Development of a Three-Dimensional Bioprinter with Inline Light Activation for Bone Tissue Engineering

Colton Alexander McElheny

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DEVELOPMENT OF A THREE-DIMENSIONAL BIOPRINTER WITH INLINE LIGHT ACTIVATION FOR BONE TISSUE ENGINEERING

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Masters of Science in The Department of Mechanical and Industrial Engineering

by

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B.S., Texas A&M University, 2012
December 2015
# Table of Contents

Abstract......................................................................................................................................... iv

Chapter 1: Introduction................................................................................................................... 1
  1.1 Tissue Engineering................................................................................................... 1
  1.2 Stem Cells............................................................................................................... 3
  1.3 Extracellular Matrix and the Cellular Microenvironment...................................... 4
  1.4 Cells Scaffolds and Contracts............................................................................... 6
  1.5 Additive Manufacturing in Bioengineering............................................................ 8

Chapter 2: Bioprinting....................................................................................................................10
  2.1 Rationale............................................................................................................... 10
  2.2 Inkjet Bioprinting................................................................................................... 12
    2.2.1 Thermal Inkjet Bioprinting............................................................................... 12
    2.2.2 Piezoelectric Inkjet Bioprinting........................................................................ 16
    2.2.3 Acoustic Inkjet Bioprinting...............................................................................16
  2.3 Laser-Assisted Bioprinting................................................................................... 17
    2.3.1 Laser-Guided Direct Write Systems................................................................17
    2.3.2 Matrix-Assisted Pulse Laser Evaporation Direct Writing (MAPLE-DW).... 18
    2.3.3 Absorbing Film-Assisted Laser Induced Forward Transfer (AFA-LIFT).....19
  2.4 Microextrusion Bioprinting.................................................................................. 21
  2.5 Biomaterials for Bioprinting in the Third Dimension...........................................22
  2.6 Summary............................................................................................................... 25

Chapter 3: Design and Manufacturing of a Bioprinting Platform................................................ 26
  3.1 Constraints and Goals........................................................................................... 26
  3.2 Prior Art................................................................................................................ 26
    3.2.1 Bioscaffolder.................................................................................................... 26
    3.2.2 Bioplotter.......................................................................................................... 27
    3.2.3 RepRap.............................................................................................................. 27
    3.2.4 DIY CNC Mills................................................................................................. 27
    3.2.5 Inkjet Printers.................................................................................................... 28
    3.2.6 Experimental Setup of Guillotin et al. (2010)...................................................28
  3.3 Concept Generation and Selection....................................................................... 28
    3.3.1 Print Modality................................................................................................... 28
    3.3.2 Material Selection............................................................................................ 30
    3.3.3 Axial Actuation................................................................................................. 32
    3.3.4 Control Program...............................................................................................33
    3.3.5 Configuration....................................................................................................34
  3.4 Machining............................................................................... ............................... 38
  3.5 Assembly Features.................................................................................................39
  3.6 Maintenance........................................................................... ............................... 40

Chapter 4: Validation of Bioprinting Platform............................................................................. 42
  4.1 Calculated Motor Values....................................................................................... 42
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.1</td>
<td>Z Axis Calculations</td>
<td>42</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Pump Output</td>
<td>44</td>
</tr>
<tr>
<td>4.2</td>
<td>Calibration</td>
<td>45</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Linear Calibration of X, Y and Z Axis</td>
<td>46</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Volumetric Calibration</td>
<td>48</td>
</tr>
<tr>
<td>4.3</td>
<td>Design Processing</td>
<td>50</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Two-Dimensional Geometries</td>
<td>51</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Three-Dimensional Geometries</td>
<td>52</td>
</tr>
</tbody>
</table>

Chapter 5: Conclusion and Future Work ............................................. 56

- 5.1 Conclusions ................................................................................. 56
- 5.2 Cell Printing ............................................................................ 57
- 5.3 Osteogenic Differentiation ..................................................... 58

References ......................................................................................... 59

Appendix A – Bill of Materials ............................................................ 65

Appendix B – Drawing Files ................................................................. 70

Vita ....................................................................................................... 119
Abstract

Bioprinting offers exciting possibilities for tissue engineering related to tissue and organ failure due to trauma, disease, or aging. This is of substantial societal value as the world populace mean age rises. Current needs for tissue and organ repair far exceed availability. A strategy being explored to counter this imbalance is the single step fabrication of cellular constructs. This paper explores various bioprinting techniques and suggests a custom design for a cost-effective, low-maintenance bioprinting platform for academic research. The platform functionality was validated via two-dimensional image transfer and simple three-dimensional geometries.
Chapter 1: Introduction

1.1 Tissue Engineering

Tissue and organ failure due to trauma, disease, or aging accounts for hundreds of billions of dollars in national healthcare costs in the United States alone. Current treatment solutions involve organ/tissue transplantation (both human and animal), surgical reconstruction (non-transplant based), artificial prostheses, or mechanical devices (Milliman, 2008). In 2012, there were 118,520 patients on the organ recipient waiting list with only 28,432 transplant operations performed. Additionally, 8,420 patients died waiting for organs, while another 3,250 patients were too sick to undergo an operation when an organ became available (OPTN/SRTR, 2014).

By 2020, the world populace will see a transition as people 65 years or older become the dominant global demographic. This shift will also correspond with a worldwide trend, both in developed and developing countries, of noncommunicable diseases replacing infectious diseases as the primary cause of mortality and quality of life loss (Dobriansky et al., 2007). While the current needs for tissue and organ repair outstrip the existing means of correction, the demands of a populace with increased longevity necessitates innovative new treatment vectors.

Tissue engineering may help with this unmet need by offering alternative or complimentary avenues to address organ/tissue failure via development of natural, synthetic and natural-synthetic hybrid biomimetic tissue and organ surrogates. Langer and Vacanti (1993) defined this nascent subject as “an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.” Furthermore, they offered three foundational strategies for the creation of tissue in vitro:
1. *Isolated cells or cell substitutes.*

Some injuries and diseases can be mitigated or treated via the infusion of cells, a process generally known as cell therapy. Mesenchymal stem cells (MSCs), for example, not only are able to differentiate along the mesoderm lineage (bone, cartilage, tendons, fat and muscle), but may also be able to differentiate, at least partially, along the endodermal and ectodermal lineages (Newman et al., 2009). MSCs have been used to aid in heart repair after myocardial infarctions (Assis et al., 2010), improve redevelopment of tendons after tears (Correia et al., 2013), and direct local anti-inflammatory responses in inflammatory diseases (Newman et al., 2009).


The regenerative ability of the body originates from undifferentiated, pluripotent stem cells that, in conjunction with external stimuli, can differentiate and replace injured or necrotic tissues and organs. These stimuli include small signaling molecules, growth factors (GF), proteins, as well as mechanical stimuli unique to each organ/tissue system. Unfortunately, many published studies utilizing growth factors provide contradictory results and seem to have a low methodological quality to their reports, inhibiting the full potential such substances have shown (Chen et al., 2010; Correia et al., 2013). While treatment with these substances alone have encouraged dubious results, most researchers recognize advantages of utilizing GFs, signaling molecules and proteins in conjunction with cell scaffolds and cultures to promote a healthier artificial microenvironment (Chen et al., 2010; Lee et al., 2011).
3. Cells placed on or within matrices

Traditionally used as a top-down approach in tissue engineering, biocompatible materials (synthetic, natural, or decellularized tissue/organ) are designed, fabricated and subsequently seeded with cells. This has become the main approach for the development of implantable, replacement devices and is especially effective with thin and/or avascular tissues and organs such as skin, bladder, cartilage and connective tissue, and of tissues with a high level of remodeling, such as bone (Place et al., 2009).

While tissue engineering has greatly expanded upon these original three strategies, success ultimately is most closely related to the properties of the scaffolds used. The development of three-dimensional printing of cells and biomolecules represents a fundamental shift in tissue engineering paradigms and the potential realization of lab-grown replacement tissues, both simple and complex (Carvalho et al., 2013).

1.2 Stem Cells

As Langer and Vacanti set forth in their 1993 paper, the manufacturing of tissue substitutes is dependent upon directed cellular growth, oftentimes within matrices. The type of tissue formed and the potential applications for these cell seeded matrices depends upon the type of cells used. Stem cells provide the most versatile of platforms as several tissue types are differentiable from a single source.

Embryonic stem cells (ESCs) possess the highest potential for tissue engineering applications due to their ability to form all three germ layers found in development (endoderm, mesoderm, and ectoderm). From these three basic layers, every tissue type arises. The endoderm layer lends itself towards the formation of insulin positive cells and hepatocyte-like cells. The mesoderm layer differentiates into cardiomyocytes, hematopoietic progenitors, leukocytes and
endothelial cells. While the ectodermal layer primarily gives rise to neuroprogenitors (Atala, 2010).

The limited availability and, more pressing, the ethical ambiguity of ESC procurement resulted in restrictive regulation, both in the United States and in Europe. Only within the last five years has the United States government lifted its ban on federal funding of human embryonic stem cell lines created after August 9, 2001.

While ESCs represent a potential “all-in-one” source of replacement tissue and organs, multipotent stem cells are a much more readily available and equally viable option for more specific applications (Mironov et al., 2009). Whereas ESCs are totipotent stem cells, able to differentiate into any cell found in the body, multipotent stem cells possess limited stemness. Stemness refers to a cell’s ability to self-renew and to differentiate into various cell types. The multipotent label indicates a cell’s ability to form multiple cell types. Mesenchymal stem cells, for example, can differentiate into cells that form fat, cartilage and bone (Blitterswijk, 2008).

1.3 Extracellular Matrix and the Cellular Microenvironment

Cocktails of various growth factors traditionally are used to induce stem cell differentiation, however there is an increasing link between differentiation and mechanobiological, biochemical and bioelectrical cues from the cellular microenvironment (Reilly and Engler, 2010). The cellular microenvironment is composed of soluble molecules (growth factors, ions, and proteins), adjacent cells, and the extracellular matrix (ECM).

The ECM is a by-product of cellular activity and secretion of resident tissue and organ cells. It contains a mixture of structural and functional proteins, composing a mainly fibril structure. Transduction of the various cellular signals employ an immense range of protein mechanisms that couple the intracellular and extracellular domains. Function often follows form
for cells, and the resistance to deformation the ECM exerts on the cellular components effect protein production, cell motility, cell phenotype, differentiation and several other factors. The secreted molecules and proteins form into fibrillar networks via proteins, like integrin, which attach to the Arginine-Glycine-Aspartic acid domain of fibronectin. Fibronectin is a connective protein that also binds to collagen, fibrin and other proteoglycans (Reilly and Engler, 2010; Trappmann et al., 2012).

