INTERNALIZATION OF F-ACTIN MONOMERS INTO 3T3 FIBROBLASTS VIA THERMAL INKJET PRINTING FOR INVESTIGATION OF CYTOSKELETON INCORPORATION AND MECHANICS

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ABSTRACT

We will review the conversion of a standard thermal inkjet printer into a bioprinting system and the effects of printing F-actin monomers with cells. The use of any printing system along with biological material or for biological or medical use has been termed bioprinting. Bioprinting has been used in vascular grafts, scaffold design, gene transfection, micro patterning and many other applications and is very diverse. Specifically we will look at the internalization of F-actin monomers into 3T3 fibroblasts as a result of cell membrane disruption from thermal inkjet printing. If the actin monomers were internalized and then incorporated into the cytoskeleton, further investigation of cytoskeleton organization, construction and response to mechanical loading from atomic force microscopy could be conducted.

First, a bioprinter had to be modified from a standard printer. An HP Deskjet 500C and an HP Deskjet 500 were used. The only difference is that the HP Deskjet 500C is a color printer and has a different type of cartridge. Both the printers themselves and the ink cartridges that accompanied them had to be modified to accommodate cells and F-actin monomer solution. The printer and cartridges were customized for the application of printing cells. A proof of concept was performed first to see if the converted HP Deskjet 500 could indeed print viable cells without any marked decrease in viability and function.

After finding that the cells that were being printed were not only viable, but also continued to grow until confluence it was decided to print the cells along with the
fluorescently tagged F-actin monomers to see if monomers could be internalized by the printed cells. Fluorescence microscopy confirmed that the monomers could be internalized by the cell before the damage to the cell membrane could be repaired.
DEDICATION

This work is dedicated to my parents, Gordon and Susan, who supported and encouraged me to achieve as much as I could and allowed me to overcome all of the obstacles that I encountered.
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CHAPTER 1
INTRODUCTION TO BIOPRINTING

The field of bioprinting is vast and has many applications. Bioprinting can be defined as “the selective deposition of ‘boinks’ of biologically active components including proteins, peptides, DNA, cells, hormones (including cytokines, growth factors and synthetic hormonal signaling peptides), ECM molecules and native or synthetic biopolymers” [4]. This means that the application order of magnitudes range from small molecules such as DNA up to implantable scaffolds. Many different types of printers have been used. Some of the setups are 2-Dimensional and some are even 3-Dimensional. Some bioprinters are printers that have been modified from preexisting printers to accommodate their new functions while others have been completely customized from scratch to fulfill a specific niche.

Bioprinting has been used in tissue engineering such as in formation of implantable scaffolds to printing of actual vascular tissue. Direct cell application therapies, and biosensor microfabrication have also been functions of bioprinting [1-6]. In addition, more recently, gene transfection has been conducted using bioprinting systems [7-9]. To summarize, bioprinting can be used in many facets of the bioengineering landscape. The reason that bioprinting has been helpful in a variety of fields of study is the techniques ease of uses; it is sometimes as easy as just supplying a simple document for printing. Figure 2.1 shows an example of a three dimensional bioprinting system.
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Figure 1.1 3-Dimensional Bioprinter

BIOPRINTING IN TISSUE ENGINEERING

Tissue engineering is a broad fast growing field that is producing therapies for different pathologies present in medicine. “Tissue engineering, also referred to as regenerative medicine, represents the convergence of science, engineering and clinical disciplines in order to understand the underlying biology of tissue development, homeostasis and repair, and then apply this knowledge to develop therapies that re-establish tissue and organ function impaired by disease, trauma or congenital abnormalities” [4-5]. There are many different approaches in tissue engineering; however most approaches utilize a combination of cells, extracellular matrices or scaffolds, and hormones or other signaling molecules. These are what are considered tissue building blocks [4]. Currently, researchers are investigating the use of tissue engineering for the treatment of pathologies to many different tissue systems such as
skin, cartilage, bone, blood vessels, skeletal muscle, bladder, trachea and myocardium. Basically, in current research, almost all types of tissues are trying to be substituted or regenerated via tissue engineering. There are three main approaches to tissue engineering [5]:

1. Biomaterials are used to direct host cells to differentiate and regenerate injured site.
2. The pathological site is supplemented with cells and factors that induce regeneration and healing.
3. Utilization of scaffold with seeded cells via in vitro bioreactor to simulate the architectural and microenvironmental implanted condition.

Recently bioprinting has emerged as a useful tool in tissue engineering. This comes as no surprise as printing in general can create specific patterns in a controlled manner. Bioprinters are able to create biomimetic constructs based on spatial organization that can be produced by computers. The capability of producing complex biomimetic implantable tissue constructs is essential in the tissue engineering field.

SCAFFOLD BASED TISSUE ENGINEERING

Though bioprinting is not perfect in the realm of tissue engineering, it has its advantages when compared to approaches from earlier methods of regenerative medicine. The first methods that were used in tissue engineering were attempts at combining cells, hormones and growth factors with implantable scaffolds with no complex inherent design as a sort of blue print for surrounding tissue for repair. The problem is that these simple
scaffolds have no spatial complexity and are limited by shapes. Bioprinting allows for spatial complexity and patterning that could be more biomimetic [4]. Computer aided deposition of cells allows bioprinting to create biomimetic structures. One example of how spatial control is important is the orientation and differentiation of musculoskeletal tissue. Geometric and biochemical cues that reside in the extracellular matrix in the form of growth factors are essential in the differentiation of stem cells in vivo [10]. In this application fibers that are similar to fibers that are present in vivo that direct stem cell differentiation are printed by a bioprinter to try and mimic the nature differentiation of the stem cells. Without the use of the printers, the stem cells would not have the necessary geometric and cellular stresses provided by the printed fibers and would probably differentiate differently in vitro than without the polymers providing stresses and substrates for cell differentiation.

As stated before, scaffolds are an important part of tissue engineering. Scaffolds can provide structural integrity while potentially providing the necessary growth factors and stimuli that cells need to differentiate properly and to function correctly in vivo. There are many different materials that are used in the production of tissue engineering scaffolds. Biopolymer scaffolds use materials such as alginate, fibrin and chitosan are popular because of how each of these materials can help mimic the microenvironment of cells that are seeded onto them. Scaffolds function in a critical role of extra cellular matrices for cell growth and differentiation. The idea of scaffold based tissue engineering the use of scaffolds in conjunction with the patient’s own cells to provide a structure for new tissue ingrowth and proliferation in pursuit of therapeutic healing. Figure 1.2 below
shows poly(lactic-co-glycolic acid) porous scaffold that can be used in bone tissue engineering.

![Scaffold for bone tissue engineering](image)

**Figure 1.2** Scaffold for bone tissue engineering

Lately bioprinting has become a method for scaffold creation. Bioprinting has shown promise over other scaffold formation methods such as fiber bonding, solvent casting, particulate leaching, melt molding, gas foaming, and soft lithography by its capability of high resolution shape control and consistency [11]. This is evident in one study in which bioprinting was used for endothelial cells with alginate to create 3D tissue constructs. A multi nozzle computer-aided modeling system was formulated to create heterogeneous tissue scaffolds. The resolution of the system that was created was 10 µm.
The computer model was converted into layers by software created by the authors. The layers together created a biomimetic scaffold [11].

Cell viability tests were conducted of the seeded cells on the construct. This is because during the printing process cells go through shear forces that could be detrimental and cause harm. The standard calcein and ethidium homodimer (EthD-1) were used for the cell viability study. The authors found the viability of cells as around 75-85% for cells that were encapsulated onto the alginate hydrogel scaffold. This finding showed that cell viability after printing is not a huge concern. Although the property of cell viability varies between bioprinting systems, it is generally not a concern. The authors concluded that bioprinting was a unique tool in scaffold construction by its ability to incorporate the scaffold along with controlled amounts of cells, growth factors, or other bioactive compounds with precise spatial position to form finalized constructs [11].

Mass transport is also a major consideration in the design of tissue engineered scaffolds. Most cells can only be viable when they are capable of nutrient exchange mostly in the form of vascularization. Cells can only survive as far as diffusion can take oxygen and nutrients. This critical distance is around 100 µm. Computer aided design and the high resolution (micron scale) that can be achieved with bioprinting can help to ensure that no part of the scaffold that is being printed will go without blood supply and eventually fail.

Even though bioprinting can replicate the spatial structure of biological tissue it leaves much to be desired. Duplicating the complex nature and functionality of in vivo conditions is difficult to accomplish by just using the ‘building blocks’ of tissue even
when spatial structure can be duplicated by means of bioprinting. Although structure follows function or vice versa, replicating structure in itself is not enough to create regenerated tissue engineered structures that will function properly in vivo. Some of the problems with scaffolds are their degradation and the immunogenic response that follows [12].

