -- and consequently the higher the printing resolution (Dharmadasa, 2016). The finer the tip, the higher the shear stress for the cells, resulting in an increase in cell mortality. The pneumatic system employed by commercial bioprinters helps compensate for this by direct control of extrusion pressure. This approach unfortunately also results in a higher incidence of tip clogging. For the sake of this project, larger tips were selected to compensate for the pressure control issues of the simpler screw drive. Stainless steel tips of 13- and 16-gauge were selected as they were already on hand and produced extrusions of suitable resolution for basic fabrication (Figure 14).

For tip optimization, the 3T3 cell-laden bioink was extruded in concentric circles into six-well polystyrene plates. After the addition of 2 mL of bioink into each well for both 13- and 16-gauge syringe tips, two other wells were filled with bioink from an untipped syringe to act as a control. The wells were then filled dropwise with 4 mL of DMEM media. Cells were incubated at 37 °C for six days.

Later syringe tip optimization investigated finer-gauge syringe tips as printer instability issues were resolved during the PSP3 printhead trials. Stainless
Steel syringe tips were purchased and tested ranging from 12 - 28 gauge. These tips are commonly available at hobby stores as they are used for precision adhesive applications. Tip optimization was then performed again this time investigating 18 and 20-gauge syringe tips. 20-gauge, 1-inch tips were determined optimal as they allowed for extrusion of the higher viscosity bioink necessary for print integrity while maximizing cell viability. 20-gauge also closely matches the printer’s factory 26-gauge print tip extrusion size, eliminating the need for factory software modification. The easily available, low-cost, stainless steel luer lock fitting and factory-cut, 1-inch length made these adhesive-applying syringe tips ideal for this project.

The initial protocol developed for syringe loading involved intermixing the gelatin and enzyme and allowing incubation for three hours at 37 °C, followed by intermixing the bioink with 3T3 cells resuspended in DMEM. The mixture was then drawn into 5 mL disposable syringes and placed into a Pyrex petri dish for an additional hour until reaching a semi-viscous state. The extrusion tips were then attached to the disposal syringes.

Later, syringe tip assembly and system sterilization protocols were optimized. Sterilization of the printer platform and exposed surfaces applied 70% ethanol spray while male and female luer locks, the printing syringe tip, and polyvinyl tubing all required submersion in 70% ethyl alcohol. These components were then flushed via syringe with additional alcohol to ensure there were no internal air pockets. After approximately one-hour submersion (i.e. the time
required for the sterilized printer hardware to dry to the point of safe system power-up) the submerged components were then assembled and flushed with 20 mL of sterile DMEM to ensure no remaining alcohol was present in the assembly. This sterilization protocol was chosen as it did not require the use of an autoclave and as such would greatly reduce the secondary equipment cost of the printing system, allowing it to be more accessible to underfunded facilities.

The optimized bioink mentioned earlier was then loaded into the disposable syringes and relocked to the alcohol-sterilized and DMEM-flushed print tip assembly. Bioink was then extruded, flushing out any remaining inline DMEM. The loaded printhead assembly was then allowed to air incubate for 45 minutes, or until bioink reached sufficient viscosity that syringe air bubbles could not migrate when the syringe was inverted. Room temperature bioink incubation proved to greatly cell-laden bioink gelation time from the original 3 hours at 37 °C protocol down to 45 minutes while also reducing the possibility of accidental contamination.
CHAPTER THREE

PROJECT DATA

Optimization Data

Initial experiments with bioink in DMEM displayed repeated and prolific contamination with gram positive bacilli bacteria. The source of contamination was determined to be MooGloo MTGase, after incubation confirmed that both gel, DMEM, and gel plus DMEM displayed no contamination.

The experiment was repeated with a new supply of MooGloo, with results showing MTGase wells as contaminated but DMEM and gel controls as uncontaminated. A method was then developed for enzyme solution filtration to eliminate the bacterial contamination.

![Figure 15: Bioink Sterilization Optimization.](image-url)
Preliminary experiments with 3T3 cells in the presence of the bioink showed no sign of retardation in growth rate or of abnormal morphology, for both the 30 mg/mL and the 15 mg/mL MTGase concentrations. Congealed 15 mg/mL and 30 mg/mL bioink was added into two-day-old flasks of 3T3 cells. Flasks were cultured for an additional two days at 37 °C. Cells not only formed a confluent layer on the flask, but they began to colonize the bioink fragments (Figure 13).