Described by Bissell and Aggeler (1987) as a dynamic reciprocity, the ECM transmits signals to resident cells, which will respond with protein synthesis and secretion. This in turn changes the ECM response to, and signal transduction from, external cues. The ECM is considered to have three main components for concurrent signal generation: structural, mechanical and biochemical (Reilly and Engler, 2010). The changing microenvironment and constant ECM remodeling couples its structural and functional components modulating important factors (proliferation, differentiation, apoptosis, cellular motility, etc.) in a feedback loop towards establishing and maintaining homeostasis (Bissell and Aggeler, 1987; Reilly and Engler, 2010).

The dynamic reciprocity further influences cellular development via the mechanical properties it imparts to the ECM. Depending on desired tissue, the cellular niche experience elastic moduli ranges from 0.1 kPa for soft brain tissue to greater than 30 kPa for bone (Reilly and Engler, 2010). Trappmann et al. (2012) showed that it is not the physical stiffness of the ECM that regulates cell fate, but cell-ECM tethering capacity, often mediated via collagen linking. Microenvironments with reduced collagen expression tend to exhibit lower elastic moduli due to the distance between fibers and limited crosslinking resulting in decreased
mechanical feedback and disallowing of integrin complexes to complete signal processing (Trappmann et al., 2012).

The signal transduction dictated by the biochemical composition, such as adhesion domain distribution, can impair or improperly enhance signaling pathways. Cells, reacting to stimuli, can pull on the ECM release sequestered biomolecules allowing for cellular uptake and modulation. If the cell is unable to generate great enough mechanical force due to, for example, collagen anchor locations too distal to each other, the biomolecule remains trapped. The structural, mechanical and chemical properties of the ECM work in concert to generate proper biosignal transduction. The coupling of these parameters beget some innate challenges in developing cellular scaffolds (Trappmann et al., 2012).

1.4 Cells Scaffolds and Constructs

Ultimately, success in tissue engineering, including cellular seeding, is intrinsically determinant on the properties of the cellular scaffold (Carvalho et al., 2013). Traditionally, cellular scaffolds are prefabricated and then seeded with cells forming constructs. Due to the reciprocal nature of the ECM and constituent cells, a heavy emphasis is placed on using biodegradable polymers, with mechanical and chemical composition closely matching the host tissue environment. As Williams (2008) comments, “Of equal note is the requirement for suitable biodegradation parameters. There is no point in designing a system that will facilitate complex tissue regeneration if that tissue is ultimately destroyed by the influx of inflammatory cells associated with the degradation process or if the material stimulates the immune system as it degrades and releases antigenic material” (p. 2950).

Mironov et al. (2009) expand on this concept by delineating basic assumptions that guide traditional scaffold-based tissue engineering: “1) cell growth is substrate attachment-dependent;
cells need a solid substrate for attachment and proliferation; 2) tissue constructs must have an organo-specific shape; a solid scaffold is essential to keep the desired shape; a tissue construct could not maintain its shape without a solid rigid scaffold; 3) the scaffold serves not only as an attachment substrate, but also as a source of inductive and instructive signals for cell differentiation, migration, proliferation and orientation; 4) the porous structure of a solid scaffold will allow optimal cell seeding, tissue construct viability, and vascularization; and 5) mechanical properties initially provided by the rigid solid scaffold after its biodegradation will be maintained by controlled neomorphogenesis of parenchymal and stromal tissue synthesized in vitro or in vivo in the tissue construct” (p. 2165).

While many of these assumptions have proven true, their application towards solid scaffolds present fundamental challenges. A main contention these assumptions allude to is the scaffold’s necessity to be biomimetic to the native tissue/organ microenvironment. Towards this end, scaffolds exhibit an interconnected porous structure, allowing for seeding and ingrowth. Porous openings are generally optimized to be in the 50 µm to 500 µm range (dependent on cell type), encouraging an optimal opportunity for invasion and seeding. However, many cells, including human adipose derived stem cells (hADSCs), have diameters of 20 µm – 30 µm and fail to completely span scaffold openings when anchored. On the cellular level, the cell is still experiencing a two-dimensional culture, limiting its use as a biomimetic model (Mironov et al., 2009).

Beyond the limitations of recreating a true three-dimensional microenvironment, vascularizing large constructs remains problematic. Oxygen diffusion becomes ineffectual for constructs with cells more than 200 mm from a source, either vascular or media/construct-atmosphere interface. In vitro, perfusion-based bioreactors can act as a stopgap solution as the
neovasculature forms. These systems force nutrient media through the construct, while simultaneously removing waste. *In vivo*, nutrient supply and waste removal are dependent solely on proximal capillaries, necessitating the incorporation of these structures in future vascular tissue implants (Kaully et al., 2009; Rauh et al., 2011).

Precise placement of multiple cell types within the three-dimensional scaffold is extremely challenging and achieving cell densities comparable to organ specific values remains unsolved in traditional seeding. With this approach, cells from the patient are isolated and expanded in culture. Their attachment sites are removed thermally or chemically and suspended in media. These suspended cells are then introduced to a porous scaffold in which they can reattach. Cellular attachment sites are statistically driven. Beyond cell placement and penetration, preformed solid scaffolds tend to be more rigid, not lending themselves to contractile tissue replacement such as the heart or vasculature (Boland et al., 2006). With such intrinsic challenges connected to traditional scaffold development and cellular association, tissue engineering is undergoing a paradigm shift, adopting methodologies more associated with the electronics industry than biotechnology. In the last twelve years, additive manufacturing has become a new frontier for tissue engineers to explore.

1.5 Additive Manufacturing in Bioengineering

Additive manufacturing (AM) in bioengineering presents not only a paradigm breaking approach to the construction of tissue constructs, but a clear and feasible path towards up-scalable, high throughput, and personalized generation of medical implants. In manufacturing there is often a compromise between production speed and customization. With rapid prototyping techniques this tradeoff is becoming less distinct.
The advent of three-dimensional printing in the 1980’s resulted in an engineer’s ability to quickly model and develop personalized end products through computer-aided design and automated construction. By adapting commercial additive manufacturing processes to biomedicine, medical professionals can and have begun practicing truly individualized medicine. Tissue engineering, at the clinical level, is a highly personalized field, aiming at the restoration or replacement of an individual’s tissue or organs. Much like a tailor making a suit to perfectly fit a customer, medicine is striving to create implants and treatments to perfectly fit their patients, resulting in minimal negative side effects. It is already extensively noted within pharmaceutical trials that individuals with similar profiles may have different responsiveness to the same agent (Hoffmann-La Roche LTD, 2013.) Tissue constructs face additional geometric, compositional, and biological constraints dependent on injury location, severity, and patient predisposition. The combination in personalized flexibility of design, as well as the reliability and reproducibility of automated assembly, engenders additive manufacturing to the biomedical field (Melchels et al., 2012).
Chapter 2 Bioprinting

2.1 Rationale

The central motivation for bioprinting is the creation of three-dimensional cell cultures in which cell-cell and cell-extracellular matrix interactions dominate cellular regulation, allowing for viable *in vitro* tissue and organ surrogates. One marker on the path to realizing this goal is the development of biomimetic models via three-dimensional microenvironment-mediated cell cultures.

While two-dimensional cell cultures played a paramount role in developing an understanding of cell/tissue morphology, disease mechanisms, drug discovery and several aspects of tissue engineering and regenerative medicine, their use as predictive biomimetic models is inherently handicapped due to oversimplified microenvironments. In two-dimensions, the chemical and physical properties are more micro-manageable, and imaging and characterization tend to be easier (Sayal, 2014). However, the information gleaned from these studies has been shown to be inadequate. Sayal (2014) states that the fundamental differences in two-dimensional and three-dimensional microenvironments influence cell behaviors ranging from cell attachment, growth, differentiation, cellular metabolism, stimuli response, and overall function. Indeed, cells adopt dimensional and polarized shapes based on the orientation of integrin mediated attachments. In the case of two-dimensional cultures, cell attachment occurs only on one side of the cell (the culture plate) allowing expansion and motility within minutes. Conversely in three-dimensional cultures, attachment and spreading is preceded by proteolytic breakdown of the physical microenvironment occurring over the course of several hours or even days (Melchels et al., 2012; Sayal, 2014).
Growth, motility and expansion are just three of the many factors that change depending on the cellular environment. Dunn et al. (1989) showed that hepatocytes maintain many of their liver-specific functions when cultured between two layers of collagen, but lose these functions within days in two-dimensional cultures. Cells in two-dimensional cultures often divide atypically, become progressively flatter and lose any differentiated phenotype. However some cells, like chondrocytes and mammary epithelial cells, can regain their physiological function, form and phenotype when redeployed in three-dimensional cultures (Melchels et al., 2012; Sayal, 2014).

With the ability to incorporate cells into a computer based fabrication system, researchers are able to address the three main shortcomings of current scaffolding approaches: the inability to mimic cellular organization of natural tissues; challenges in up-scalability to economically feasible clinical application; and neovascular incorporation (Melchels et al., 2012). As previously mentioned, the current paradigm of cellular constructs is to construct solid scaffolding supports, relying on stochastically driven seeding processes. These scaffolds are further challenged by pore size optimization, in which cells need to be able to penetrate and attach to the scaffold. Additionally they experience three-dimensional microenvironments providing space for future proliferation. Bioprinting, or the computer-aided spatial deposition of cells and biologically relevant materials, looks to overcome these challenges by creating supporting structures in line with cellular seeding. Use of computer-aided fabrication processes in bioprinting provides avenues towards good manufacturing practice, quality control and the foundation for regulatory legislation (Melchels et al., 2012; Mironov et al., 2008).

While many two-dimensional cell patterning systems exist, with mechanisms ranging from chemical to mechanical to optical, Wilson and Boland (2003) et al. showed that
inexpensive, noncontact based printing platforms with the potential for three-dimensional control was possible. Defined by the First International Workshop on Bioprinting and Biopatterning as “the use of material transfer processes for patterning and assembling biologically relevant materials (molecules, cells, tissues, and biodegradable materials) with a prescribed organization to accomplish one or more biological functions,” (Carvalho et al., 2013, p. 307) bioprinting utilizes three main strategies in material deposition: inkjet printing; laser assisted printing; and microextrusion (Murphy and Atala, 2014). These methodologies each have their own advantages and shortcomings, which will be reviewed in the following sections, leading ultimately to the design of a cost-effective, adaptable cellular printing platform.