**NON SCAFFOLD BASED TISSUE ENGINEERING WITH BIOPRINTING**

Self-assembly and self-organization are approaches that are starting to be used in regenerative medicine as an alternative to formation of tissue scaffolds and in vitro culturing for implantation. Self-assembly and self-organization are a part of a new wave of methodology being pursued in the tissue engineering field. It is based on the harnessing of the regenerative capabilities already present in the body. Histogenesis and organogenesis are examples of self-assembly processes that are already present in the body. This new wave of tissue engineering hopes to utilize the body’s capabilities that are already present in differentiation of cells from undifferentiated cells such as the three main germ layers during development. The liver’s outstanding regenerative capabilities are also a major caveat in the new self-assembly avenue.

Possibly the best example of self-assembly comes from L’Heureux and colleagues with the use of sheet based tissue engineering [12] and [13]. In sheet based tissue engineering sheets of human smooth muscle cells and fibroblasts were cultured in the presence of ascorbic acid in efforts to increase the production of collagen. The sheets were then detached and wrapped around a tubular mandrel to create a tube form mimicking the parts of blood vessels including discernible media and adventitia as
illustrated in Figure 1.3. Endothelial cells were then seeded onto the lumen of the construct creating an autologous small diameter blood vessel that is currently in clinical trials [13]. Bioprinting could also be used in this application. Instead of culturing sheets of the different types of cells used in the construct, printers could be used to deposit cells in a more in vivo architecture than just in vitro culturing.

Figure 1.3 Representation of blood vessel organization

In work by Karoly Jakab et al. self-assembly of printed cells into topologically defined structures were used in tissue engineering applications [14]. ‘Bio-ink’ particles that consist of multicellular spheroids were placed onto ‘bio-paper’ or a biocompatible environment by a bioprinter. The bio-ink particles were taken from Leghorn chicken embryos and isolated into single cells. Human endothelial cells were used. The two types of cells were then centrifuges at a ratio of 4:1 with the larger proportion being the cardiac cells. The cell pellet was the incubated for 10 minutes and transferred to an in
house apparatus that extruded the cell pellet. The cell pellet was then cut into equal sized cylinders which rapidly changed conformation into spheroids. These spheroids are what were used in the printing process as bio-ink particles. The researchers wanted to mimic early morphogenesis by physical mechanisms in genetic control of patterning through self-assembly. After the bio-ink particles were printed they fused and formed more complex structures. This is analogous to embryos in early structure forming processes. Multiple types of tissues were created using this method but functionality tests were conducted on printed cardiac and endothelial cell bio-ink particles. When tested the fused cell particulates showed signs of primitive blood vessels with the endothelial cells exhibiting vascularization. The printed bio-ink multicellular spheroids that fused were also able to synchronously beat like normal heart tissue.

BIOPRINTING IN GENE TRANSFECTION

Gene transfection is the introduction of plasmid DNA or siRNA into the cell. Transfection is usually done by creation of a hole in the cell membrane that allows for the transfected material to be integrated into the transfected cell. Transfection can occur from many methods such as: chemically and non-chemically based, particle based and viral methods. Some researchers recently have been exploring the use of inkjet-mediated gene transfection as an alternative to the previously stated methods. Inkjet printing was theorized to be a good alternative to other methods of transfection because of the stresses put on printed cells could create transient membrane pores in which the transfection material could be incorporated. Figure 1.4 shows the cellular response that creates
efficient transfection of genetic material and a standard method in which transfection is conducted.

Figure 1.4 Illustration of genetic transfection along with standard method of transfection injection.

A group with Xu, T. et al created simultaneous gene transfection and cell delivery with inkjet printing [7]. They used plasmids that were fluorescently tagged with green fluorescent protein (GFP) and printed with live porcine aortic endothelial cells. The porcine aortic endothelial cells were cultured, trypsinized, and suspended in a buffer at a concentration of 1.5-2×10^6 cells/mL. The plasmids along with the cells were directly placed into the ink cartridge of a standard inkjet printer and printed onto collagen substrates. Results show that GFP expression was over 10% and the viability of cells after transfection occurred was over 90%. The cell viability was evaluated by a standard tetrazolium compound (MTS) assay. The transfection efficiency was calculated after twenty four to forty eight hours of incubation. The cells were fixed and stained with DAPI at 10 mg/mL and then imaged for GFP expression. The calculated proportion of
transfected cells was found by comparison of live cells present against live cells present that also had GFP expression evident. Also, the transfected porcine aortic endothelial cells were successfully delivered to target sites in a scaffold made of fibrin gel and was implanted into mice. The GFP was expressed both in vitro and in vivo in the mice.

The means by which effectiveness was tested in vivo was direct printing of cells and plasmids onto fibrin glue solutions into the subcutaneous tissues of athymic mice. The fibrin glue and cells then formed in situ. The implant was then retrieved after one week of implantation. Fluorescent microscopy showed that the GFP was still being expressed even after a week of implantation and by direct printing into subcutaneous tissue of mice.

The authors found that print conditions have a profound effect on transfection efficiency. In their experimental design, two different cartridges were used, one was found to be much more effective at creating the disturbances necessary for incorporation of the DNA plasmid.

DIFFERENT TYPES OF BIOPRINTING

Inkjet printing is a rapid, versatile, and easy technique for bioprinting. In addition, it is relatively low-cost compared to other bioprinting techniques. Inkjet printing is also considered rapid and versatile. Small molecules, single cells and clusters of cells are easily printed by using inkjet printing. This makes it easy for smaller structures to be evaluated by themselves or in close packs without much outside influence. One outcome
that can be considered an advantage or drawback depending on the application is be that during the process of printing, the cell or molecule being printed will experience some damage due to the high speed of deposition, mechanical and heat stresses applied. Inkjet printing is the type of bioprinting that was utilized in our research for this reason as thermal inkjet printing and its effects were desired in our application. Inkjet printing also lacks the ability to spatially organize compared to more complex systems.

The other main approach in bioprinting is the ‘mechanical extruder’ approach [14]. Particles and multicellular aggregates are placed into a supporting environment. The aggregates are of known composition and act as tissue fragments in three dimensional forms. These aggregates can be then printed into a certain topology and structure with the aid of computer generated templates [14]. The advantages of being able to print aggregates are that when the cells are printed, they remain together in a more physiological form than in inkjet printing. They are still in contact with neighboring cells and intercellular signaling is possible

Bioprinting has also created an alternative in gene transfection that can improve efficiency and cost. In our work we will try to utilize bioprinting in a way to incorporate fluorescently tagged F-actin into fibroblasts to characterize the internalization and incorporation of the F-actin monomers. A standard inkjet printer will be used to create transient membrane pores. We will also attempt to characterize cytoskeletal response to mechanical loading from atomic force microscopy by utilization of the F-actin monomers.
In conclusion there are really limitless possibilities for the applications of bioprinting. Bioprinting has also only begun to show its potential in the field on Bioengineering. Bioprinting is just an emerging field but already has numerous applications and will only have more in the future. Tissue engineering and gene transfection are two fields that are vastly benefiting from bioprinting. In tissue engineering better scaffolds are being created to increase the compatibility and effectiveness of constructs that are implanted into the body. This increase of effectiveness can be explained by the spatial resolution and organization that can be produced by accurate bioprinting systems.
CHAPTER 2

THE ROLE OF F-ACTIN AND FIBROBLASTS

Before continuing it is important to know the characteristics and roles of the two major components of the research being conducted. The two main structures that were evaluated and utilized were the F-actin monomer and the 3T3 Fibroblast.

F-ACTIN

Actin in one form or another is found in almost all eukaryotic cells. Actin has many abilities and functions in the cell. Actin mainly resides in the cytoplasm portion of cells with a few exceptions such as nuclear actin. Monomers of actin have a relative molecular mass of around 43,000. Figure 2.1 shows the molecular structure of the actin protein along with its subdomains and functional groups. Actin monomers have the ability to self-assemble into filaments which are important for cytoskeleton organization and assembly. Actin monomers in globular form, or G-actin, can polymerize to form actin polymers which can be called F-actin, actin filaments or microfilaments. The filaments that are formed from the polymerization of G-actin monomers are a double helix shape. These actin filaments can go through even more bundling or cross-linking into more complex structures as a result of signal transduction pathways. The organization of bundles and groupings of actin filaments is essential to the overall integrity of the cytoskeleton [15]. Actin is present in the cell in two main categories. The first category is a fine meshwork in the cytoplasm. The second is as bundles of parallel filaments that are attached to the cell membrane via alpha-actinin.
Figure 2.1 Actin monomer

Although the actin filaments are essential to the mechanical structure of the cytoskeleton, their half-life can be measured on the order of minutes. This large amount of overturn in actin filaments make the polymerization and depolymerization of actin monomers and filaments a precious balance. The influences that control the balance of equilibrium in actin are hormones, growth factors, internal differentiation signals and extracellular matrix signals. The polymerization and depolymerization mechanics are dictated by the proteins cofilin and profilin. Cofilin is the protein that causes actin filaments to depolymerize while profilin can enhance or encourage the polymerization of actin monomers. Figure 2.2 shows the mechanisms of actin polymerization and how it is an energy dependent process.
Actin and the cytoskeleton are important in the viability and functionality of cells. It provides a structural framework around which cell shape and polarity are defined, it allows cells to move and divide, conducts receptor-mediated endocytosis, secretion, protein sorting, mRNA localization, spatial ordering of glycolysis, spindle formation and organization and signal transduction [16] and [17]. It is obvious that actin monomers and their polymerized counterparts are very important in a vast amount of applications in the cell. Actin filaments are present in the cell cytoskeleton as stress fibers. The stress fibers are oriented in directions of applied stresses onto the cell [18].

