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ASPIRATION-ASSISTED BIOPRINTING

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by

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ABSTRACT

Each year there are approximately 130,000 organ transplants conducted worldwide. Although a substantial number of organ transplantation, the current situation is far away from meeting the demand so that many people on the waitlist die each day. The supply of transplantable tissue faces the same complications and relies on a series of grafting techniques. Allografts (tissue from another human) and xenografts (tissue from an animal) are used to increase supply but these methods have a variety of issues. These problems include the possibility of rejection due to the presence of specific histocompatibility antigens that are targeted by antibodies in the recipient, and for the later there is a risk of microbial or viral transmission resulting in novel diseases. Autografts (tissue from the patient) eliminate the concerns of the previous two methods thanks to the use of genetically identical tissues. However, Autografts are only applicable in a small number of cases due to a lack of supply and large cost associated with the procedure. There is a clear need for cheap, accessible, and biocompatible tissues and organs for transplantation with a possible solution lying in bioprinting. Bioprinting offers a promising technology to fuse the high precision and time efficient qualities of additive manufacturing with tissue engineering to replicate morphogenesis with the ultimate goal of producing artificial tissues and organs on an industrial scale.

This dissertation has centered on understanding, designing, and prototyping a new hybrid bioprinting process, called "Aspiration-assisted Bioprinting (AAB)," which enables to pick and precisely bioprint a wide of range of biologics, with dimensions in an order-of-magnitude range (80-800 μm), into/onto a gel substrate with minimal cellular damage. In order to better understand the response of biologics to the bioprinting process, the underlying mechanism explaining the physical behavior of viscoelastic spheroids and their interactions with physical governing forces during aspiration, lifting and bioprinting were unveiled. Compared to existed methods, the approach that was explored in this dissertation facilitated the bioprinting of spheroids in higher

positional precision and accuracy, ~11 and ~15% with respect to the spheroid size, respectively. In addition, it enabled bioprinting in 3D with flexibility of bioprinting of non-uniform, any-size spheroids into (I) a functional gel as a scaffold-based approach or (II) a sacrificial gel as a scaffold-free approach. The newly-developed AAB platform, modified from a Makerbot Replicator I (<\$1,000), operates a custom-made glass pipette, which is used to ‘pick up’ biologics and ‘3D bioprint’ them into or onto a gel substrate. This new approach paves the way for bioprinting several tissue types and a wide range of spheroids in 2D and 3D in gel substrates with novel applications including (I) the development of micro-physiological systems to demonstrate the collective angiogenic sprouting behavior of spheroids in a scaffold medium, (II) fabrication of osteogenic tissues to decode the role of mid-term osteogenic induction of stem cell-based spheroids (prior to bioprinting) on the mineralization and assembly behavior in a scaffold-free environment and (III) fabrication of the 3D osteochondral interface with biologically-relevant morphologies, which is quite challenging using existing technologies.

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Chapter 1

Introduction

1.1 A brief introduction of Tissue Engineering

According to the Organ Procurement and Transplantation Network (OPTN), there are currently over 112,000 people on the waitlist for a replacement organ in the United States [1–3], but the donor number is just 60,000. People on the waitlist suffer from nonstop pain, discomfort, and a plethora of other inconveniences. More importantly, there are approximately 20 of those on the waitlist who die each day [1–3]. There is a clear disparity between the number of donors and people in need of life saving organs, and this gap continues to grow [4]. Even when it comes to just the donation of tissue, there is no stable source. While the tissue and organ supply today is purely reliant on donors, there are multiple grafting options to help meet the demand for tissue [5,6].

Grafting is a common medical procedure that involves the movement of tissue from one area to another. The three types of grafting are xenograft, allograft, and autograft [7]. A xenograft is when tissue is taken from a non-human donor and implanted into a human recipient. Porcine tissue harvested from the small intestine is the most commonly used for xenografts today. Xenografts are the cheapest of the three options due to the availability of the tissue. However, xenografts are the most likely to result in immune rejection by the recipient due to the presence of specific histocompatibility antigens that are targeted by antibodies in the recipient causing hyperacute rejection. Furthermore, the majority of non-histocompatible antigens present from the donor are also immunogenic [8]. There is also a risk of viral or microbial transmission between animals and humans resulting in novel diseases. The next type of graft is an allograft, which occurs

between two members of the same species that are genetically different. Since the donor and recipient are the same species, the risk of non-histocompatible antigens being immunogenic disappears, and, as a result, the risk of rejection diminishes. However, the genetic differences mean that the donor-specific histocompatibility antigens will be attacked by the recipient's antibodies, leaving the possibility of hyperacute rejection present. Allografts are also much more expensive than xenografts due to the lack of available donors and the added difficulties of its harvesting. The final category of graft is an autograft, which is the ideal type of graft because the donor tissue comes from the recipient so that both the donor and recipient are genetically similar. This eliminates the concern of an immune rejection. The increased safety from the biocompatibility makes autografts ideal, but the availability of donor tissue makes its widespread use impossible [9,10].