When cells were removed from their culture flask by trypsinization and integrated into the bioink, the cells took on a new morphology. Cells on surface layers of the bioink showed a spread similar to the morphology of 3T3 cells in 2D culture. However, cells embedded in the bioink maintained a suspension

![Figure 16: 3T3 Cells in Bioink.](image)
morphology. The cells did not stretch out and remained spherical. After seven days in incubation, networks of fungal hyphae began to spread out from surface layers of the bioink. The fungal contamination was present on both the 15 mg/mL and 30 mg/mL MTGase conditions, but not in the 3T3 cell culture flasks. As there was no observed difference in cell proliferation or morphology between the 15 mg/mL and 30 mg/mL MTGase conditions, the 30 mg/mL MTGase concentration was selected as it congealed in less time (Table 2). The experiment was duplicate, with the same results. For the next experiment, bioink and cells were extruded into cell culture flasks in addition to a six-well plate. The flask bioinks did not result in fungal contamination, while the six-well bioinks did result in fungal contamination. This indicated that the fungal contamination was likely related to laboratory, and incubator sterility issues (Figure 15).

Next, extrusion tip optimization was performed with untipped, 13-gauge, and 16-gauge extrusions. The 30 mg/mL 3T3 cell-embedded bioink was extruded

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Table 2: Syringe Tip and MTGase Concentration Optimization.
cultured and stored for all future work. The GFP 3T3 variant line was chosen as live cells produce the green fluorescence protein and prior research indicated that 3T3 cells proliferate on the bioink surface in a similar fashion as on a conventional polystyrene petri dish. However, embedded cells appeared to maintain a suspended morphology. Whether these cells were still alive and whether they would be able to proliferate and expand within a 3D printed bioink construct remained unknown. Utilizing the GFP 3T3 cell line (Figure 17) enabled distinguishing between living embedded cells and dead cells.

Repeating the optimization experiments demonstrated that embedded cells survive the extrusion process with the 16-gauge syringe tip, as well as with the 18- and 24-gauge tips. However, during the printing process the 15 mg/mL MTGase concentration was deemed unusable as it had not provided sufficient viscosity for the simple 1 cm ring-printing tests during the PSP2 printhead trials. The 30 mg/mL concentration when combined with the 20-gauge syringe tip represented the minimum viscosity and syringe tip gauge required to print successfully 1 cm bioink rings. Finer gauge or higher viscosity prints were deemed infeasible with the existing hardware as motor overheat became an

![Figure 17: GFP 3T3 Cells in Optimized Bioink.](image-url)
apparent issue. A 20-gauge syringe tip with 30 mg/mL concentration comprised the minimum requirement for stable, adequate resolution bioprinting as well as the upper limit of the current hardware's extrusion capabilities. This combination of print tip and bioink formulation was chosen for testing with both the simple 1 cm ring-printing tests as well as the eventual, more complicated ear-printing tests.

Test rings were printed as a method for optimizing printer hardware configuration as well as for fine tuning printer software settings. The 3D models were imported into the native factory slicing software FlashPrint developed for use with the Creator Pro series desktop 3D printers. 3D models could easily be manipulated in this software environment while print settings such as build speed, printhead deposition rate, and infill could be adjusted and reported directly to the printer (Figure 6). These features greatly simplified the process of
system and model optimization. Although printhead and bioink viscosity formulations were optimized by looking at cell survival, ultimately, they represented the mechanical limits of the system (i.e. the best resolution and material integrity that could be printed without overtaxing the printer’s extrusion stepper motors).

Cell survival was compared between extruded and non-extruded bioinks to find parameters that yielded sufficient survival. GFP 3T3 cells were later investigated to obtain a better understanding of embedded cell survival within the 3D printed constructs. GFP cells were chosen for assessing cell survival and proliferation as conventional staining methods like tryphan blue are toxic to cell survival and would likely require multiple constructs to be produced and cross sectioned over time. Alternatively, cell tracking fluorescent probes such as thermofisher’s CellTracker Blue CMAC allows for live cell tracking over time, with a portion of the probes being transferred over several cell divisions, however this method is costly with the least expensive probe being 270$ for 5 mL. Additionally, it was unknown if complications would arise, such as reduced cell count accuracy as each generations probe concentration decreased, or if the probes function would be effected by the presence of the MTGase.