**ACTIN IN CELL MOTILITY**

Almost all cell types can move one way or another. Some cells move more than others by design, but excess or lack of movement can also be a sign of pathology.
Nevertheless the actin cytoskeleton is considered to be the primary means by which cells perform crawling motion.

![Diagram of cell crawling modality of movement](image)

Mitchison T J.

Figure 2.3 Cell crawling modality of movement

Cells may move for many different reasons. Inhibition of motion such as in bone and cartilage, motion during the wound healing process in the form of granulation tissue and endothelial cells and readily moving cells such as neutrophils may be possible. Cell motility involves a combination of the cytoskeleton, cell membrane, and adhesion. Coordination of these mechanisms and structures are necessary for cell motility. The standard means by which a cell moves by crawling can be described as a forward protrusion creating adhesion and traction to generate intracellular and extracellular forces for movement. The result is de-adhesion and tail retraction as shown in Figure 2.3 [19].
High concentrations of actin filaments organized into arrays characterize the protrusion that begins cell motility. The simplest form of the region with actin arrays that protrude away from the main cell body can be named a filopodias. A more complex form of cell motility structures come in the form of a lamellipodia. Lamellipodia motility is evident in cultured fibroblasts. The difference between lamellipodia (Figure 2.5) and filopodia (Figure 2.4) is that lamellipodia have actin filaments “organized as orthogonal cross-weaves” between different sets of filaments while filopodia only have actin filaments protruding in the same direction of movement [19]. Fibroblast lamellipodia are punctuated by microspikes, or small filopodia containing parallel arrays of actin filaments.

Figure 2.4 Filopodia created by actin filament network
Figure 2.5 Lamellipodia created by actin filament network

The presence of actin filaments at the leading edge of the cell necessitates constant polymerization of actin to continue movement. The only two ways in which the leading edge can extend is by either new actin filament polymerization or by extending existing actin filaments. There is a great debate on which one of these methods is used in the extending of cell motility structures [19]. Whichever method is predominant in cell motility a high amount of actin monomers for polymerization is needed in the area of the protrusion. The most likely candidates from which these actin monomers are harvested is the depolymerization simultaneously occurring and from other portions of the cell. For the cell to proceed forward, the rate of polymerization in the cell motility structure must be higher than the rate in which the rate of depolymerization of actin chains is occurring.

Much emphasis has been given on actin filament organization and deposition for movement but not much has been covered on how the actin helps create the forces needed for cell movement. It is theorized that the force needed for movement is
produced in the leading edge [20]. It is proposed that the force alone created by the extending of actin filaments can cause membrane deformation and eventually cell movement. Without going into too much detail, actin filaments rectify a thermal motion of a load to produce an axial force. The force that is created in the actin filaments present in the leading edge allows cell crawling motility [20].

ACTIN IN RECEPTOR-MEDIATED ENDOCYTOSIS

Receptor-mediated endocytosis is a process in which material is internalized into a cell by inward budding of the cell membrane in response to site specific receptors on the outside of the cell membrane being triggered by a ligand. Signals from the receptors covering the extracellular side of the cell membrane start a signal transduction cascade into the cytoplasm of the cell. The result in a clathrin coated vesicle. Opsonization of the vesicle allows from the vesicle to become an endosome. Figure 2.6 illustrates the mechanisms of receptor-mediated endocytosis.
Figure 2.6 Illustration of receptor-mediated endocytosis from ligand binding to uncoated transport vesicle.

As stated before in the review of actin in the cytoskeleton, the plasma membrane’s functionality is closely related to the neighboring and attached actin cytoskeleton or the cell cortex [21]. It should therefore come as no surprise that since these two structures are so closely related, the process of receptor-mediated endocytosis hinges on the involvement and function of cortical actin filaments. The actin’s involvement in receptor-mediated endocytosis is twofold. It is an important part of the directing of vesicle budding and fusion while also reorganization of actin filaments is essential in removing physical barriers of the inward budding cell membrane wall. Figure 2.7 shows the actin-myosin complex of the actin cortex stained in green. The red that is visible is blebbing from apoptosis. The blebbing and degrading of the cell allowed
the authors to gain more insight on the structure of the actin cortex along the cell wall of cells. This image shows how closely related the two structures actually are.

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Figure 2.7 Image of the actin/myosin cortex stained green

It has been found that receptor-mediated endocytosis is inhibited in the yeast S. cerevisiae that have had mutations in actin. Also, yeasts that were deficient in three different types of genes (END3, END5/VRP1 and END7/RVS167) important in the organization of actin filaments also proved to be inept at conducting receptor-mediated endocytosis. Evidence shows that the activation of Rho family GTPases has been shown to inhibit receptor-mediated endocytosis. This is probably because the Rho GTPases
stimulate the deposition of cortical actin filaments and create a physical barrier for the inward budding of the cell membrane [16]. More on the effect of Rho GTPases on the actin cytoskeleton will be discussed later. To make a final judgment on how important actin filaments are in the execution of cell-mediated endocytosis the examiners utilized drugs that sequestered actin monomers shifting the polymerization/depolymerization equilibrium vastly in the direction of depolymerization [21].

A perforated cell assay was used to finally confirm that actin filaments are indeed necessary in receptor-mediated endocytosis. High doses of Thymosin β4 or Tβ4 were introduced to cultured cells. Tβ4 is an actin monomer binding protein that reduces that amount of free actin monomers available for polymerization into actin filaments as shown in Figure 2.8.

![Graph showing the internalization of TfR vs Thymosin β4 concentration.](Modified from J Biol Chem 1997, 103, 20332-20335)
Figure 2.8 the effects of different Thymosin β4 concentrations on formation of clathrin pits compared to a control sample.

The assay was designed to measure the budding of preformed clathrin coated pits. The data shows that actin filaments are required for late events such as coated pit constriction from the cell membrane or vesicle detachment from the cell membrane. It is quite obvious from the schematic that as the concentration of Thymosin β4 increases the internalization of transferrin into the cell decreases steadily. The decreasing does not actually even flatten out at the highest concentration of Thymosin β4 that the authors used. This shows the extreme relationship between the actin filament network and the process of cell-mediated endocytosis. The higher proportion of clathrin pits present at low doses of Thymosin β4 has been theorized to be the effects of less physical barriers due lower concentrations of actin filaments at the cell membrane [21]. In conclusion, actin filaments are required in receptor-mediated endocytosis.

**RHO GTPASES AND THE ACTIN CYTOSKELETON**

As stated before, actin organization and functionality can be greatly affected by outside signals. One of the mechanisms by which outside signals effect actin filament formation is the activation of the Rho family guanine triphosphatases (GTPases) [16]. The Rho family GTPases that will be covered in this section are Rho, Rac and Cdc42. In 3T3 fibroblasts Rho activation leads to actin-myosin complex assembly and creation of focal adhesions and further organization of actin filament structures. Another form of
actin filament activation that has been discovered is the activation of Rac, another
member of the Rho family. Rac can be activated by platelet derived growth factor
(PDGF) or insulin which results in the formation of an array of actin filaments creating
lamellipodia. Activation of Cdc42 which is related to Rac results in the less complex
filopodia [16].

Fibroblasts are not the only cells that Rho GTPases have effects on in relation to
the creation and organization of actin filaments. Neuronal cells increase the length of
their axon with structures at the tip of their axons called growth cones. Actin
polymerization is thought to be driving the axon growth in the growth cone.
Macrophages also seem to be susceptible to actin filament formation for movement based
on signals through focal adhesions and Rho family GTPases activation [16]. In
conclusion the Rho GTPases act on extracellular signals to coordinate changes in the
organization of the actin cytoskeleton.

The activation of Rho GTPases and resulting actin filament formation is just one
of the mechanisms in which actin is effected by outside signaling pathways and illustrates
how actin and the cytoskeleton are susceptible to outside influences. The Rho family
GTPases and actin filament formation has also been shown to have importance in smooth
muscle differentiation [22]. The authors believe that RhoA, yet another part of the Rho
GTPases regulates both “SMC-specific transcription and stress fiber formation in SMC.”
ACTIN IN MITOSIS AND CYTOKINESIS

Mitosis is one of the most important parts of the cell cycle. Dividing and proliferating is paramount in tissue viability. All students learn of the mitotic cell cycle that includes prophase, metaphase, anaphase, and telophase or PMAT. Students also learn that chromosomes are duplicated and then distrusted equally during the mitotic process. As it turns out actin has a profound influence on the distribution and assembly of both the spindle fibers and the chromosomes that are being divided into the two new cells [23]. Cytokinesis is the last step of mitosis during telophase in which the cytoplasm of the two new distinct cells is divided and finally segregated from each other. Figure 2.6 shows the stages of mitosis with actin stained green.