2.2 Inkjet Bioprinting

2.2.1 Thermal Inkjet Bioprinting

Inkjet printing is a contact free, forward transfer method of deposition in which a small droplet of liquid is ejected from a small opening on the print head to a waiting substrate. Inspired by and originally adapted from the commercially available office tool, inkjet technology mainly utilizes thermal resistors, piezoelectric elements, or acoustic waves to achieve fluid jetting (Nishiyama et al., 2009; Wilson and Boland, 2003). Thermal inkjet printing systems may be some of the most widely explored platforms for early bioprinting applications. Droplet formation and ejection occur through a micropulse to circuitry that superheats a small fraction of the ink volume, vaporizing it and generating a pressure wave (Ringeisen et al., 2006). Bubble formation occurs near the print head opening, and the expansion and collapse of the bubble causes a small volume of ink, in the range of 10 pL to 150 pL, to be ejected out the nozzle. The droplet volume is dependent on several factors, including heat gradient, pulse frequency, and “bioink” viscosity; a parameter directly coupled to cell density (Cui et al., 2010; Owczarcz et
Despite its successful adaptation as the first inkjet bioprinting platform, researchers were wary of thermal inkjet systems due to concerns of thermal shock from the superheating and vaporization process. In 2010, Cui et al. explored the heat shock damage intrinsic with thermal systems. The Hewett-Packard (HP) Deskjet 500 printer that was adapted to bioprinting operated at 3.6 kHz, resulting in an ejection event every 2 µs, and utilized $1.3 \times 10^{-5} J$ of energy. However, the heating process occurs in each of the 50 print head nozzles before repeating, giving a total cycle time of roughly 120 µs. This 120 µs window allows the ink within the channel to return to ambient temperatures before a new heating regime begins. Cui et al. (2010) calculated that the temperature of the droplet could increase a maximal 24°C based on the equation:

$$\Delta T = \frac{E}{C_p \times V_{drop}}$$  \hspace{1cm} [Equation 1]

They determined the heat capacity of the ink to be $4.18 J/kg \cdot K$, the volume of the droplet to be 130 pL, and assumed 100% energy dissipation, making $E = 1.3 \times 10^{-5} J$. With the ambient temperature being 22°C, the maximum temperature cells would be exposed to is approximately 46°C. In Wang et al.’s (2003) related heat shock protein expression study, cellular response of protein expression was noted after ninety minutes of exposure to 43°C. Due to the vast timescale differences, the few microseconds’ thermal stress was determined to be insignificant. 22°C was estimated as the maximal temperature rise, however previous studies indicate that the heating process is significantly less than ideal, and ambient temperature rises are restricted to only 4°C – 10°C (Xu et al., 2005).
While thermal stresses are the most apparent concern with the inkjet process, cells are also subjected to hydrostatic and inertial forces associated with pressure wave formation, as well as significant shear as the fluid near the walls of the channel travel much slower than at the center. Post ejection, cells experience the mechanical stresses of impacting the substrate. Despite these concerns, several studies show significant cellular viability, comparable to normal handling values (Boland et al., 2007; Cui et al., 2010; Murphy and Atala, 2014; Nakamura et al., 2005; Roth et al., 2004; Saunders et al., 2008).

Considering the unique forces cells face during a typical inkjet process, this is a somewhat surprising trend. While Xu et al. (2005) indicated thermal stressors were minimal, the orifice related shear stress is more difficult to assess and post-ejection stresses seem to be dominated by the impact of the cell into the waiting substrate. Saunders et al. (2008) showed the initial velocity, and overall kinetic energy, of the droplet greatly influenced cellular printing viability.

Tirella et al. (2011) showed that strain energy of the droplet absorbed by the substrate is inversely related to its elastic moduli. Substrates with higher elastic moduli undergo little if any deformation, forcing the bioink to absorb all the forces associated with impact, which decrease cell viability. While printing into fluid substrates offers the highest dissipation of kinetic energy, bioinks consisting only of cells and culture media coalesce, losing any spatial orientation or information (Tirella et al., 2011), suggesting that a hydrogel layer be placed in between the printing surface/orifice and cell plates. Wang et al. (2008) show that across various ejection velocities, cell viability is challenged by von Mises stress and shear strain, as well as stress/strain related to impact(s). To mitigate mechanical damage, the lowest possible ejection velocities should be utilized. Additionally, any hydrogel substrate should be sufficiently thick enough to
completely absorb the kinetic energy of the droplet preventing penetration causing a secondary impact with culture dishes or any support structure.

When Cui et al. (2010) tested CHO cell viability remained near 90% with no significant change in apoptosis ratios versus unprinted cells. However, enlarged membrane pore sizes were observed post print. Fifteen minutes after being subjected to bioprinting, the exaggerated pore size allowed 40,000 MW dextran to enter the cell, roughly equating to a 90 angstrom diameter. The cellular membrane demonstrated recovery, with pore diameter continually decreasing post print until they blocked the invasion of 16Å wide molecules (propidium iodide) two hours after being subjected to print stress. The transient nature of the pores allow for potential drug delivery during the printing process.

While inkjet printing seems to offer high cell viability and high throughput, it is restricted by cell density, with a limit around $8.2 \times 10^6 \frac{cells}{mL}$ (Cui et al., 2010; Xu et al., 2005). For the HP Deskjet 500 print head, a linear relationship exists between cell concentration and number of printed cells on the range of $2.1 \times 10^6 \frac{cells}{mL}$ and $8.2 \times 10^6 \frac{cells}{mL}$. Researchers noticed that higher cell concentrations tend to cause nozzle clogging. Increasing the concentration from $8.2 \times 10^6 \frac{cells}{mL}$ to $16.5 \times 10^6 \frac{cells}{mL}$ decreased the number of printed droplets with at least one cell present by 33%. Optimal conditions for bioprinting are system dependent, and each new platform will face its own optimization process. A bioink composition of 1x Dulbecco’s phosphate-buffer saline solution and cells with a print head nozzle diameter of ~50 µm, $7.7 \times 10^6 cells/mL$ should result in one cell per droplet (Cui et al., 2010). Using an “optimized” value of $8.2 \times 10^6 \frac{cells}{mL}$, every droplet contained at least one cell with approximately 15% containing more than one (Cui et al., 2010).
2.2.2 Piezoelectric Inkjet Bioprinting

Piezoelectric inkjet systems operate on a very similar basis as the thermal inkjet. Instead of utilizing thermal resistors for bubble nucleation, a controlled voltage pulse is administered to a polycrystalline ceramic in each nozzle, which acts as an actuator to provide a transient pressure flux via expansion/contraction to eject fluid. Once the voltage peak subsides, the piezoelectric crystal or plate relaxes into its original configuration, refilling its ink stores and enabling a new ejection event (Ringeisen et al., 2006). While piezoelectric printers have been utilized to print biologicals with some success (Lemmo et al., 1998; Guillotin and Guillemot, 2011; Nakamura et al., 2005; Saunders et al., 2008), the lingering concern about sonification-related cell lysis and membrane damage occurring within the 15 kHz – 25 kHz piezoelectric operating range have stymied its use in favor of the more trusted thermal inkjet method (Cui et al., 2010).

2.2.3 Acoustic Inkjet Bioprinting

Acoustic inkjet systems offer several distinct advantages over thermal and piezoelectric systems, utilizing ultrasonic sound waves to generate jetting events. The ultrasound field allows for droplet formation without the heat induced or shear stressors of piezoelectric or thermal inkjet systems. The ink is able to maintain its cohesion over the orifices in acoustic systems, until the sound waves constructively interfere, overcoming the surface tension and inducing droplet formation. By adjusting the pulse frequency, duration, and amplitude, researchers are able to maintain a constant droplet volume, while influencing directionality as well. Without a cyclic heating regime, nor narrow-channeled nozzles to push cells through, acoustic bioprinting has the potential to use higher viscosity bioinks while maintaining higher-throughputs with higher viability (Murphy and Atala, 2014). Due to commercial availability of thermal and piezoelectric
inkjet systems, the ease of adaptation, and the ability to print via well-known programs such as Microsoft Office, the development and experimentation with acoustic inkjet has been limited. The main disadvantage of acoustic inkjet printers is their steep development and learning curve compared to other inkjet systems (Boland et al., 2006; Murphy and Atala, 2014; Tasoglu and Demirci, 2013). However, with higher throughputs, a wider arsenal of biomaterials, and great flexibility with bioink viscosity, acoustic inkjet printing may be a superior choice over its piezoelectric and thermal cousins.

2.3 Laser-Assisted Bioprinting

2.3.1 Laser-Guided Direct Write Systems

Laser assisted bioprinting began with simple laser-guided direct write (LGDW) methods to place single cells on a receiving substrate with micrometric accuracy. Conceived before Boland’s experiments with inkjet bioprinting, LGDW transfers cells via optical entrapment (Odde and Renn, 2000). A laser is passed through a fluid with cells suspended within it. The difference in the refractive index between the cells and media scatter photons, radially trapping the cell within the laser’s beam and propelling it axially along its path. The light is often limited to the near-infrared spectrum of wavelengths to avoid damaging the printing materials: cells.

Nahmias et al. (2005) noticed that the refractive difference also provided issues with throughput due to the limited optical driving forces. They evaluated the refractive index of multipotent adult progenitor cells (MAPCs) to be 1.40 while endothelial cells exhibited a refractive index of 1.35. This 3.6% decrease in refractive index quartered the radial trapping power and decreased the axial force by 25x (Nahmias et al., 2005). While the refractive index of cells can be normalized with the attachment of bioinert VEGF-linked polystyrene beads, LGDW faces numerous issues.
Throughput is the main issue LGDW systems face when attempting tissue and organ synthesis. Odde and Renn (2000) reported deposition rates of 2.5 cells/min, and while Nahmias et al. (2005) achieved printing rates on the order of hundreds of cells per hour. Several approaches have been suggested to increase print speeds, ranging from increasing cell concentrations to increasing the laser power. Each of these solutions have their own intrinsic challenges. Increased cell concentrations risk clogging optical channels used to guide cells to the substrate, whereas a higher powered laser risks causing addition cellular damage. Current techniques are wholly inadequate for bulk tissue development (Nahmias et al., 2005; Odde and Renn, 2000), but can be highly useful for application-specific in vitro cultures models, such as cancerous microenvironments (Schiele et al., 2010).

2.3.2 Matrix-Assisted Pulse Laser Evaporation Direct Writing (MAPLE-DW)

While directly placing cells became more of a liability than an asset for LGDW systems, the application of focused light in bioprinting found other methods of contributing towards viable bioprinting solutions. Matrix-assisted laser evaporation direct writing (MAPLE-DW) is a modified laser-induced forward transfer method (LIFT) in which a laser pulse causes substrate vaporization, ejecting material to a waiting substrate. MAPLE-DW utilizes a three layer printing ribbon which is optically transparent to the laser wavelength. A biopolymer is spread over a supporting quartz ribbon. A cell suspension is then spread across the biopolymer. A laser pulse is focused on the support/biolayer interface, and a portion on the matrix absorbs the incident wavelength of light, producing an energy transfer. The transferred energy results in material being ejected forward (“forward transfer”) towards a receiving substrate (Barron et al., 2004b).

The absorption and ejection events are highly energetic phenomena and are the primary concern related to cell viability. Thermal and optical radiation effects have the potential to
damage the cellular printing material. The risk is minimized via control of various parameters such as laser pulse wavelength, duration, and intensity. Additionally, the absorption of the laser incident energy results in a portion of the biopolymer transforming into a small pocket of plasma. The plasma formation enhances the energy absorption, causing in a highly localized thermal spike and protecting approximately 95% of the surrounding material from harm (Ringeisen et al., 2006).