Final Project Data

The final experiment investigated cell survival and proliferation between extruded and non-extruded GFP 3T3 cell-laden bioinks. The bioprinter and
extrusion assembly sterilization protocol as stated in previous sections was conducted. 3T3 GFP second passage cells were cultured and integrated with the bioink at a concentration of $1.83 \times 10^7$/mL at a ratio of 95% living cells. 5 mL of cell-infused bioink was loaded into a syringe for printing purposes (Figure 18).

The remaining 5 mL was transferred into a 6-well plate. The printer incubated the bioink at room temperature for 45 minutes until printing viscosity was reached, then bioink was deposited by the bioprinter at a rate of 1 mL per 10 minutes. The resulting ear was completed after 50 minutes. Once the ear was completed, it was removed using a sterile spatula and placed in the 6-well plate. Both the bio printed ear and non-extruded bioink was then submerged in 10 mL of DMEM media with 20% serum as recommended by the cell line manufacturer. The 6-well plate was then cultured for 17 days under standard mammalian tissue culturing conditions. Cell survival was assessed via GFP cell counting with ImageJ. Specific locations were imaged repeatedly over the 17-day period at 3-

**Figure 19:** ImageXpress Z-Stack Well Locations Diagram.
intervals with the use of ImageXpress micro-robotic microscope system by Molecular Devices. 5 separate locations for both the unprinted bioink control and the printed construct were selected (Figure 19). Each location had 15 images taken on different focal planes. Each location and focal plane were then reimaged via a computerized imaging protocol on subsequent days. All resulting GFP images were then analyzed by ImageJ to create Z-stacks for each location (Figure 20). These image stacks were then subdivided into three zones -- bottom, middle, and top. Each Z-stack was then analyzed via a custom cell-counting macro created for ImageJ by Terisa Ubina (Bournias-V. lab). Resulting data was then exported into a spreadsheet for analysis.

Previous experiments demonstrated that the optimized bioink was sufficiently biocompatible as to allow 3T3 cell proliferation from within extruded constructs however they did not track cell counts over time or compare cell survival at relative depths within the bioink material.

Figure 20: ImageJ Z-Stack Cell Counting Macro.
The initial cell counts between the extruded bioink and the non-extruded control bioink had a noteworthy difference in initial cell concentrations. This difference was likely due to settling of cells in the bioink prior to pipetting, with the extruded bioink having on average 40 to 50 more cells in the 20x viewing area of the microscope. Unprinted bioink had only a modest average increase in cell count from day 3 to day 6, with an overall decreasing trend in living cell counts after day 9 (Figure 21). Printed bioink, however, demonstrated overall GFP cell proliferation until day 9 when cell counts declined. The trends in both the experimental and control group were obtained by averaging each of the three depth zones. Similar trends were found in both the bottom (Figure 22) and the middle zones (Figure 23) when analyzed individually in either group. However, the experimental top zone average cell count displayed a reduction in living GFP cells between day 3 and day 6, with cell proliferation increasing in the view field between days 6 and 9. Cell loss was also less pronounced between days 9 and 17 than during prior periods. Cells for experimental bottom and middle zones had the greatest GFP density, likely indicating a greater cell density. However, between days 12 and 15 the average cell loss in these zones outpaced the top zone. Top zone averages for the experimental group had an average GFP cell count of 80 cells in the visible area, with middle and bottom zones averaging between 70 and 75 GFP cells in the visible field (Figure 24). Extruded control cells followed a more consistent decline than their experimental counterparts while middle zone cells between day 12 and day 15 rebounded with an average
Figure 21: Average Cell Differential Count Experiment Vs Control.

Figure 22: Average Bottom Cell Differential Count Experiment Vs Control.
Figure 23: Average Middle Cell Differential Count Experiment Vs Control.

Figure 24: Average Top Cell Differential Count Experiment Vs Control.
cell count ranging between 35 and 40 versus bottom and upper zones with an
average cell count between 30 and 35.