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Figure 2.6 Cell Undergoing Mitosis with Actin Stained in Green (160x).
Although actin does not directly control the position and assembly of spindles that control the location of chromosomes during mitosis it does have a profound effect. Spindles and spindle-associated astral microtubules dictate where the actin-myosin contractile complex is located during cytokinesis. Alternatively during mitosis actin filaments can have an effect on the assembly and position of the mitotic spindle [23]. Overall actin filaments have been found to “regulate astral microtubule growth and spindle migration during syncytial divisions in Drosophila, control spindle positioning in Caenorhabditis elegans zygotes and mouse oocytes, influence spindle form and function in early Xenopus divisions, and have a crucial role in chromosome congression during meiotic division in starfish oocytes.”

Most eukaryotic cells that are preparing for mitosis ‘round up’ into a more spherical form before mitosis [23-26]. This rounding up that occurs simultaneously with nuclear envelope breakdown and chromosome condensation is a dramatic shape change. This shape change is only possible by the remodeling of cortical actin cytoskeleton neighboring the cell membrane. The coordinated change of the cell membrane and the cortical actin creates this drastic shape change in a matter of minutes [27-30]. The rounding occurs by the reeling in of lamellipodia protruding from the cell body which leaves actin rich extensions connecting to the substrate. The overall rounding of mitotic cells is necessary for correct mitosis.

The means by which actin filaments were found to be integral in the synthesis of spindle fibers during mitosis was by their inhibition. Inhibition of the Rho family
GTPase Cdc42 that was reviewed earlier caused defects in spindles [31-32]. In addition, a decrease in cell rounding can cause loss of cortical rigidity and further result in malformation of spindle which can lead to the misplacement of chromosomes [33]. After evaluating the inhibition of actin during the formation of spindle fibers, and noticing that correct spindle fibers cannot be formed without during inhibition, it is safe to say that actin filaments are crucial during the formation of spindle fibers for mitosis.

As stated before the actin cortex also has some control over the spindle positioning that are responsible for chromosome location. In order for correct division of chromosomes and cytoplasm for the two daughter cells the spindle position must be correct. If the spindle position is incorrect chromosome distribution can be unequal and cause mutations in cells that could cause functionality problems [34-35]. The actin fibers that connect the cell to its substrate act as a quantitative memory of the adhesion patterns generated in interphase and dictate spindle orientation [36].

Actin is not only important in preparation for mitosis but is essential in the last step of mitosis [37]. Figure 2.6 easily shows the formation of what is called a contractile ring with the use of actin monomers expressing GFP. Actin is a vital part of the contractile ring that generates the forces needed to finally separate the two daughter cells [38]. A complex of actin, myosin II and other structural and regulatory proteins assemble at the equatorial cortex of the daughter cells and contract in a manner similar to a purse string to divide the two daughter cells [37]. Contractile ring assembly has commonly been studied by the use of fluorescence recovery after photobleaching (FRAP) by
studying the turnover of actin monomers that express GFP. Results showed that there was actin recruitment to the site of contractile ring assembly by GFP-expressed actin returning to the site of photobleaching. The results inevitably show that there is a necessity for actin to be present in the contractile ring to perform cytokinesis [39-40]. Combine these results with the knowledge that actin is also an important factor in earlier stages of mitosis it is fair to say that actin plays an important role in cell mitosis though it does not normally receive this distinction.

INTRODUCTION TO FIBROBLASTS

The cell type that was printed the most in our research was the 3T3 Fibroblasts. This section is for a basic understanding of fibroblast structure and function. Fibroblasts have an elongated shape with a centrally located oval nucleus. Fibroblasts can be extremely active and mobile such as during wound healing or immobilized such as how they are in dense collagenous tissues such as tendons. Fibroblasts have many functions and differ greatly in these functions but their most important one seems to be the ability to synthesize extracellular components such as the proteins collagen, elastin and glycosaminoglycans (GAGs) which are structures that contain amino acids, amino sugars and other sugars [41].

The attribute of being able to make collagen makes fibroblasts very helpful during the wound healing process. The fibroblasts actually synthesize and secrete procollagen, the unpolymerized form of collagen that is present in most extracellular matrix capacities.
The secreted procollagen is converted into α-collagen which in turn cross-links into functional collagen. This process takes place just outside or at the cell membrane of the fibroblast [41].
CHAPTER 3

CONVERSION OF HP DESKJET 500 AND CARTRIDGES

Portions of this Chapter were published in:

ABSTRACT

Bioprinting has shown great promise in many fields of study. It is also a very diverse field of both hardware and applications as illustrated in Chapter 1 on Bioprinting in general. As stated before there are mainly two different types of bioprinting systems with the first being inkjet printing and the second being extruders. This chapter will exhibit the conversion process of a standard Hewlett Packard Deskjet 500 printer and its matching Hewlett Packard (HP) Black 26 Cartridges.

INTRODUCTION

The Hewlett Packard Deskjet 500 or HP Deskjet 500 is an inkjet printer that only prints in black and white. There is an HP Deskjet 500C that doe have color capabilities but was not used extensively in this research. It is 17.2 inches in width by 7.9 inches in depth and the height is 14.8 inches. Its documented print speed is up to 240 characters/second and connects to the computer using a 1.0 x parallel cable with 36 pins.
The printer is also capable of 300 dots per inch (DPI). DPI is a standard measuring technique for printer resolution. Inkjet printing has a resolution of around 300-600 DPI while more modern laser printing is more in the range of 600 to 1800 DPI. The HP Deskjet 500 is capable of handling paper sizes of 7.25 in x 10.5 in, 8.5 in x 14 in, 8.5 in x 11 in and 8.25 in x 13.7 in. The paper feed mechanism is mechanical and nature and for our applications will need to be bypassed. Drivers for the printer are preloaded in most computers but can also be found easily on the internet by simply going through the Hewlett Packard software page.

MATERIALS AND METHODS

This section will provide the protocol for the conversion of the HP Deskjet 500 and the HP Black 26 Cartridges. Figure 3.1 and 3.2 are the hardware before the conversion process has taken place. The protocols below were taken from previously published work in the Journal of Visualized Experiments (JoVE) on March 16, 2012 entitled “Creation of Transient Membrane Pores Using a Standard Inkjet Printer.”
Figure 3.1 HP Deskjet 500 unconverted
Conversion of HP Deskjet 500 Printer

It should be noted that this technique should work with many commercially inkjet printers. However, older printers tend to work better as they use ink cartridges with larger diameter nozzles, which do not clog as easily. In addition, older printers tend to use mechanical paper feed sensors that are easier to bypass. Printers with optical sensors can be tricked but using a small strip of paper on the far edge of the printer during each cycle, but is much more difficult than the mechanical system to “trick”. In order for current commercially available printers to work the resolution (DPI) must be low. The resolution of the HP Deskjet 500 is 300 DPI. The cheapest printer in the HP Deskjet series has a resolution of 600 DPI. This printer could be used but can cause more problems with clogging of printer head.

1.1. Remove the top plastic case of the printer by unlocking several plastic clips from the bottom base of the printer and slowly lifting the top off.

1.2. Unscrew button/display light panel from top of printer, leaving it connected to the printer’s motherboard.

1.3. Clean the inside of the printer, especially the areas where the ink cartridge rests and where printing occurs.

1.4. Locate the cables supplying power to the paper feed mechanisms and unplug them from the motherboard.
1.4.1. In the HP Deskjet 500, these are found just below the paper tray towards the left of the front

1.5. Locate paper detection mechanism. Bypass the mechanism by affixing a string or wire loop to serve as a manual pull handle

1.5.1. In the HP Deskjet 500, the paper detection mechanism is a gray plastic lever found above and behind the printing/paper feed mechanism

1.6. Create a stage in front of the paper feed mechanism (where the paper would be fed from and deposited) in order to bring the desired printing slides to a level just below the cartridge print head.

1.6.1. For our experiments, the foam shipping holder for 15mL centrifuge tubes was used with several microscope slides taped in place in the printing region to bring the final level of the slides to the desired height.

1.7. To maintain aseptic technique, the printer can be placed inside a standard biohazard cabinet or tabletop laminar flow hood.

1.8. It is important to understand that modifying the HP Deskjet 500 and using it not for its intended purpose will compromise the warranty.

Conversion of HP Black Cartridge

1.1. Remove the cartridge from its packaging, leaving the protective tape covering the printer contacts and print head for the time being.
1.2. Stabilize the body of the cartridge (black portion) either with a clamp or vise (simply firmly gripped the cartridge by hand usually worked as well), leaving the green top clear of any obstacle.

1.3. Using pliers or an adjustable wrench, grasp the green top of the cartridge and twist back and forth several times until it breaks free.