MAPLE-DW suffers from limited reproducibility and resolution. While the biolayer matrix is homogenously mixed, microstructural differences can result in variations in the ejection events. Ringeisen et al. (2002) reported MAPLE-DW printing groupings of ~50 cells/spot a resolution of roughly 150 µm. Additional studies were able to transfer cells with greater than 95% viability and overcame the adhesion issues Ringeisen et al. witnessed in their 2002 studies. Matrigel was used as a basement membrane to print onto, safely adhering a range of various cell types (Schiele et al., 2010). The inconsistent nature of the biopolymer matrix creates issues of inconsistency with ejection spot size, droplet satellite formation and resolution. MAPLE-DW has several issues to overcome before significant gains can be made with this system (Ringeisen et al., 2006).

2.3.3 Absorbing Film-Assisted Laser Induced Forward Transfer (AFA-LIFT)

Another modified laser-induced forward transfer modality operates on a very similar basis as MAPLE-DW. Absorbing film-assisted laser-induced forward transfer (AFA-LIFT) utilizes a thin metal coating (Ag, Au, Ti, TiO₂, etc.) that acts as a sacrificial layer instead of a biopolymer matrix for incident light absorption. The sacrificial layer thickness exceeds the optical skin depth of the material used, completely protecting the biological material from optical irradiation interactions (Guillemot et al., 2010). Moreover, the crystalline matrix of the metal
film engenders reproducible, uniform jetting events that are dependent on laser pulse energy and the rheological properties of the bioink (Ali et al., 2014).

Several cell types have been printed with near 100% viability. Barron et al. (2004a) even printed multiple cell types adjacent to each other with near native tissue resolution of 10 µm – 100 µm. AFA-LIFT demonstrates higher versatility that inkjet counterparts. Bioinks with a cell concentration of $10^8 \frac{\text{cells}}{\text{mL}}$ and a viscosity of 120 mPa have successfully been processed (Guillotin et al., 2010). This compares to the $8 \times 10^6 \frac{\text{cells}}{\text{mL}}$ maximum concentration of inkjet systems (Cui et al., 2010). While higher viscosity would translate into dangerously high shear stresses with thermal and piezoelectric inkjet systems, AFA-LIFT is an orifice/nozzle free system that greatly mitigates shear stressors during transfer processes.

Also called biological laser printing (BioLP) dependent on the research group, droplet formation is only limited by the physical constraints of micrometric stage positioning rates and laser pulse frequency. BioLP has been operated with laser pulse frequencies of 5 kHz. While vastly outstripping LGDW systems, AFA-LIFT is comparable to the low end of inkjet systems, which generate droplets on the 3.6 kHz – 500 kHz frequency range (Barron et al., 2004a; Cui et al., 2012; Nakamura et al., 2005).

Many of the concerns associated with thermal, piezoelectric, and acoustic inkjet processes translate to AFA-LIFT and MAPLE-DW systems. Biological materials jetted from one surface into an awaiting substrate are subjected to shear flow and thermal stressors from the ejection process, as well as shear and impact related mechanical damage due to cell-substrate interactions post-ejection (Ali et al., 2014; Wang et al., 2008). With AFA-LIFT, much like MAPLE-DW, most thermal stressors are mitigated via the sacrificial metal layer and the insulating vapor bubble formed. Shear stressors, as well as most of the post-ejection stressors,
are primarily influenced by the initial ejection velocity. Ali et al. (2014) showed that, dependent on laser pulse power, sub-jetting regimes, laminar jetting regimes, or turbulent jetting regimes were possible. At lower power settings, but greater than those associated with the subthreshold regimes, Ali et al. (2014) achieved “slow” ($97 \frac{m}{s}$) jetting plumes, which in turn enable higher survivability and finer resolution. AFA-LIFT has achieved viability rates comparable to, or surpassing, inkjet systems (Ringeisen et al., 2006).

### 2.4 Microextrusion Bioprinting

Microextrusion printing is an adaptation to the most commonly available commercial three-dimensional printers. Often, these systems comprise of a temperature-controlled dispensing head and stage, with robotic spatial control along the X, Y, Z, and/or A axes. While previous printing methods can be classified as “drop on demand” platforms, microextrusion is a continuous printing modality capable of handling a much broader range of materials with much higher viscosities than witnessed with inkjet or laser-assisted techniques. Microextrusion has successfully incorporated the standard materials of three-dimensional printing such as acrylonitrile butadiene styrene (ABS) plastic or polyactide (PLA) plastic, as well as several biologically relevant materials such as hydrogels, biocompatible copolymers and cell aggregates with viscosities ranging from $30 \frac{mPa}{s}$ to $6 \times 10^8 \frac{mPa}{s}$ (Chang et al., 2011; Peltola et al., 2008).

Microextrusion can be achieved through various methods. Most commonly it is achieved through pneumatic actuation, screw mechanisms or via pistons. Pneumatic and screw-based systems display a greater capability for dispensing higher viscosity fluids, indicating potential adaptation to a wider range of materials for bioprinting processes (Chang et al., 2011; Murphy and Atala, 2014). Mechanical systems benefit from quicker feedback response due to the delay in pneumatic gas compression. Additionally, screw-actuation based systems benefit from a higher
degree of spatial control, allowing finely tuned volumes to be dispensed. The complexity of mechanically driven microextrusion bioprinting systems often result in a tradeoff with limited maximum driving force (Murphy and Atala, 2014).

Microextrusion systems possess the capability to match or surpass laser-assisted bioprinting, with nonbiological three-dimensional printing recorded with a resolution of 5 µm and linear speed of at 50 µm s\(^{-1}\) (Murphy and Atala, 2014). This translates to a 5 mm cubic structure developed in approximately 40 minutes, microextrusion printing, dependent on delivery method, may be able increase its print rate (Chang et al., 2011). Nonbiological three-dimensional microextrusion requires the heating of plastic filaments before printing processes can occur. Cellular deposition may be coupled with thermosensitive hydrogel placement, cellular homeostatic responses to heat shock should be mitigated for most cultures. This suggests that biological three-dimensional printing could potentially operate at higher operational rates. As is, microextrusion has already been used to develop clinically relevant structures such as aortic valve conduits and branched vascular networks (Duan et al., 2013; Murphy and Atala, 2014).

Microextrusion also tends to exhibit lower cellular viability post printing. As discussed with inkjet printing, nozzle diameter seems to impart some influence on viability (Chang et al., 2008). However, it is the shear stresses and pressure force necessary to extrude highly viscous fluids that damages cells (Nair et al., 2009). While these can be countered with larger diameter nozzles and lower viscosities, these measures incur loss in print resolution (Murphy and Atala, 2014).

2.5 Biomaterials for Bioprinting in the Third Dimension

In essence, each of the cellular printing methods discussed thus far are two-dimensional patterning modalities. They are able to achieve three-dimensional control by actuating the Z axis,
allowing repeated layering of two dimensional arrays. Equally important in developing the third dimension, is proper material selection. Commercially available home three-dimensional printers generally use plastics, such as ABS or PLA. Other rapid prototyping systems use resin, polymers, ceramics, powders, or metals to stack two-dimensional layers into a three-dimensional final product, utilizing organic solvents, high temperatures or nonbiocompatible crosslinkers.

One of the greatest strengths in the application of rapid prototyping to the biomedical field is the inline ability to create and seed cellular constructs with patient specific geometries. One of the main challenges bioprinting faces is characterizing and developing printing materials that provide structural, mechanical, and functional properties desired (Murphy and Atala, 2014). The field currently focuses its efforts on using naturally-derived polymers or synthetic molecules.

Naturally-derived polymers are advantageous due to their similarity to native ECM, whereas synthetic polymers can be specifically tailored to match desired biomechanical properties. Both types of materials need to be matched for biocompatibility and degradation profiles (Murphy and Atala, 2014). Both synthetic and naturally-derived materials are usually utilized as hydrogels. Hydrogels are networks of crosslinked polymer chains that are able to absorb significant amounts of water, while maintaining their three-dimensional structure. Hydrogels present an ideal opportunity for bioprinting application to encapsulate cells within printed material. While providing a tridimensional microenvironment, the hydrogel also maintains high enough stability for layered structures (Carvalho et al., 2013; Henmi et al., 2008; Melchels et al., 2012).

Trying to print fully crosslinked hydrogels would necessitate high pressure requirements for nozzle based systems and fusing mechanisms for drop on demand printers. As previously
stated, high pressure printing regimes are detrimental to cell viability (Nair et al., 2009). To utilize hydrogels in bioprinting processes, the hydrogel is deployed in its precursor complimentary components, with a base suspended with the cells in the bioink and the crosslinker present in the substrate. Most natural hydrogels used in additive manufacturing are chemically crosslinked. In chemical gelation methods, cells are suspended in a hydrogel precursor solution and deposited on/in a complimentary substrate that causes crosslinking via chemical diffusion. This results in a microenvironment that severely limits cell mobility and proliferation of embedded cells due to meshes that are orders of magnitude smaller than the cell. However, several different strategies have been developed that allow degradation sites to be incorporated via cell-mediated matrix degradation, optically driven photo-degradation or hydrolytic breakdown. With sufficiently high cell density, or with an ECM production focus, natural gel degradation may be permissible (Melchels et al., 2012).

Many hydrogels have been used in experimentation: collagen, gelatin, Matrigel, agarose, alginate, as well as many synthetic polymers (Melchels et al., 2012) Of these, alginate is one of the more common due to its favorable biocompatibility, cellular support capacity, and drug delivery potential (Wüst et al., 2014). Alginate also benefits from being able to be used cross-platform, whether with inkjet, laser-assisted, or microextrusion based systems (Billiet et al., 2012). Alginate crosslinks in the presence of calcium ions. Boland et al. (2006) performed experiments determining optimal concentrations of alginate and calcium for effective gelation and structural fidelity, in which an 8% sodium alginate solution combined with 0.4 mmol/ml calcium chloride allowed for continuous and distinct hydrogel channels. Alginate hydrogels have been successfully used with several cell types including MSCs, endothelial cells, neural cells,
and hepatocytes (Billiet et al., 2012; Boland et al., 2003; Cui and Boland, 2009; Hong et al., 2013).

Wüst et al. (2014) conducted a study in which alginate’s properties were modified and tailored to fit specific applications with additives such as gelatin or hydroxyapatite. These two additives were incorporated to exploit a reversible thermal gelation at the print head. Just before extrusion, the cell-laden hydrogel precursor was heated to 50°C, inducing gelation. This imposed a stable external structure. While the structure cooled to room temperature, calcium ions crosslinked the alginate. As the thermal gelation was reversed, the chemical crosslinking maintained structural integrity. Multiple gelation methods used to create extended solidification may allow for a wider variety of biomaterials to be used as initial “fast” and reversible crosslinkers to impart a stable biophysical environment as more long term cell structures develop.