The ear was cross-sectioned on day 17 prior to data-analyzing GFP cell
dispersal. Surface layers of the ear displayed 3T3 GFP cells confluent at several
layers deep. Bottom layers also displayed strong GFP, indicating high cell
counts. Middle sections of the ear displayed clusters of cells with strong GFP;
however, there were also large empty spaces between cell clusters. The clusters
themselves could indicate cell expansion, as cells were likely originally evenly
dispersed during the printing process.
CHAPTER FOUR
DISCUSSION AND FUTURE WORK

Discussion

The project achieved several of its primary goals in that it represents the lowest-cost bioprinter currently available. All components for this printer are commercially available and require only basic tools to modify and construct. The protocols developed over the past year afford any under-funded and or under-equipped lab the opportunity to investigate bioprinting with a sterile hood and basic bench-top centrifuge being the only major pieces of equipment. The protocols were also developed with material cost in mind, the bioink formulation and sterilization requiring low-cost, readily available materials such as syringe filters, disposable syringes, ethanol, MTGase, and porcine gelatin. The quality of the bioprinter itself would likely be considered introductory by current industry standards with its limited bioink pressure control. Ultimately the printer represents the most cost-effective, adaptable, and versatile system in that it has been developed using open-source components and software, with underfunded STEM student focused school laboratories in mind.

The system is of course not without its limitations. Due to instability, stable direct injection was never achieved, resulting in additional sterilization steps and a decrease in the system’s printable viscosity range. Without pressure control during bioink injection, cultured cells likely experience a range of undefined mechanical stresses during injection, which may affect cell differentiation and
propagation. Mechanical stresses that the cells are exposed to during the printing process vary from print to print as temperature, humidity, starting bioink viscosity, and print duration have the potential to change the compressive and sheer stress profile the cells are exposed to during the printing process.

One obvious issue during the final experiment was cell dispersal variation in the bioink itself. As the cells settled in the bioink, there was a large discrepancy between the unprinted control bioink and the printed bioink. This variation was likely found in the prints themselves with the lower layers having higher initial cell counts in a gradient from the initial layers of the bio printed construct to the final upper layers. Indeed, this effect was indicated in the data with the lower and middle layers having a greater initial GFP density on average than the upper layer. Ideally, a multi-injection tip would be utilized to minimize this effect, with bioink and cells being intermixed during the printing process. This approach would allow for smoother cell distribution while permitting the cells to be kept in the cell-friendly environment of a bioreactor until they are needed, thus improving cell viability. Earlier attempts to use the 3D printer’s heating elements were unsuccessful as the 37 °C environment impeded initial gelation of the bioink.

Future Work

This project did not pursue several important avenues of investigation. Defining the mechanical forces, the cells are exposed to in both the pre and post
printing environment. Quantitative measurements of these mechanical stresses and better control of environmental and procedural variables could be potentially used to further optimize and differentiate bioink formulations for specific stem cell line applications.

Additional modifications to the printer could also be pursued. With a combination of Arduino controlled leveling stepper motors and optical positioning sensors, the build platform could be automatically leveled during the printing process potentially further improving print quality. Piezoelectric sensors could be implemented to monitor bioink pressure in the current system allowing for bioink injection pressures to be maintained within a predefined range, reducing the potential variation in mechanical stresses cells are exposed to during the printing process.
APPENDIX A

AVERAGE CELL COUNT DATA GRAPHS
Total average cell count graph depicting printed 3T3 GFP cell laden bioink vs unprinted control over 17 days culturing. Cell counts calculated from fluorescent 20x ImageXpress Images using ImageJ cell count algorithm.

Average cell count graph depicting printed 3T3 GFP cell laden bioink vs unprinted control over 17 days culturing. Cell counts calculated from fluorescent 20x ImageXpress Images captured at bottom layers.
Average cell count graph depicting printed 3T3 GFP cell laden bioink vs unprinted control over 17 days culturing. Cell counts calculated from fluorescent 20x ImageXpress Images captured at middle layers.

Average cell count graph depicting printed 3T3 GFP cell laden bioink vs unprinted control over 17 days culturing. Cell counts calculated from fluorescent 20x ImageXpress Images captured at top layers.
Average cell count graph depicting printed 3T3 GFP cell laden bioink over 17 days culturing. Cell counts calculated from fluorescent 20x ImageXpress Images captured at bottom, middle and top layers.

Average cell count graph depicting unprinted control over 17 days culturing. Cell counts calculated from fluorescent 20x ImageXpress Images captured at bottom, middle and top layers.
APPENDIX B

IMAGEXPRESS AND IMAGEJ DATA
Cell count data for printed 3T3 GFP cell laden bioink vs unprinted control over 17 days culturing. Cell counts calculated from fluorescent 20x ImageXpress Images using ImageJ cell count algorithm.

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