1.3.1. This should not take much force, but the top is no longer needed, so it is okay if it breaks when removing it.

1.4. Using screwdrivers, pry off the clear plastic piece now exposed.

1.4.1. Again, this should not take much force, but it will not be needed, so it is okay if it breaks during removal.

1.5. Empty any remaining from the reservoir.

1.6. Remove the plastic protective tape covering the printer contacts and print head.

1.7. Thoroughly flush the reservoirs with water.

1.7.1. Use a pipette or syringe to push water through the channels

1.7.2. Water will likely leak from the print head during this process; this is acceptable, and does not cause any damage to the functionality of the cartridge.

When the water runs clear, allow the cartridge to dry

RESULTS

The result of both of the conversion processes protocolled above are a printer and cartridge that have the ability to print cells and other materials onto a cover slip
positioned in the printing zone. The outer shell of the printer was taken away exposing
the innards of the printer. The paper feed mechanism was bypassed to allow for the
printer to work without sensing there was no paper constantly. Finally, a stage was built
for which the cover slip could reside and have cells deposited onto it via the printing
process. The cartridge that accompanies the printer was also formatted so that it can
accommodate cells suspended in solution as well. Figure 3.3 shows the final bioprinting
complex and Figure 3.4 shows the final product of the cartridge conversion process.

Figure 3.3 Final product of conversion process on HP Deskjet 500
DISCUSSION

The process to convert a standard inkjet desktop printer for bioprinting is not particularly difficult. The most challenging step is determining how to bypass the paper feed mechanism, which is dependent on the brand and model of printer used. However, this is relatively simple when the paper feed sensor is mechanical as described here. For models with optical feed sensors, other techniques may need to be employed to trick the printer into thinking it is using paper; for instance, one can run a small piece of paper through the printer while it prints on the microscope slide. Bypassing the paper feed mechanism is likely to be the most difficult step in applying these procedures to different printer models.
When constructing a stage to hold the coverslips for printing, it is important to ensure proper alignment and height. The stage should allow for the coverslip to be placed in the middle of the printing area. In addition, it should place the coverslip at an adequate height to allow clearance for the print cartridge to pass over the slide without disrupting it. The height of the stage must also not be too low for the printed solution to deposit immediately onto the cover slip. The exact height of the stage will depend on the printer model.

A major limitation of the design described in the above steps is that this printer is not capable of printing in more than one dimension. This limits the potential for use in patterned applications such as scaffold printing. To allow for 3D printing, a specialized stage needs to be used. The stage needs to have incremental height adjustments for layer-by-layer deposition of material. The height adjustment would allow for the printer head to be moving in only two dimensions but would have the stage create the third dimension of the printed material by creating deposition of material in the height dimension.

CONCLUSION

The conversion process had its fair share of trial and error in trying to figure out the slightly dated technology but was eventually concluded. Reliability of this technology was also suspect and was thought to create some problems in being able to replicate experiments later down the line in the research of bioprinting. The next portion of the research had to do with the application of the newly developed system.
ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Dean for helping us find the printer online. The process was much more difficult than anticipated. I would also like to thank Alexander Owczarczak for helping in the conversion process.
CHAPTER 4
INTERNALIZED G-ACTIN MONOMERS AND CYTOSKELETON

ABSTRACT

The conversion process of the hardware was completed. This process was illustrated in the previous chapter. This part of the thesis will cover the application of the converted bioprinting system through the use of fluorescently tagged g-actin monomers and 3T3 fibroblasts. It was thought that the use of inkjet printing could deposit single cells onto a substrate. The term inkjet printing is interchangeable with the term Drop on Demand (DOD) printing. The term defines the technology. It means that drops can be placed in an orderly manner based on the file that was submitted. This capability goes back to previous sections about how bioprinting and its inherent special organization can be useful in precise deposition of material.

Before even attempting to print cells the new printer needed to be tested to see if it could actually perform the application that we wanted it to. We were worried that its advanced age and condition would prevent us from being able to print live cells. We were also somewhat skeptical of the source in which we were receiving the printer. The cartridges also needed to be tested to see if they would clog. A simple solution of Phosphate Buffered Solution and food coloring was used to test the six cartridges in our possession to evaluate the effectiveness of each cartridge.
Inkjet printing was used as a mechanism to incorporate g-actin monomers into the 3T3 fibroblasts by disturbing the cell membrane. This disturbance was caused by the inkjet printing process itself. The work of Xiaofeng Cui et al. with the use of inkjet printed Chinese hamster ovary cells was used as a template [42]. It was shown that though there is damage to the cell membrane, the damage is transient and in certain cell types is repaired within a few hours. The ability to create membrane pores without causing lasting damage and cell lysis was thought to be a very useful tool for internalization of g-actin monomers. Although the applications were similar, much of the data verified using the different cell type and printing solution.

Printing began with just 3T3 fibroblasts suspended in PBS alone to investigate if there would be any problems associated with printing cells with the particular printer in our possession. It was found that the cells were easily printed for a while but clogs quickly because a problem in the older technology. This made sense because ink would regularly cause clogs in the printer during normal operating, adding masses to the printing solution only made things more arduous for the old printer and cartridges that were being used. Next we wanted to test the viability of the cells that were printed through the new bioprinter.

It was found that cell viability was around 78%. The reason that a live/dead assay was performed was because it was theorized that the inkjet printing could cause too much damage to the cell membrane for the cells to remain viable after printing. The value of 85% seemed to be comparable to other cell viability studies that had been conducted after
inkjet printing. The high viability of cells that were printed also allowed us to continue on in the attempt to internalize g-actin monomers.

INTRODUCTION

The entire project hinged on whether the inkjet printer could create sufficient damage in the cell membrane for g-actin monomers to be internalized. It was obvious that it was important to understand how the inkjet printing process works and how the membrane damage occurred during the printing process. The inkjet process is mainly dependent on the print head, the cartridge, the horizontal track, and the media in which the printed material is deposited. Successful inkjet printing depends on all four components to coordinate. The entire process leads to a vertical row of pixels being deposited as the print head and cartridge move in a horizontal manner. This allows for the printer to be able to function in two dimensions, compared to just printing a horizontal line of pixels as the cartridge-print head complex moves horizontally. This functionality allowed the printer we converted to be able to print in two dimensions instead of just one.

More important for our research was the thermal component that the HP Deskjet 500 could offer. This thermal energy that is used in the printing process is what would create the membrane damage that was exploited in the internalization of small molecules. In thermal inkjet printing heat is used to release the ink for the cartridge through the nozzle onto the substrate which in our case was cover slips more often than not [43].
thermal inkjet printing small drops of a size ranging from 10-150 picoliters are ejected by pressure created by the formation and collapse of small air bubbles from heating [44]. The heat that is applied to create the force to eject ink droplets is created by current being ran through a resistor. When doing this there is an inherent amount of heat that is given off due to the resistivity applied. Figure 4.1 shows the actual process of thermal inkjet printing as it may be easier to understand with visual aids.

www.encyclopedia2.thefreedictionary.com/inkjet+printer

Figure 4.1 Process of Ink Ejection via Thermal Inkjet Printing

There are other methods to get the same Drop on Demand technology such as the use of piezoelectric printers. Unfortunately the process of piezoelectrical printing cells causes cell lysis due to the operating frequency that ranges between 15-25 kHz [45].
Obviously piezoelectric printing was ruled out because of the amount of lysis caused during the printing cycle. Cell wall damage was desired, but not at the magnitudes in which piezoelectric printing created. It was therefore confirmed that thermal inkjet printing was the modality that would be used for printing of cells.

MATERIALS AND METHODS

Testing of Printer and Cartridges

Printing was conducted using an entirely black Microsoft Office 2010 Word file to test the efficacy of the printer, print head and cartridges. There were six cartridges in all. All were tested first without the use of the cleaning methods outlined in Appendix A. Initial tests began with trying to print with Dulbecco’s Modified Eagle’s Medium (DMEM) with the absence of cells. Secondary testing used a solution consisting of Phosphate Buffer Solution with blue food coloring. After the initial printing, the cleaning procedures were enacted and then all of the printer cartridges were tested again for efficacy. Printing was conducted on standard letter paper of 8½ in by 11 in paper. Results were based off of amount of visible color deposited onto the paper in a uniform manner.
Cell Culture

3T3 Fibroblasts were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Fisher Scientific) with 10% fetal bovine serum (FBS) (Sigma) and 1% antibiotic-antimycotic solution (Sigma). 3T3 Fibroblasts were maintained in polystyrene T-75 flasks in an incubator at 37°C and 5% CO2. The cell media was replaced every 48 hours with fresh media. Cells were used between passage 3 and 8 to try and maintain consistent results. Cells were passaged at around 80% confluence and were trypsinized with 0.25% trypsin with 0.02% ethyldiaminetetraacetic acid (EDTA). Cells were then centrifuged at 1000 rpm for 5 minutes to create cell pellet. Cells were resuspended and then reseeded in polystyrene T-75 flasks at a density of 50,000 cells/mL.