2.6 Summary

The exploration of the multitude of options and directions of bioprinting as a biological and tissue engineering resource provides a foundation for the considerations in developing a cost-effective, versatile printing platform. This review covered a brief blush of the total body of work concerning bioprinting but should impart insight and understanding in the design considerations and decisions associated with the development of a three-dimensional bioprinter with inline light activation for bone tissue engineering.
Chapter 3: Design and Manufacturing of a Bioprinting Platform

3.1 Constraints and Goals

The overarching goal of this development is the delivery of a cost-effective printing platform able to dispense a cell-laden cube measuring 1 cm x 1 cm x 1 cm with a minimal resolution goal of 100 µm and reasonable printing speed. Future applications of the printing system will utilize mesenchymal stem cells, which in suspension exhibit a diameter of approximately 30 µm (Toma et al., 2002). Therefore, if a nozzle or orifice based approach is used, it needs to be able to allow these cells to pass through without clogging. Furthermore, as with all bioprinting platforms, cell viability should be as high as possible, with a lower end goal of 70% viability.

This bioprinter is being developed for a small-scale academic research lab, where it will be placed in a biological safety cabinet allowing a workable area of 70” x 20” x 27” (W x D x H). The safety cabinet is a Sterilgard III Advanced (Model SG603A) procured from The Baker Company. Since the platform will be placed in a clean room environment, it needs to be portable and with the ability to be sterilized. Additionally, academic research laboratory groups experience higher turnover and greater temporary employment than most dedicated research facilities. With the influx of untrained assistance, being able to visually inspect a mainly stationary system for signs of contamination would be advantageous.

3.2 Prior Art

3.2.1 Bioscaffolder

As seen in the previous chapter, several systems have been developed for the application of rapid prototyping in biotechnology. The BioScaffolder, developed by GeSim couples nonbiological pressure driven (100 kPa – 800 kPa) microextrusion, with optional piezoelectric cell deposition. It possess 2 µm step sizes in the X and Y direction and 10 µm control in the Z.
With an included heating pad, it can process biopolymers, hydrogels, bone cement paste, biocompatible silicones and polymer pastes. It has a work envelope of 100 mm x 346 mm x 40 mm, allowing different configurations of culture dishes and plates.

3.2.2 Bioplotter

The Bioplotter by EnvisionTec operates at $0.1 \frac{mm}{s}$ to $150 \frac{mm}{s}$ that transverses a work envelope of 150 mm x 150 mm x 140 mm. The BioPlotter has a 0.001 mm resolution along the X, Y and Z axis. Five unique material cartridges can be used per job and the system maintains an automatic tool changer. Also equipped are high and low temperature nozzles and switchable base plates to allow operational temperature ranging from 0°C – 250°C.

3.2.3 RepRap

RepRap is an open-source, low-cost home three-dimensional printing platform. One of its main selling points is that once a main unit is set up, it is able to print and replace all of its mechanical parts. Work envelopes and resolution are system dependent as drive electronics can be upgraded to improve resolution. While the RepRap is a community based open-source environment, it allows for a lot of flexibility in design. However, this comes at the price of each RepRap build being slightly different, increasing troubleshoot times. Additionally, the software controls for RepRap are developed with Arduino. While Arduino is a versatile I/O and language system, it does present another hurdle to overcome in the learning process.

3.2.4 DIY CNC Mills

Many hobbyist have created their own custom mills for home machining. Recognizing that three-dimensional printers are similar computer numeric control (CNC) devices with a print head instead of a drill tool, a great deal of insight could be gain from related forums. Additionally, CNC mills and lathes have long-standing industrial uses which have prompted the
development of dedicated software for three axis control. Software such as Mach3, Omax Layout or LinuxCNC made by third parties are designed to make utilizing CNC devices as user friendly as possible.

3.2.5 Inkjet Printers

Wilson and Boland (2003) showed that stock inkjet printers could be modified for use as a bioprinter, with the advances made in biomaterials enabling three dimensional control as well (Nakamura et al., 2005). Inkjet systems are the most cost effective, with the lowest end HP printer, the HP Deskjet 1010, costing only $29.99 as of January 2015. With a commercial inkjet system, the power, X and Y motor control and interfacing are already established. Additionally, two-dimensional representations of a design can be loaded into basic home office software (i.e. Microsoft Word PowerPoint, etc.) and printed without the need to purchase or learn new software. And while not necessarily being used for its originally intended purpose, Wilson and Boland (2003) found that HP technical support was willing to lend troubleshooting aid.

3.2.6 Experimental Setup of Guillotin et al. (2010)

AFA-LIFT provided the most consistent, positive results of the laser-assisted bioprinting methods. Guillotin et al. (2010) showed that high cell densities could be used to statistically print physiologically relevant cell concentrations with native tissue resolution. However, high upkeep, laborious sample preparations, and highly specialized equipment impose a substantial barrier to field entry.

3.3 Concept Generation and Selection

3.3.1 Print Modality

The first decision to make with the development of a printing system is which modality to pursue. With the majority of the design work already completed by a manufacturing firm,
modifying an inkjet printer seemed to be the ideal choice. The printers from the mid-1990’s possessed 300 dpi resolution, approximately 2 µm, well under the desired 100 µm goal. Additionally, wireless capabilities could be added by means of a universal serial bus (USB) adapter. The inkjet printer could be completely enclosed within a biological safety cabinet, without the need to run an outside line into a controlling computer.

The HP Deskjet 1010 is the lowest end thermal inkjet HP sells. It is able to print with a 600 dpi x 600 dpi resolution, allowing for ~1 µm. However, this is managed via smaller print head nozzle. As seen in Figure 1, the nozzle print head used by Boland’s research group had an orifice diameter of roughly 50 µm, whereas the Deskjet 1010 utilizes a nozzle approximately 20 µm across. Toma et al. (2002) commented that MSCs exhibited diameters of 20 µm to 30 µm across. While cells are able to undergo significant deformation, the shear stresses of ejecting cells through an opening two-thirds their size could prove catastrophic. While there is the potential that the cells will be able to recover from this stress, no literature could be found to support this. As viability is one of the few stipulated design goals, this uncertainty removes inkjet technology from design considerations.

Figure 1. Comparison between the print head of the HP Deskjet 500 and the HP Deskjet 1010.
With cell viability excluding inkjet technologies from consideration, AFA-LIFT seems like the next best choice. Guillotin et al. (2010) reported cell viability near 100% post print with single cell resolution at 5 kHz operating speed. Furthermore, the use of a laser system for cell deposition could be utilized as a catalyst for the light initiated differentiation of hADSCs into bone (Qureshi et al., 2013). However, laser-assisted bioprinting systems are expensive and are over-engineered for the intended immediate use of this system. The disadvantages of a laser-assisted bioprinting setup also include maintenance, laborious sample preparations and steep learning curves.

The last modality discussed in Chapter 2 was microextrusion. Microextrusion is a relatively inexpensive option for three-dimensional printing. Dependent on extrusion methods, cell viability ranges of 40% - 95% have been achieved (Murphy and Atala, 2014; Nair et al., 2009; Walker et al., 2010). Depending on the system configuration, microextrusion printing has a resolution ranging from five micrometers to millimeters wide deposition. Second only to inkjet technologies, microextrusion has fairly simple sample preparation protocols, decent throughput potential and is easy to learn. With sufficient resolution, manageable costs, and excellent viability potential, microextrusion is the chosen modality to design a printing platform.

3.3.2 Material Selection

The material used to construct the structural components of the printer will have a profound impact on the overall design. Several professionally manufactured CNC devices utilize stainless steel for structural components. These products have the benefit of tightly controlled Good Manufacturing Practices allowing for easily developed, high quality pieces. The general hobbyist or end-user do not have access to such facilities. While academic machine shops are
widely equipped and can handle most machining needs, this often necessitates outsourcing the work to machinists, putting the researcher at the mercy of their timetable.

Instead of a metallic superstructure, plastics were instead considered. Poly(methyl methacrylate), also known as acrylic or PMMA, is a strong, light-weight thermoplastic that can be easily machined. While acrylic may shrink or expand with temperature change, the fairly stable ambient room temperatures should mitigate this. Acrylic Plexiglas sheets also resist ultraviolet light degradation seen with other clear plastics. While optically clear, acrylic does block wavelengths below 400 nm – 300 nm. While the working area will be sterilized, UV sterilization is prevented on interior surfaces. However, this is the same issue stainless steel or aluminum would face. With acrylic however, if a bacterial or fungal colony develops outside the UV sterilization range, it will be visually apparent to the end-user.

The gold standard for biological sterilization is a process called autoclaving in which specimens are subjected to saturated steam under a pressure of approximately 15 PSI reaching temperatures of at least 121°C. Many plastics, PMMA included exhibit glass transition temperatures, which are temperature ranges in which a polymer transitions from a hard, rigid material to a soft, elastic material. Acrylic exhibits this switch over the range of 85°C to 165°C dependent on the copolymer composition. As such, dependent on the manufacturer, PMMA could be subjected to autoclaving. However, as previously mentioned, acrylic will expand or contract dependent on temperature change. Used as a structural component, such thermal warping could cause catastrophic failure or greatly reduce cycle life of the parts.

Fortunately, alternatives are available. Ethylene oxide (EtO) is a potent alkylating agent and classified by the Environmental Protection Agency (EPA) as a possible carcinogen. EtO
requires a sub atmospheric pressure chamber, moisture and the EtO gas administration. PMMA and steel are both compatible with EtO processing.

Within academic research institute machine shops, there is a continual competition to use machining equipment. Most notably, units such as water-jet cutters are constantly in use by various senior design teams or graduate researchers. Thus materials with versatile machining options are preferred. While water-jetting is one option for part development with acrylic, laser-cutting is another option. Mainly used as an engraver, the 60W laser CNC machine is able to cut through 1” thick acrylic. While some ½” thick pieces will be cut, in the interest of mitigating machining time, layered pieces of 0.22” thick acrylic will be used to create an adaptable bioprinting construct.

3.3.3 Axial Actuation

Motion control in CNC devices, such as three-dimensional printers or automated mills, is achieved with either stepper motors or servo motors. Which motor is used is dependent on the power required. Geckodrive, a leading provider of both stepper motors and servo motors, suggest the following formula for calculating the power required by the design to achieve motion.

\[
\frac{(\text{Heaviest Object}) \times (\text{Idealized Inches per Minute})}{531} = \text{Watts Required} \quad [\text{Equation 2}]
\]

If the resulting value is below 100 W, then the load setup is well within the range of stepper motor function. Above 200 W would require the power output of servo motors, while in between the two values would be at the top end of stepper motor functionality and the low end of servomotor domain.

Acrylic is a fairly light-weight material with a density of 0.0434 \( \frac{\text{lbs}}{\text{in}^3} \). Assuming stepper motor use, many NEMA-23 stepper motors weigh between 1.5 lbs. and 2.5 lbs. With a 4- or 5-axis system, motor weight would still only account for a maximum of 12.5 lbs. Doubling this
value for total weight of the heaviest part should allow for a conservative estimate to determine if stepper motors or servomotors are most appropriate. With an idealized inches per minute of 500 \( \frac{in}{min} \), the equation becomes:

\[
\frac{25 \times 500}{531} = 23.54 \text{ W}.
\]

This result shows that this application should use lower-end stepper motors, and that servomotors would be vastly inappropriate. While at this point more design is necessary to determine the necessary holding power of the stepper motors, these calculations occur once a design is established to validate stepper motor procurement.