Proof of Concept

A proof of concept test was done as stated earlier to ensure that the printing system could print live cells. Multiple concentrations of cells were used to find the optimum amount of cells that could be printed without clogging. Amounts of $5 \times 10^4$, $1 \times 10^5$, $2 \times 10^5$ and $5 \times 10^5$ cells were used. The different cell concentrations were suspended in PBS along with blue food coloring. Testing of printing with cell culture media was also performed with very poor results. The media seemed to be too viscous for the printer cartridge to handle. Even when media was diluted with 25, 50, and 75% Phosphate Buffer solution the results were subpar. The decision was made to use only PBS and to expedite the printing process to ensure cell viability. Efficacy of printer cartridges at different cellular concentrations were evaluated again based on the amount
of colored material deposited. Color would indicate the amount of cells/printed solution that were being printed.

Actin Optimization

The type of actin monomers that were used were actin from rabbit muscle. They were an Alexa Fluor® 488 conjugate. It came in an 8.6 mg/mL in G buffer, 10% sucrose. The total amount received was 200 µg. The monomers are taken from the rabbit and conjugated with the Alexa Fluor® 488, then allowed to polymerize again to form F-actin filaments to ensure that they still have polymerization capabilities. The F-actin filament composed of the fluorescently tagged monomers is then degraded back down to monomer form. The amount of actin that should be included in the printing solution was unknown. No previous literature had printed with actin monomers. The optimum ratio between actin and cells must be found. Due to the lack of previous literature this process was a lot of trial and error. Arbitrary concentrations of 1, 2, 5, 10 and 20 µg/mL were chosen. Actin solutions of these concentrations were combined with cell concentrations discussed in the previous section to try and find the optimum actin and cell concentration to be used for printing in the future.

Control Group

We wanted to make sure that the g-actin monomers could not selectively enter the cell without any damage to the cell membrane. To ensure this, 3T3 fibroblasts were
printed and allowed to incubate for 4 hours. The g-actin monomer concentration of 10 µg/mL solution was added to the sample and then incubated again for 4 hours.

Printing Actin Monomers and 3T3 Fibroblasts

The final quantities for cells and g-actin monomers that were chosen were:

- Fibroblasts: $1 \times 10^5$ cells
- G-Actin Monomers: 10 µg/mL

Printing always took place immediately after cells were passaged. The solution that contains 100,000 cells suspended in Phosphate Buffer Solution and actin monomer concentration of 10 µg/mL was considered to be the “bioink”. Creation of bioink is outlined in Appendix A along with the special passaging that is required to make the bioink. All printed cells after the proof of concept and actin optimization stages of research were printed with this exact composition of cells and actin monomer concentration. The printing process is also documented in Appendix A for those trying to replicate. The process became very repeatable. It was important to also make sure that once the g-actin monomers were taken from the protective packaging that all processes that included the g-actin monomers were done in the dark or as dark as possible to keep some visibility. This was to hopefully avoid photobleaching of the fluorophore which would make imaging of the g-actin internalization into the 3T3 fibroblasts much more difficult.
Live/Dead Assay

A Live/Dead Assay was performed to corroborate the viability results that were published in literature. We wanted to be sure that this particular printer and application would not significantly change the results of normally good cell viability data that was being published from thermal inkjet printing. No literature was found on printing of fibroblasts so the assay was performed.

3T3 fibroblasts were printed onto 10 sterile glass cover slips and placed into well plates. Well plates were then incubated for four hours to allow potential damage to the cell membranes to heal. Cells were washed with Dulbecco’s phosphate-buffered-saline before assay was performed. Solutions of 2µM calcein AM and 4 µM of EthD-1 solutions were created. Historically these have been the concentrations that have been used for NIH 3T3 fibroblasts [46]. Cells were then covered with the combined Live/Dean assay reagents and then incubated again for 30 minutes to avoid contamination and drying of samples. Samples were then washed with PBS. Samples were inverted and placed onto microscope slides.

Samples were viewed with fluorescent microscopy with excitation wavelengths of 485 nm for calcein AM and 530 nm for EthD-1. Emissions were captured of 530 nm for calcein AM and 645 nm for EthD-1. Viability was evaluated by the amount of live cells over the amount of live plus dead cells.
Imaging

Multiple forms of imaging were performed but all were done with live cells. To avoid false positive results on actin integration into cells, samples were washed multiple times with PBS to remove any free fluorescently tagged g-actin monomers. The only stain that was ever used outside of the Live/Dead Assay was 4', 6-diamidino-2-phenylindole (DAPI). No staining was ever done for actin or any other part of the cytoskeleton. This ensured that the only green fluorescence that was being produced would be from the internalized actin monomers. Confocal was attempted but with unsatisfactory results. This was probably because confocal works much better with fixed cells than live cells.

RESULTS

Cartridges

Some of the cartridges that were in our possession were holdovers from a previous printer and had already been converted but not used in some time. We wanted to make sure that the old and the new ones were still in working order. The evaluation included the amount of material deposited onto the substrate. Most of the initial testing was done with paper. The results showed that cell culture media could not be printed by the cartridges and printer. The predominant thought on this is that the media was too viscous and the pressure gradient created by the thermal inkjet printing was not creating enough force to eject the ink. Even after mixing the cell culture media with various amounts of PBS results were still lacking. Only when strictly PBS was being printed
were satisfactory results obtained. Also through preliminary testing two cartridges were concluded to be unable to be used in future research due to lack of printing.

Proof of Concept – Initial Attempts to Print Cells

It was next necessary to make sure that the printer could handle the printing of live cells. Mathematically when the size of the printer nozzles and the size of fibroblasts were taken into account it was theorized that the printer should not have a problem printing single cells. Cell concentrations of $5 \times 10^4$, $1 \times 10^5$, $2 \times 10^5$ and $5 \times 10^5$ cells/mL were evaluated. All concentrations worked but once we reached the highest concentration clogging became prevalent and changing of cartridge often was necessary.

We also wanted to see if the deposition of the cells were in the Drop on Demand format or if they were randomly being ejected or even just dropping out of the printer cartridge without being ejected. Light microscopy after printing showed that the cells were indeed being dropped on demand as was hoped. Figure 4.2 shows three printed fibroblasts on a glass cover slip in a perfect line.

![Figure 4.2 Printed Fibroblasts on Glass Cover Slips in a Straight Line (20x)](image-url)
Actin Optimization

Arbitrary concentrations of 1, 2, 5, 10 and 20 µg/mL were chosen for evaluation to find the best amount of actin to use. The actin concentrations were combined with the cellular concentrations to find the best proportion of each component for printing. For imaging purposes it was found (images not included) that the best concentration of g-actin monomers was around 10 µg/mL. The formation of the actin solution and the cell suspension was then termed “bioink.” This bioink is what would later be used in the printing process.

Control Group

The control group was imaged using fluorescent microscopy and was found that there was no actin incorporation into cells (images not included). This was confirmed through the use of DAPI stain. The actin monomers that were still present after the washing conglomerated into pockets of fluorescence.

Bioprinting of 3T3 Cells and G-Actin Monomers

Although printing in complex two dimensional patterns was possible using the converted bioprinter this was not explored extensively. Simple unorganized single cell deposition was needed, not intricate designs that could be created. To get the amount of cell deposition that was desired the simple word pattern in Figure 4.3 was created and used during all of the printing processes used in the evaluation of internalization of g-actin monomers in 3T3 Fibroblasts.
Figure 4.3 Print Pattern Used for Evaluation of Internalization of G-actin Monomers

The image has been reduced but the outline that is present is the outline of a normal 8 ½ in by 11 in piece of paper.

Imaging

The first images that were evaluated were of cells that had been printed only 15 minutes before. A DAPI stain was also incorporated. Figure 4.4 shows the multichannel images and then finally the overlay of the two images.

Figure 4.4 Fluorescence Imaging of G-Actin Monomers and DAPI with Overlay (20x)
Next another sample was allowed to incubate for around three hours after printing. Figure 4.5 shows the image captured from a cell that was incubated for three hours and then imaged. A higher magnitude of magnification was used in this image. The scale bar present in the image represents 50 µm.

Figure 4.5 3T3 Fibroblast After Three Hours of Incubation (40x)

More images were then taken of 3T3 fibroblasts with higher resolution. Figure 4.6 shows these images were also taken after three hours of incubation with the scale bars representing 50 µm. These cells were live while imaging took place.
More images of cells that had printed and incubated for three hours were taken and are shown in Figure 4.7 and 4.8. Scale bars represent 50 µm. These cells were not alive during imaging as they did not have the normal shape associated with fibroblasts. They are of a more rounded conformation.
Next, images were taken of samples that had a longer time to incubate. These samples were printed and then left overnight to incubate. The images shown had sixteen hours to incubate after printing. The images below in Figure 4.9 show the multichannel DAPI showed the nuclei, while the FITC channel showed the green expressed from the g-actin monomers. Finally, there is an overlay of the two images to show the actin incorporation into the cells with the nuclei shown blue with DAPI staining.