### 3.3.4 Control Program

Along with stepper motors, a breakout board, power supply and stepper motor drivers are necessary. Many breakout boards facilitate direct communication between the CNC software running on a computer and the stepper motor drivers. This can cause issues with many modern computer systems as DB-25 parallel port connections are becoming rarer. Unfortunately, using a USB to DB-25 parallel port adapter is not a viable solution. Parallel ports facilitate direct communication between the computer and the stepper motor drivers. Both the signal frequency and the signal amplitude convey input parameters for stepper motor coordination. While USB to DB-25 converts the signal, proper signal timing is lost.

The pulses are generated based on the computer’s internal central processing unit (CPU) clock, which in turn is based on its kernel processing speed. This means that when the CPU slows down due to heavy processing cycles, the timing of the pulses will lag behind as well. To reduce the chance of that happening, desktop computers should be used over laptops due to their more stable power supply and a dedicated system should be used for processing and actualizing jobs. Another concern with stepper motor use is basic interfacing errors due to driver
firmware/computer software incompatibility. Driver firmware should be flash updated and synced with CNC controller. With this in mind, Probotix’s 4-Axis Lightning Stepper Motor Kit was purchased with Mach3 firmware already installed.

Mach3 is a CNC software usually dedicated to mill, lathes or plasma cutters. However, Mach3 has a very active community, many of which are interested in expanding their mill utility into potential three-dimensional printing. One such community member, Nuri Erginer, developed an add-on to the Mach3 program that translates a fourth axis, the A axis, as an extruder. While some calibration is necessary to translate the linear distance of an extruded filament into the volumetric extrusion from a pump, this provides a quick and viable solution to a potential laborious coding concern.

The add-on to Mach3 decomposes STL model files in printable layers and then generates and posts G-code to Mach3. G-code is the programming language utilized by stepper motors to map and execute pathing. The combination of Mach3 and modified code mitigate the need to learn a new programming language to successfully operate the bioprinter.

3.3.5 Configuration

Now that the structural material and drive components have been decided, the overall configuration of the platform needs to take shape. Many three-dimensional printers and CNC mills have a similar. For this novel system, two structures on either side of the working envelope support a gantry that is able to traverse the Y-plane. These two structures are able to travel along to X-plane, imparting two-dimensional control. These side pieces also need to allow the gantry to travel in both directions along the Z axis imparting full tridimensional positioning capability. Within the gantry, a syringe pump will extrude cell-laden fluids into a waiting substrate. The
base is held stationary to mitigate and tidal and inertial forces experienced by the substrate associated with moving the working point.

The bioprinter should have a working area large enough to manage common culture containers such as Petri dishes, multi-well culture plates, centrifuge tubes or glass slides and cover slips. To establish reasonable print speeds, miniature extra light (MXL) timing belts will be used to drive the gantry in the X and Y directions. To generate movement along the X-axis, the MXL timing belt is linked around a cap screw in the on gantry support, looped around a timing-pulley coupled to the drive shaft, passed through the center of the support structure, circled around an idler pulley and held in place by another cap screw. The Y axis belt is cut in half, and passed through an opening in the shuttle, redirected by a 10-tooth idler pulley, connected to a 20-tooth MXL pulley directly coupled to a stepper motor, diverted by another idler pulley to loop around a vertical cap screw. The ¼” wide MXL timing belt is clamped on both terminal ends by a #4 machine screw drilled through the belt, two #4 washers and a nut. The Kevlar reinforcing fibers are able to maintain belt tensile strength, allowing the stepper motor to pull the gantry back and forth along the Y axis.

While printing may transverse several inches along the X and Y axis in a single command, the Z direction will only raise a fraction of that height before another layer is deposited in the XY plane. Dependent on pump flow rates, the diameter of the extruded fluid, and thus the Z intervals, may be on the order of a few micrometers. This necessitates a higher resolution, reproducible method of controlling the Z axis position. A leadscrew application will allow for greater control than belt actuated movement. A coupling nut, restricted from rotational movement, can easily be implemented for such a task. With a ¼”-20 thread coupling nut and a stepper motor with a 200 step/revolution resolution, a 6.35 µm step travel distance is achieved.
The stepper motor drivers obtained from Probotix also offer the option for micro stepping, a method for increasing motor resolution at the cost of travel speed. Micro stepping increases the requisite pulses per revolution via jumper resistor manipulation. The proper configuration allows these stepper motor drivers to change from 200 steps per revolution to 25,000 steps per revolution.

Four motors are used to actuate three-dimensional control. Two motors are used to power vertical movement due to the torque associated with two drive shafts on distal ends of such a wide work area. The current design utilizes a gantry of approximately 15 lbs. that imposes variable torque on the Z axis stepper motors due to its relative position along the Y axis. While a belt was initially considered to couple the two drive shafts, issues with maintaining belt tightness and cohesion prompted a change in design. Due to the constraints of working with a 4-axis breakout board, physical coupling via wiring was chosen. By wiring the two motors, and each of the solenoid driving coils, to be in parallel with each other, the load tolerance of the motors was essentially doubled. The parallel wiring mitigates the current values that power each coil, reducing power generation at lower speeds.

A fifth motor acts as a pump to extrude material into a substrate of interest. This motor is a non-captive stepper motor that allows a threaded rod to pass all the way through it. Purchased from Nanotec alongside with a M10-2 threaded, the non-captive stepper motor has a 10 µm step distance. A flanged shaft collar and linear bearing constrain the threaded rod from rotation, inducing linear vertical movement. Extrusion occurs when the threaded rod extends downward from the motor, driving the plunger of a full syringe forward. With micro stepping, the distance traveled by the plunger can be reduced to negligible. However, the inner diameter of syringe is a static variable, and thus has the greatest impact on print resolution.
While individual part drawings are available in Appendix A, Figures 2 through Figure 4 supply several model views of the completed assembly, the base, gantry, and supporting structures.

Figure 1. (Above) Isometric, XZ plane view and YZ plane view of completed assembly.

Figure 2. Isometric (left), XZ plane view (center) and YZ plane view (right) of shuttle assembly.

Figure 3. Isometric (left), YZ plane view (top) and XZ (right) plane view of gantry assembly.
3.4 Machining

A laser cutter was used to generate acrylic parts. Utilizing a 1” working distance, the 60W laser was inhibited to 0.7 speed and 75% power to cut through 0.22” thick acrylic. Most passes created clean cuts on the first attempt. Spatially large parts, such as the base cover, required multiple cuts. This is due to some minor flexing of the thinner acrylic that skewed the focal distance from the surface of the material, causing inefficient cuts. To overcome this, the laser cut the same path multiple times until a clean break occurred. Acrylic is a flammable thermoplastic and capable of combustion during laser cut procedures, in which a laser melts through the plastic to create separation. Passing the laser over previously exposed material increased probability of combustion and greater care needed to be exercised. While batch to batch variation was usually small enough use uniform speed and power settings, occasionally fine tuning these parameters resulted in better cuts.

For ½” thick acrylic pieces, the laser was set to its slowest speed setting of 0.1 and a power setting between 30% - 35% power. The PPI, or irradiated points per inch, was set to its
maximum value for acrylic of both thicknesses. With these setting, the parts labeled “Rocket Side” took four hours to complete. Replacement parts for these items will be redesigned with 0.22” thick acrylic, greatly reducing both the price and time commitment required for cutting.

Cross drilling was performed on a standard drill press equipped with micro positional encoders. A rotation based edge-finder was used to identify the leading edge, then the encoder readout ensured accurate positioning in relation to the edge. Holes were also counter sunk using the same drill press. When available, a water based coolant was used to manage thermal stresses from the drilling process. Otherwise, WD-40 was used as a coolant instead.

A lathe was used to machine the drive shafts for the X-directional stepper motor. Two ½” diameter x 8” long zinc-plated cap screws were machined down to 0.45” diameter, with a 3/8” long, ¼” diameter extrusion from one end; and a 2” long, ¼” diameter at the other. The lathe was also used to thread 1060 Carbon Steel rods with ¼”-20 thread for two inches on one end.

3.5 Assembly Features

The complete assembly utilizes just over 40 unique custom designed parts occupying a footprint of 22.25” x 16.875” x 20.4” (compared to the 70” x 20” x 27” available space) and a working envelope of 8.5” x 5.5” x 5” in its current configuration. It is calibrated to travel the X and Y axes at 100 mm per minute, while the Z axis is limited to half that at 50 mm per minute. A bill of materials and all custom drawing files can be found in Appendix A. In its fully assembled state, the entire platform weighs approximately 35 lbs.

This design offers flexibility for future use. While the current setup utilizes a 5 cc syringe, this could be switched for a larger volume model or adapted into a self-refilling syringe pump from an onboard reservoir for large scale print jobs. This reservoir could replace some of the counter weights currently located to offset the torque generated by the two stepper motors.
located on the gantry, allowing future additions to further stabilize the printing assembly. The counterweights are three 12-oz lead fishing weights. They have 1/8” bore holes that allow for easy attachment. Because only two #4 socket head machine screws were available, a ¼” bore was drilled through the third weight and attached via ¼”-20 cap screw.

Additionally, while the laser currently hangs below the carriage to activate cells within the syringe (see Figure 5), this can easily be rearranged to shine vertically into the substrate in the case of heating or cooling pads obstructing the syringes optical clarity. While Figure 5 shows the laser irradiating cells near the top of the syringe, the area of effect can be adjust by the addition or removal of spacers. Most likely, the laser will be focused just above the nozzle, ensuring all printed cells are exposed.

![Figure 5. A laser is mounted on the underside of the gantry to activate cells waiting to be dispensed.](image)

3.6 Maintenance

Many biological labs utilize various concentrations of ethyl alcohol for disinfection purposes. In the presence of this bioprinting platform, this practice needs to be modified. Alcohol induces stress crazing in PMMA, causing a network of cracks throughout its structure. As
expected, the propagation of fracture networks in a structural material closely precede defunct functionality. An alternative to alcohol sanitation is the use of dimethyl benzyl ammonium chloride, better known commercially as Clorox Disinfecting Wipes. Clorox reports that Clorox Disinfection Wipes will not harm acrylic products. Twenty four hour continuous exposure resulted in no visible change in a machined PMMA piece’s appearance. Comparatively speaking, a machined piece exposed to 100% alcohol for the same time period showed significant surface crack presence. Other non-alcoholic based disinfecting products could be used as well, such as Lysol Disinfecting Wipes.
Chapter 4: Validation of Bioprinting Platform

4.1 Calculated Motor Values

Sections 3.3.5 and 3.5 discuss prominent aspects of the bioprinting platform and Appendix B reveal the complete design. Finalized models allow the motor output requirements to be determined. From these values, the appropriateness of the motors can be assessed, and new components purchased if necessary.