Figure 4.9 DAPI and FITC Channels and Overlay of Overnight Incubation (40x)

Figure 4.10 DAPI and FITC Channels and Overlay of Overnight Incubation 2 (40x)
Live Dead Assay

Images not shown.

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<th>3</th>
<th>4</th>
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<th>7</th>
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<td>70</td>
<td>82</td>
<td>86</td>
<td>76</td>
<td>83</td>
<td>67</td>
<td>90</td>
<td>75</td>
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Table 4.1 Viability After Printing Data

Mean: 78.4% ± 7.14 Std Dev.

DISCUSSION

The printing process seemed to create the desired results in respect to the internalization of g-actin monomers into the 3T3 fibroblasts but results were varied. Figure 4.11 shows an illustration of how the g-actin monomers eventually become incorporated into the cell and can offer more insight if the process is still not understood.
The figure above shows the conversion of the printer cartridge and the inclusion of the bioink along with the results of the printing process. The final cells are shown with green cytoplasm because of the g-actin monomers that were incorporated by the printing process and deposited onto the microscope cover slip. The stage is also incorporated to alleviate any confusion that could be associated with the placement of the coverslip.

Cell viability results from Table 1 show that viability of printed cells would not be an issue. Viability was at 78.4 % ± 7.14. Although previous work with printed cells
showed that viability would probably not be a problem, it was reliving to see that this would be the case with the specific application used in this research. With cell viability being a non-issue research could then be conducted without the drawback of cells dying. One of the most interesting things that should be discussed about the actin internalization process observed is the temporal profile of actin internalization. There seemed to be a generic rule that actin became more internalized and possibly incorporated after longer incubation times before imaging. The control group showed that actin monomers were not incorporated into cells when the solution was added after printing and incubation. This proved that the actin monomers could not enter the cell through the cell membrane under normal circumstances and that cell damage was necessary.

In the first images (Figure 4) the fluorescence is locally concentrated in the lower portion of the cell. Although there is green present surrounding the nucleus, stained in blue, in the entire cell, the fluorescence is at its highest intensity in the lower portion of the cell. The fluorescence is also emanating from close to the peripheral of the cell around the cell membrane. The inclusion of fluorescent monomers at the periphery of the cell was concluded to be because of the short time after incubation (15 minutes).

The next incubation period that was tested was at around three hours of incubation. Figures 4.5-8 are all images with three hours of incubation. Three hours of incubation proved to have some mixed results. Figure 4.5 shows fluorescence localized in the left lateral portion of the cell. The results seem very similar to the cell that was imaged 15 minutes after printing. On a side not it is also possible to see an extension of
the cell in the lower left portion of the image. It is possible that this is an example of a lamellipodia discussed earlier in reference to cell motility. Unfortunately the actin that has been internalized was not in the area of this rapid formation of actin filaments. This would be been an interesting occurrence to investigate but did not materialize. Nevertheless, Figure 4.5 exhibits actin internalization, but only in the area immediately adjacent to the cell membrane.

Figures 4.6 and 4.7 show more promising results than what was exhibited by Figure 4.5. In these images actin is internalized much farther into the cytoplasm than immediately adjacent to the cell membrane. Each cell has localized fluorescence as seen before. This localized fluorescence or highest intensity of fluorescence was considered to be the highest concentration of g-actin monomers that was internalized by the cell. Image 4.7 is an overlay of the fluorescence channel with the background channel included to show the debris that is present in the bottom left corner of the image. This debris does not fluoresce due to lack of fluorescent g-actin monomers. Only the living cells present in the image are fluorescent.

Figures 4.7 and 4.8 exhibited mixed results. Although the cells were apparently dead, the cells in these two images showed actin internalization. The cell in Figure 4.7 again shows actin internalization around the periphery near the cell membrane. In contrast, Figure 4.8 shows much more diverse internalization of the monomers. Both of the cells seem to have a larger magnitude of cell damage as evidenced by monomer internalization in multiple sites around the cell. Although the cell is evidently dead,
Figure 4.7 provided possible insight into the phenotypic change of the cell membrane during cell death.

As discussed earlier there is a definite coordination between the actin cortex and the cell membrane. This coordination is important in many applications. Figure 4.7 seems to show the signs of cell necrosis. During the process of cell necrosis the cell swells and the chromatin is damaged and eventually the cell lyases [47]. The cell that is pictured in Figure 4.7 seems to be previous to cell lysing. It is theorized that the actin that is part of the actin cortex is being pushed up against the cell membrane during cell necrosis. The actin that was present may have been the only thing keeping the cell membrane intact.

The cell in Figure 4.8 does not seem to be swelling and rounding as badly as the cell present in Figure 4.7. There is evidence of more fluorescence present deeper into the cell cytoplasm but much of it is still centralized around the plasma membrane. This centralization of fluorescence seemed to be a pattern present. This was probably because of what was learned about the actin cortex. Actin is not present in microtubules which are responsible for much of cytoskeletal integrity in the central part of the cytoplasm and location of the nucleus near the center of the cell.

To further investigate the time dependent nature of actin internalization the next images included was after overnight incubation of sixteen hours. This would give the actin to be further internalized more. Figures 4.9 shows a single cell that that has internalized actin. The cell nucleus is obvious in the first image of the sequence while in the second image of the sequence a halo type structure can be seen around where the
nucleus was present. The overlay shows the nucleus surrounded by this halo of fluorescence. Unlike most of the other images discussed previously the fluorescence seems to be uniform in the structure as opposed to being highly concentrated in small areas of the cell. This could mean that the actin that has been internalized has been dispersed around the cell.

Figure 10 showed multiple cells with actin internalization. Similar results were present in the cells in Figure 10 as the cells in Figure 9, although Figure 10 seems to have a cell that is dividing. The dividing cell in Figure 10 is located in the center of the image. The nucleus is visually bilobed. Fibroblasts have single lobed nuclei thus spurning the theory that the cell is dividing.

Being able to visualize the actin activity during cell mitosis would be a very useful tool. The resolution and magnification of the image was not high enough to view small portions of actin involvement but it shows that the cortex as a whole seemed to be driving the organization of the cell membrane. Unfortunately it is believed that mitosis was not far enough along to visualize the contractile ring and whether the internalized actin was being utilized in the contractile ring complex. Being able to visualize the formation and action of the contractile ring would offer some insight into the recruitment of actin filaments that was outlined in the overview of functions of actin in mitosis. Although there are some theories the exact location from which actin is recruited to the contractile ring.

The use of internalized g-actin monomers could also put another debate to rest. This debate is whether cells create new actin filaments or simply extend pre-existing
filaments as the main source of extension/motility of the cell membrane during many functions. Using time lapse microscopy the actin monomers could be tracked during their tenure in the cell and would lead to a better understanding of their recruitment.

Although the dispersion of the actin monomers seems to be more uniform the location seems to be unchanged. It was previously theorized that with longer incubation time would come deeper internalization. This theory seems to be disproved as the time of incubation increased; the penetration depth of actin remained the same. The actin that is being internalized during the printing process seems to be only being utilized in the actin cortex adjacent to the cell membrane, if it is being utilized at all. It is entirely possible that the actin monomers that are printed incur the same damage that the cells experience. This damage could lead to less functionality of the actin or conformational change that would exclude them from their normal biological functions in the cell. It is unlikely that the fluorophore conjugate was inhibiting the actin from being utilized. Processing of the fluorescently tagged actin monomers included the actin being re-polymerized proving the functionality of the monomer remained while being conjugated with the fluorophore.

Overall the internalization of Alexa Fluor 488 conjugated g-actin monomers was a success. The utilization of these monomers remains a mystery. Further research would have to be conducted to evaluate the biological incorporation of the internalized g-actin monomers. At this stage much is unknown. The monomers could have been utilized in the images that were taken but it is unknown. It is also possible that the monomers were not being incorporated and were merely idly present in the cytoplasm. The latter seems to be less likely because of the location in which the monomers were repeatedly found in
the cell. Most of the monomers stayed around the cell membrane where the actin cortex would be predominate.

CONCLUSION

The bioprinter that was converted from a standard inkjet model performed the function that it was intended. It was successful in being able to internalize g-actin monomers into living cells. The printing process also did not have an overbearing impact on cell viability and thus its usefulness can be utilized in many other applications. Although internalization of the g-actin monomers is definite, their capacity to perform their normal function in cells is still undetermined.

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CHAPTER 5
RECOMMENDATIONS AND FUTURE WORKS

The HP Deskjet 500 was suitable for the applications that were presented in this research. It is theorized that many other printers could have also been used. The resolution of the HP Deskjet 500 was 300 DPI. Other inkjet printers could have been used that are more contemporary and would not have the problems associated with the older technology. Many of the newest computers to not even have parallel cord connectors and could not be used in conjunction with the HP Deskjet 500. It was also much more difficult than expected to find the HP Deskjet 500 that was used in this research. The newer versions in the HP Deskjet line have a resolution of 600 DPI. These were not used because of the need to avoid clogging. Once the printing solution was changed to PBS clogging was not an issue. The size of the nozzle in a 600 DPI printer is also suitable for printing cells and macromolecules.

In this research only fibroblast cell lines were used. It would be interesting to also see the effects of printing on other cell types. It would be very interested to see if printing stem cells would lead to differentiation in a desired manner. This application would have profound effects in tissue engineering. Imagine printing stem cells into a scaffold before implantation causing differentiation into the type of cell that is desired without having to include growth factors and other morphogenic factors into the scaffold. The printing could act as both a mechanism to precisely deposit cells while differentiating them into the phenotype that is desired for the certain application.
Although much was done in respect to the internalization of g-actin monomers into 3T3 fibroblasts, much more was wanted to be done. One of the main things that were desired to be explored was cytoskeleton mechanics. Actin is an important part of the cytoskeleton and its mechanics are only partially understood. In our experiments atomic force microscopy would be used to investigate the response of the cytoskeleton to applied loads. The internalized g-actin monomers would help in the investigation. Stress fibers in cells are often aligned with the stresses that are induced onto them. Tracking the monomers could help understand how stress fibers are formed and how they recruit the monomers based on these forces.

Another aspect of the research that was not completed but would be recommended would be the use of time lapse fluorescent microscopy. The research presented only took snapshots at different amounts of time after incubation. Using a microscope setup that could keep the cells that are being imaged would help with the time lapse imaging. The microscope stage would have to allow for a normal incubator environment (37° C and 5% carbon dioxide) while still being able to record the cells would give a better understanding of the temporal profile of the g-actin monomers. Being able to image cells during twenty four hour incubation could allow more insight into the utilization of the g-actin monomers. Much more would be known whether the g-actin monomers were being incorporated into the actin cortex or merely idly present in the cytosol. The AFM experiment in the paragraph before could also be helpful in figuring out whether the monomers are being polymerized and used. If they were, the
monomers would align with the stress fibers that are being formed. It would also be possible to see their recruitment as a result of mechanical loading.

Gene transfection seems to be a field that is underutilizing the bioprinting field. Bioprinting would allow for cheap an easy gene transfection and provides a high cell viability factor that is not present in many current forms of gene transfection. Many cells that are used in tissue engineering must have gene modulation conducted. Bioprinting would allow for the genes of the cells being printed to be altered. Bioprinting, specifically through the use of thermal inkjet printing, could vastly improve the efficacy of gene transfection.
APPENDIX A

EXTRA PROTOCOLS

PRINT CARTRIDGE CLEANING PROTOCOL

The cleaning of printer cartridges is very important. It helps maintain an aseptic environment while also avoiding crystallization of salts and other biological material that could cause blockages in the cartridge.

Pre Printing:

- Place all cartridges that are going to be used in a 1:1 mixture of water and Rust inhibitor
- Rinse and then place cartridges in a mixture of 1:4 instrument lubricant and water
- Sonicate in deionized water for 15 minutes

After Printing:

- Rinse thoroughly with water
- Place used cartridges in 1:4 mixture of instrument lubricant and water for 40 minutes
- Sonicate in deionized water for 15 minutes.
COVER SLIP PROTOCOLS

Sterilization

- Place cover slips in a petri dish and cover with 70% ethanol
- Place petri dish in laminar flow hood and UV for at least one hour before printing
- When cover slips are ready for use, take the petri dish out of the laminar flow hood and take them out one at a time while drying them off with a kimwipe

Plasma Cleaning

- Make sure line to oxygen is close and venting line is open
- Put slides in plasma cleaner without them touching or overlapping each other inside the cylinder
- Turn on pump that is below the cleaner
- Hold lid in place
- Turn the power to the plasma cleaner on
- Turn the pump on
- Open the oxygen a little to allow the line to fill with oxygen
- Close venting line for oxygen completely
- Allow the pressure to drop to around 150 (UNITs) which will take around 10 minutes
- Slowly turn on oxygen
- Open the venting knob near the lid and adjust to keep the pressure between 150 and 200 PSI
• Once the pressure is stable turn the plasma on high

• Leave the plasma cleaner on for 10 or more minutes at a pressure between 150 to 200 PSI. You should be able to see purple/blue plasma in the machine

• After the 10 minutes, turn the plasma cleaner from high to off.

• Turn the oxygen off on the cleaner and below the cleaner

• Open the venting knob and then venting line very slowly to allow the pressure in the cleaner to go back to atmospheric pressure and the lid will pop off. If you open the venting knob too quickly the cover slips inside will be sucked into the lid and break more than likely

• Retrieve the cover slips from the plasma cleaner and place in either deionized water depending on when the cover slips are going to be used

Covering of Cover Slips with Fibronectin/Collagen

• Make sure slides are dry from storage in ethanol or DI water

• Place the amount of collagen/fibronectin necessary to cover the cover slip. This will depend on the size and shape of coverslip that you are using. When using the smaller circular slides 5 µl of collagen/fibronectin should coat the slide

• The concentration will depend on the application, but in my research I used concentrations ranging from 1 µg/ml to 0.1 mg/ml

• Place slides in incubator until the fibronectin/collagen has dried out and polymerized
• The slides are now ready for use

SPECIAL PASSAGING FOR 3T3 FIBROBLASTS

• Aspirate the old medium from the T-75 flask in which the adherent cells are culturing

• Rinse twice with 5 ml of Dulbecco’s Phosphate Buffered Saline (DPBS) allowing the DPBS to be in contact with the cells for 30 to 60 seconds. Aspirate each time in between rinses

• Add 5 ml of Trypsin + EDTA per T-75 Flask and incubate for 5 minutes at 37° C

• Remove from incubator after 5 minutes and gently tap flask to help cells detach

• Check under microscope to confirm detachment

• Add 5 ml of fresh medium to T-75 flask to stop the Trypsin reaction

• Pipette the cell suspension into a 15 ml conical centrifuge tube. Centrifuge at 1000 rpm for 6 minutes for fibroblasts

• Carefully aspirate the spend medium while leaving the cell pellet intact

• Add 1 ml of fresh DPBS instead of normal cell culture medium into the centrifuge tube and gently break up cell pellet by pipetting up and down slowly

• Count the cells with Trypan Blue/hemocytometer to get an accurate cell concentration to create bio-ink
• If cells need to be reseeded into T-75 flask take 100 µl of cell solution and dispense into a T-75 flask and add 15 ml of fresh cell culture medium

Cleaning of Printer Before Printing

• Place printer in laminar flow hood
• Make sure printer is at least six inches away from the front of the hood to continue aseptic environment
• Wipe down printing zone with 70% ethanol for sterilization purposes
• Also wipe down the printer head with kimwipe sprayed with 70% ethanol to try and prevent clogging
• UV Sterilize for at least one hour before use

3T3 Media

10% FBS, 1% Anti/Anti (or Pen/Strep if antifungal agent must be avoided)

500 mL High Glucose DMEM

• Add 56.2 mL FBS
• 5.6 mL of Anti/Anti – 2.8 mL Penicillin/Streptomycin, 2.8 mL Amphotericin B into the 500 mL High Glucose DMEM

Making of Cell Suspension – “Bioink”
• Culture cells until ready to passage.
• Passage cells by special passaging protocol also in Appendix A.
• Starting at this point the process should be done in the dark to avoid the photobleaching for fluorophores due to light.
• Create combination solution of 10 μg/mL g-actin monomers along with 1 x 10^5 cells/mL concentration of cells.
• Note: 250 μL of bioink prints around three cover slips depending on the pattern used for the printing process.

Bioprinting

• This process should be done in the dark to avoid the photobleaching for fluorophores due to light.
• Power on the printer and let it warm up.
• Place desired printing substrate in the center of the stage below the printing zone where the cartridge and printer head translate. The substrate used in this research was glass coverslips coated in collagen and fibronectin. Many other substrates can be used and even biological tissue.
• Create desired printing pattern file for printing.
• Load the prepared cartridge with desired cell suspension. Suspension should be pipetted into the small circular well at the bottom of the cartridge compartment. Use approximately 100-120 μL of solution.
- Turn off printer

- Manually slide printer head and cartridge over sample to make sure that contact will not be made during the printing cycles that will occur during printing.

- Turn printer on and let it warm up again

- Cartridge will eventually go to rest area where it stays during times of that it is not operating

- Print the desired file with the HP Deskjet 500 selected. For large amounts of deposition needed, print the file multiple times by changing the amount of copies desired to be printed

- Printer will warm up again

- Printer head and cartridge will move to “ready” position just outside of where it normally rests during times of inoperation.

- When cartridge is in ready position, pull up on paper feed mechanism wire and printing should commence.

- If multiple copies have been selected the paper feed wire will need to be pulled in between each copy or the printer will think there is a jam.

- In the event of a jam, turn printer off and repeat all steps.

- Printer will print onto substrate that is placed in the printing zone.
References