4.1.1 Z Axis Calculations

The Z axis motors are stressed the greatest due to working against gravity to drive the gantry vertically. Stepper motors are rated not by their power output, but by their holding torque. Additionally, rotor inertia should be ratio matched against the load inertia. Having too high of a ratio increases the risk of missed steps and severely limits motor functionality. For NEMA-23 motor sizes, this ratio, defined in Equation 6, should be no larger than 5.

\[ J_L = J_W + J_S \]  \hspace{1cm} \text{[Equation 3]}

\[ J_W = W \times 16 \times \left( \frac{P_B}{2\pi} \right)^2 \]  \hspace{1cm} \text{[Equation 4]}

\[ J_S = \left( \frac{\pi}{32} \right) \times \rho \times L_B \times D_B^4 \]  \hspace{1cm} \text{[Equation 5]}

As seen in Equation 3, the total load inertia is the sum of the screw inertia and the structural load inertia. In Equation 4, \( J_W \) is the inertia due to the weighted load, \( W \) is the weight in pounds (11 lbs.), and \( P_B \) is the screw pitch (0.05). For Equation 5, \( \rho \) is the density of the steel used for the leadscrew (4.57 oz/in³), \( L_B \) is the length of the leadscrew (12”), while \( D_B \) is the diameter of the screw (1/4). Plugging these values into the above equations establishes:
\[ J_L = J_W + J_S \]
\[ J_W = 0.01116 \text{ oz} \times \text{in}^2; J_S = 0.02102 \text{ oz} \times \text{in}^2; J_L = 0.03218 \text{ oz} \times \text{in}^2 \]

\[
\text{Rotor Inertia} \geq \frac{J_L}{J_O \times i^2} \quad \text{[Equation 6]}
\]

The 280 oz\text{*in} stepper motor reports a rotor inertia \( J_O \) of 2.626 oz \times \text{in}^2 giving a ratio of 0.012, well below any threshold. With a permissible inertia ratio established, the next parameter of interest is the required output torque. The load torque is defined by Equation 7 and \( F \) is defined in Equation 8.

\[
T_L = \left( \left( F \times \frac{P_B}{2 \times \pi} \right) \times 1.1 \right) \times \left( \frac{1}{\eta \times 0.01} \right) \quad \text{[Equation 7]}
\]

\[
F = F_A + W \left( \sin(\alpha) + \mu \times \cos(\alpha) \right) \quad \text{[Equation 8]}
\]

\( W \) is the weight of the load. Alpha is the angle load and is measured in relation to the ground, in this case 90°. \( P_B \) relates to the same pitch value as used in the inertia calculation and \( T_B \) relates the breakaway torque, which is the amount of torque necessary to begin threading a screw when no other forces are present. For \( \frac{1}{4}^\prime\prime \)-20 thread screws, the minimum breakaway torque is 4.5 oz\text{*in}. Finally, \( \eta \) relates the efficiency of the system. Typical leadscrew efficiencies range from 30% to 70%. Values of 30%, 50% and 70% were used to examine potential opportunities to eventually improve the platform performance. \( F_A \) defines a force actively working against the gantry’s direction of travel and in this case goes to zero. Thus, plugging in known values to Equation 7 and Equation 8 yields:

\[ F = 0 + 11(\sin(90^\circ) + 0) = 11 \text{ lbs} \]
\[ T_L = \left( \left( \frac{11 \times 0.05}{2 \times \pi} \times 1.1 \right) + 4.5 \right) \left( \frac{1}{50 \times 0.01} \right) = 147.1 \text{ oz} \times \text{in} \]

By multiplying by the safety factor of 1.5, the required torque is assessed as 220.632 oz\(\text{in} \). This value was assessed with 50% efficiency assumed. Presuming a generous estimate, and 30% is more conservative, the required torque becomes 245.13 oz\(\text{in} \) (367.7 oz\(\text{in} \) with a 1.5 safety factor included.) However, if the threaded rods were replaced with higher efficiency rods, such as coarse threaded acme rods, the efficiency could increase to the 70% range which would only necessitate 105.1 oz\(\text{in} \) (157.6 oz\(\text{in} \) with a 1.5 safety factor.)

With the Z axis motor suitability established, the X and Y axes are also validated. While the X axis drives a heavier load, this is mitigated by the kinetic friction of steel bearing on a steel sheet. Based on the equation for \( F \), the effective force driven by the X axis motor is only 1.088 lbs due to a kinetic friction coefficient of 0.05 mitigating the force needed to move the 21.75 lbs structure. The load is likewise reduced from 9.5 lbs to 0.95 lbs due to similar kinetic friction coefficients between the steel bearing and aluminum rod.

**4.1.2 Pump Output**

This printer design utilizes a syringe clamped in place to extrude a cell-laden liquid, as seen in Figure 6. While the current design is limited to 5 mL of fluid, the clamp could be adapted to larger volume syringes. The printer emphasizes single use syringes to facilitate simple, easy printing set up and clean up environment. To achieve extrusion, a non-captive motor capable of generating 800 N of from was purchased from Nanotec.
Using digital calipers to measure the inner diameter of the syringe to be 12.35 mm, the cross sectional area was calculated to be 119.79 mm$^2$. Recognizing that pressure is merely a force applied to an area, 800 N imposed on an area of 119.79 mm$^2$ results in 6.7 Mpa of pressure. While cell printing usually occurs around 30 kPa, the operational range of this pump allows highly viscous fluids to be extruded as well (Nair et al., 2009).

4.2 Calibration

There are two ways to calibrate stepper motor motion. The first is to direct it to move a supposed set nominal distance and measure the actual distance travelled. Mach3 maintains position awareness via counting the number of steps a motor takes and comparing that value
against a steps per unit measurement. Mach3 is unitless and will maintain positional accuracy as long as imperial or metric units are consistently used. However, Skeinforge, the model slicer used by the three-dimensional printing add-on, utilizes metric units. While Mach3 attempts to convert between the two, it often will fail to translate software imposed limits, bringing print jobs to a halt. As such, it is highly recommended that motors within Mach3 be calibrated to steps per mm.

4.2.1 Linear Calibration of X, Y and Z Axis

The stepper motors along the X and Y axis should be similar in their steps per mm due to the identical set up of their drive mechanisms. Both utilize 20-tooth MXL pulleys with a pitch diameter of 0.509” to drive the gantry uniaxially. To determine the steps per unit measurement, the overall distance of one revolution is divided by the number of effective steps per revolution. With a micro stepper ratio of 1:10, 2,000 steps are required to complete one revolution. A 0.509” diameter correlates to a 1.5991” perimeter. Converted to millimeters, this gives 40.61714 mm per revolution. By dividing the steps per revolution, the steps per unit measurement are calculated to be 49.24 steps per mm, or approximately 20 μm per step. While used as a starting calibration, this value was tuned to 47 steps per mm, giving an average percent error of 0.08% when moved across a 10 mm space. The Y axis displayed similar trends. Theoretically, with a maximum ratio of micro stepping (1:125), the printer could achieve 1.6 μm positional resolution.

The second way to calibrate the printer is to use Mach3’s built in calibration tool. The user dictates the distance Mach3 should tell the motor to travel. After the motor positions itself at the designated location, the user indicates what the actual distance traveled was. Mach3 then calculates and suggests a new steps per unit value. This tool can be extremely helpful in establishing and validating motor setting, however unless high resolution tools are available, the
uncertainty error associated with measurements will cause Mach3 to suggest new values with each iteration. These proof of concept calibrations use measuring calipers with readouts to the nearest hundredth of a millimeter. Due to this uncertainty, Mach3 suggestions fluctuated 46.331 \( \text{steps/revolution} \) and 47.246 \( \text{steps/revolution} \).

The Z and A axes motors utilize threaded rods to generate motion, differing the calculations required to calibrate. Using an imperial lead screw with a ¼”-20 thread results in a 0.05” linear movement per revolution. Imperial screws utilize a nomenclature in which the first value is the screw diameter and the second value is the number of threads per in. Therefore, on a ¼”-20 screw it takes twenty revolutions to travel one inch, or 0.05” per revolution. Converting this to millimeters equates to 1.27 mm. As mentioned previously, the Z axis requires greater control than the X or Y. Thus, the Z axis utilizes a 1:125 microstepping ratio, resulting in 50,000 steps per revolution. The Z axis translates 1 mm of linear movement every 19685.04 steps imparting a step interval of 50.8 nm. Using this step count generated an average error percent of 0.04.

While the Z axis step distance is on the extremely fine side, the micro stepping by 125x is of concern. Increasing micro stepping ratios eventually transforms the linear motion profile to a nonlinear one. Nonlinear profiles greatly detract from system reliability and should be avoided. However, the microstepping ratio was chosen in part to physically limit the options associated with Z axis motor configuration. The required torque calculated in section 4.1.1 indicated that the 280 oz*in stepper motor could be operating at the edge of its performance profile with a potential torque load of ~245 oz*in. By imposing such a high number of steps per unit measurement, the acceleration and velocity profiles for this motor are limited. Additionally,
several passages along the full Z axis range of motion has maintained the linear relationship seen on its shorter journeys.

Output from the breakout board to the stepper motors is limited by the computer’s kernel processing speed. The computer used with this set up operates at approximately 27 kHz. With such a high number of steps, the Z axis speed must be below 81 mm per minute. This speed cap forces the motor to operate within a certain domain of its torque-speed curve to prevent skips maintain step coherence.

The A axis is also a leadscrew setup. However, it uses metric nomenclature. The non-captive motor actuates a M10-2 threaded rod. This means that each thread is 2 mm apart, or a 2 mm linear movement occurs every revolution. Similar to the X and Y stepper motors, a 1:10 micro step ratio was imposed. This created a linear actuator with one micrometer step size with similar error percentages to the Z axis motion. With a 1 μm step distance, the A axis is able to extrude as little as 0.12 μL of fluid.

4.2.2 Volumetric Calibration

The volumetric calibration of the pump is a more convoluted process dependent on the properties of the bioinks processed such as viscosity, swelling factors, coalescence resistance, etc. However, these manifest these various factors manifest via a single variable: the filament diameter. This diameter can be modified within the Skeinforge interface page of the three-dimensional printing add-on for Mach3 and will influence the image pathing the slicing program generates to prevent overlap from occurring. As such, the pathing for a 1 mm filament diameter will be less packed than the pathing for a 0.1 mm filament diameter. This likewise will influence the number of layers to be printed. It is assumed that a single pixel (single step extrusion event ejecting the physically limited smallest volume possible) printed into a fluid substrate will form a
spheroid structure. Thus the filament diameter is also the layer height, imposing that the 0.1 mm diameter filament will need ten times the number of layers to complete an identical structure. The optimization between resolution and speed is application dependent and will need to be assessed for each print job.

An example calibration was performed by attempting to print a 1cm x 1cm x 1cm cube. Assuming a filament diameter of 1 mm, and a packing density of 10, a STL file of the model cube was generated using SolidWorks. STL files are meshed model files used by slicer programs to generate two-dimensional layers that can be followed with G-Code. While STL files are generated from AutoCAD software, many programs, including SolidWorks, generate error-ridden STL files. Fortunately, Microsoft offers a free web service that will repair faulty STL files. Therefore, before loading any STL model file into Mach3, it is advisable to upload the files to https://modelrepair.azurewebsites.net.

Utilizing a 1.7 mm filament diameter, six layers are needed to create a one centimeter height. It is suggested that any calibration prints be conducted in Offline mode so that potential catastrophic collision can be avoided. Normal microextrusion devices deposit strings of semi-melted plastics. The add-on for Mach3 assumes this functionality and attempts to extrude the path length of the print head for each layer. The linear path traveled by the print head is much greater that the linearly-actuated extrusion of the bioprinter. Whereas checking the program reveals the pump wants to travel 39 mm over six layers, a 5 mL syringe plunger is able to travel a much shorter distance. Knowing the cross-sectional area from the maximum pressure output calculations discussed in section 4.1.2, the height containing 1 mL of fluid can be calculated with Equation 9.

\[ V = \pi \left( \frac{D}{2} \right)^2 * h \]  

[Equation 9]
Where this can be rewritten as:

\[ 1000mm^3 = \pi \times \frac{(12.35mm)^2}{4} \times h \]

Therefore, by moving 8.35 mm the pump extrudes a volume of one milliliter. A syringe fill with 5 mL of fluid would therefore be able to travel only \(~42\text{ mm},\) not the 75 mm called for by Mach3. Since the program is depositing six layers, the overall distance the pump should move in one layer is one-sixth the total distance traveled in the print. Multiplying the ratio of desired movement to programmed movement by the calibrated steps per millimeter determines the total steps the pump is supposed to travel. Dividing job step count by the number of layers dictates that the proper fraction of volume is extruded for each layer, ensuring the calibrated volume is dispensed. This relationship is demonstrated in Equation 10. Using a 5 mL syringe with an inner diameter of 12.35 mm, and an extruded filament diameter of 1.7 mm, the calibration for the pump is 214.103 \(\frac{\text{steps}}{\text{unit}}\).

\[ \frac{H_{\text{mL}}}{x_{\text{prgm}}} \times S_{\text{rev}} \times \frac{1}{n_{\text{layer}}} = S_{\text{layer}} \]  

[Equation 10]

4.3 Design Processing

With the calibrated system, two-dimensional designs were processed. Regrettably, Mach3 does not have a built in two-dimensional drawing G-code generator. Most two-dimensional drawings can be transformed into vector images with InkScape, an open-source drawing software. These vector images were then saved as .DXF files and imported into SolidWorks to form three-dimensional models. These models, saved as .STL files, were uploaded to Microsoft for repair then subjected to the three-dimension printing add-on wizard within Mach3.
4.3.1 Two-Dimensional Geometries

Multiple two-dimensional designs were attempted. Unfortunately, this printer setup was not developed for imaging via print head/substrate interaction. The pressure required to draw ink created torque forces that prevented the pen head from operating with the dexterity demanded by the printer. Figure 7 depicts efforts at overcoming this with a fine tip Sharpie mounted in a syringe. While the results lack the desired resolution, they do offer evidence of three-dimensional control, as the Z axis had to be aligned such that the Sharpie could make continual contact with the paper. The fill profile of several small letters is lacking due to the print head diameter of a sharpie. The image is connected between each letter due to the two-dimensional printing process. When able, Skeinforge will map paths that do not cross openings. When holes in the printing geometry are inevitable, as with Figure 7, the extrusion pump is programmed to retract a set distance to prevent any material deposition.

Something to note is that while Skeinforge slices the original model, the act of deposition actually creates a mirror image. While not mission critical for many operations, it does create issue when printing words.

Figure 7. Two-dimensional print of “Geaux Engineering.”
The pathing defined by Skeinforge will directly reflect the programmed capabilities of the printing tip. In Figure 8, a printing material diameter of 0.6mm is compared against the printing path of a 1.7 mm diameter filament. With a nearly threefold increase in resolution, Skeinforge is able to generate a much cleaner pathing profile and a more accurate three dimensional object.

![Figure 8: Generation of high resolution pathing (left) compared to low resolution (right) is dependent on printed filament diameter.](image)

### 4.3.2 Three-Dimensional Geometries

As a proof of concept, a simple one milliliter cube (10 mm x 10 mm x 10 mm) was printed. To achieve three-dimensional samples, Neosporin was used as a printing material due to its viscous gel nature. A food coloring additive was also used with the printing fluid to aid in visualization. While the drawing functionality was challenged by the torque generated by dragging the ink tip across a page, three-dimensional printing experienced no such issues. Utilized, as intended, as a noncontact based printing platform. It is recommended that the generated G-code for three-dimensional deposition be manually altered slightly. The initial
coding generated by Skeinforge often actuates the Z axis to the printing height before the moving the X or Y axis to position. This can cause catastrophic collisions, damaging the motor couplings or destroying structural components.

Using the method described in Section 4.2.2, the 39 mm extrusion length desired by Skeinforge was modified to match the 8.35 mm travel distance required to extrude 1 mL. Pre-print volume can be compared against post-print volume in Figure 9.

Figure 9. 1.6 mL were present in the syringe prior to printing while only 0.6 mL remained post print.

Figure 10 shows the printing in process. Figure 11 presents a printed cube with approximate dimensions of 1 mL x 1 mL x 1 mL. The filament diameter and extrusion height, programmed as 1.7 mm, were estimated from an extruded sample of Neosporin.
Figure 10. A third cube is printed in the same Petri dish as two previous printing jobs.

Figure 11. A printed construct measured at approximately 10 mm across the X (left), Y (center), and Z (right) axes.
Figure 12 shows the repeatability of the platform as five cubes were printed, with three of the cubes placed in the same petri dish. While the current set up allows only for a single source to print at once, multiple syringes printed from in a serial manner within the same culture plate could allow for co-culturing of different cells types.

Figure 12. Five cubic structures printed as a proof of concept for three dimensional control with a fourth axis acting as a micro extruding pump.
Chapter 5: Conclusion and Future Work

5.1 Conclusions

The work presented in this thesis aims at developing a cost-effective bioprinter for academic research. Background considerations of tissue engineering were explored and the three main bioprinting technologies (inkjet printing, laser-assisted bioprinting and microextrusion printing) discussed. Ultimately, a variation on microextrusion was deemed appropriate and selected as it seemed most appropriate for the established goals and constraints.

The developed platform delivers three-dimensional spatial control with movement step intervals of approximately 20 µm in the X and Y axes and roughly 51 nm in the Z axis. Due to the relatively low power requirements of the novel bioprinting system, stepper motors were used to actuate tridimensional motion. To control the Z axis, two motors were slaved to the same driver, creating parallel motion.

By incorporating a non-captive stepper motor into the gantry, a simple, yet effective syringe pump was implemented. The syringes used to dispense fluids are regarded as a single use attachment to the platform. This mitigates maintenance overhang as all biohazardous or biowaste materials are contained to the single syringe. Care should be observed when choosing syringe needles as both the gage and length can have deleterious effects on cells.

Associated with the pump and cellular deposition is the laser light activation attachment. Connected to the underside of the gantry and directed along the X axis, a laser pen 365 nm wavelength is attached. Angled such that the cells are irradiated with light before being printed, it offers inline capability to active nanoparticle functionalized cells. While this design was chosen for ease of implementation, in the case of future studies in which the optical line of sight between the laser and syringe is inhibited, the laser can be placed such that it irradiates vertically into the waiting substrate.
Acrylic was chosen as a structural material due to the ease of machining and its optical clarity. While acrylic blocks the UV spectrum of light, preventing interior surface sterilization, it allows for visual inspections to detect possible contaminant colony growth. In the case of such growth, or before its initial installation, the bioprinting platform needs to undergo sterilization. Due to the glass transition temperature of PMMA, autoclaving is not an option. Instead, Ethylene Oxide treatment is suggest. During day to day operations, dimethyl benzyl ammonium chloride (Clorox) disinfectant wipes should be used for sanitation purposes.

To mitigate the learning curve associated with utilizing a new system, Mach3 was chosen as a control program. While probable most researchers in bioengineering fields have not worked with Mach3 before ArtSoft, the Mach3 developers, offer extensive documentation to ease the barrier to entry. Additionally, Mach3 supports a robust and enthusiastic community that is constantly offering technical support and additional utility such as the add-on that enables the use of a 4th axis as a pump. Mach3 generated G-code was used in the creation of two-dimensional patterning and simple three-dimensional geometries.

5.2 Cell Printing

While time constraints did not allow for the printing of cellular constructs, literature shows that the dispensing of cells through a syringe (the incorporated printing method) imposes minimal stress and can reasonably deliver 85% - 95% viability in comparison to controls (Aguado et al., 2011; Amer et al., 2015; Mamidi et al., 2012; Walker et al., 2010). Aguado et al. (2011) explore the potential of viscoelastic fluids and gels such as calcium cross-linked alginate, which improved viability significantly. Mamidi et al. (2012) showed that ejection through a 26 G bore size needle imparted no detrimental effects to the cells. However, at gages 30 and higher, a trend appeared in which viability started dropping the smaller the diameter (Amer et al., 2015).
The literature has shown that cellular deposition is possible via syringe and needle. To garner the best results, interactions between the bioink, print speed, and substrate need to be optimized.

5.3 Osteogenic Differentiation

This printer was designed as a preliminary project for single step deposition and activation of mesenchymal stem cells. Qureshi et al.’s (2013) study can be reproduced with single step seeding and light mediated osteogenesis. In brief, functionalized silver nanoparticles will be light activated via the onboard laser mediating microRNA delivery resulting in increased alkaline phosphatase activity and mineralization of the MSCs. Due to the reduced power of the laser pointer, an extended period of approximately 30-40 seconds of exposure will occur before cellular printing. Additional future work may incorporate a higher resolution laser device, allowing for side by side co-culturing of multiple light activated tissues.
References


Appendix A
Bill of Materials
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**Title:**

A

**Phase 5 Shuttle Spacer**

**Size:** 4:1

**Weight:**

**Sheet 1 of 1**
Vita

Colton McElheny received his Bachelor of Science in Biomedical Engineering from Texas A&M University in 2012. That same year he applied and was accepted into the Louisiana State University Graduate School for Mechanical Engineering. During his tenure at LSU he worked as a Research Assistant in the School of Mechanical and Industrial Engineering and as a Teaching Assistant working with undergraduate students in the School. He will graduate with a Master of Science in Mechanical Engineering (MSME) in December 2015, with the intention to gain practical experience in industry before pursuing a Doctoral degree at a later date.