There is a clear need for new technology or systems to help bolster the number of both donor tissue and organs which, in turn, could aid those suffering by increasing the success rate of operations [10,11]. Tissue engineering aims to replicate morphogenesis in the laboratory to create living structures using the same processes that occur during embryonic development. Applying tissue engineering to grafts would allow for the large-scale production of biocompatible tissue taken from the donor, thereby decreasing the cost and increasing the applicability of autografts [12].

1.2 A brief introduction of Bioprinting

Patients in need of organ transplants currently wait extended periods of time for life-saving treatments due to the shortage of organ donors. Therefore, several researchers have developed diverse methods to build engineered tissue for transplantation [13,14]. The bioprinting system is the most widely used of these methods to develop three-dimensional (3D) functional living tissue with the patients' own stem cells using layer-by-layer additive robotic deposition [13,15–18].

Scaffold-based bioprinting and scaffold-free bioprinting approaches are two primary tissue biofabrication methods [19].

1.2.1 Scaffold-based Bioprinting

Bioprinting can be defined as the spatial patterning of living cells, tissues, and other biologics by stacking and assembling them layer by layer [14]. In scaffold-based bioprinting, cells can be bioprinted in a scaffold matrix such as hydrogels to support cell growth, proliferation, and interactions [20,21]. Extrusion-Based-Bioprinting (EBB), Droplet-Based-Bioprinting (DBB), and Laser-Based-Bioprinting (LBB) are the main bioprinting methods to fabricate design-specific tissue constructs using scaffold-based bioprinting (Figure 1.1) [14,22,23].

EBB incorporates a fluid-dispensing and automated system in tandem in order to achieve the bioprinting. Computer-Aided-Design (CAD) is used to define the paths along which cylindrical filaments containing cells and hydrogel are continuously deposited. It is essential that the hydrogel used for bioink possesses shear thinning, low adhesion and surface tension, and thixotropic behaviors to make the filaments bioprintable [24]. After the extrusion, the bioink is exposed to cross-linking schemes such as ionic, photo, and enzymatic cross-linking in order to solidify the gels into a scaffold-like structure. In EBB, the fluid systems used for dispensing bioink can be controlled by various options including valve-based or valve-free configurations. Mechanical systems also exist in the form of a piston and screw-driven configurations with stepper motors. The piston configuration grants the user direct control over the bioink flow. The screw-driven method allows the user more spatial control and generates a higher pressure, which is suitable for more viscous bioinks. Overall, EBB offers great advantages due to depositing more viscous bioinks: however, bioprinting resolution is a major drawback [24].

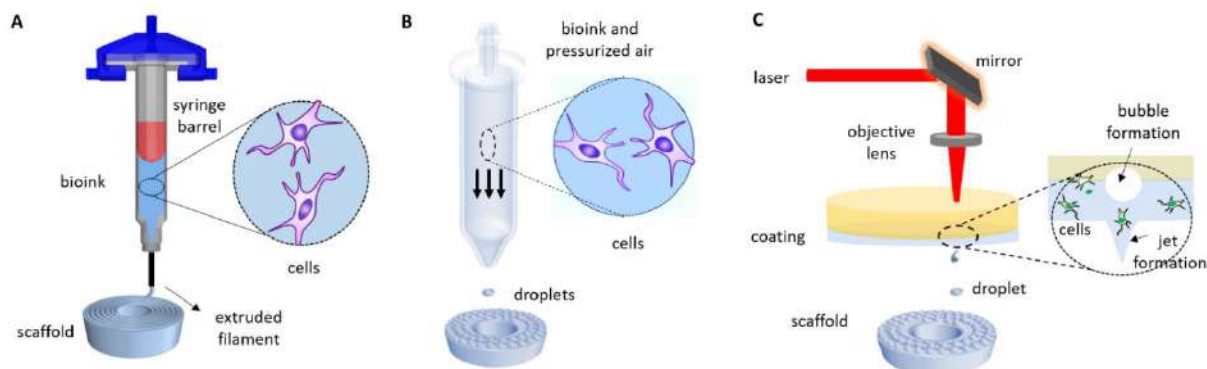


Figure 1.1: Schematic illustrations of bioprinting methods including (A) Extrusion-based Bioprinting (EBB), (B) Droplet-based Bioprinting (DBB), and Laser-based Bioprinting (LBB).

In DBB, bioink can deposit in a droplet form onto a substrate in predetermined 3D architectures. It offers unique advantages owing to its simplicity, versatility, and high resolution [25]. In order to generate micro-droplets, thermal, piezoelectric, and micro-valve actuations have been utilized. The thermal actuation induces a pulse through a heating element to create air bubbles which then collapse to generate pressure pulses. In piezoelectric and micro-valve actuation, a sinusoidal wave is used to eject droplets through the nozzle where pressure and frequency of applied signal are the essential parameters to tune their size [23,25].

LBB incorporates three stacked layers including the bottom consisting of the bioink, the middle being a donor layer, and the top being a metal. During the bioprinting process, the pulse of a laser focuses on a small area of the donor layer and bioink, resulting in a following local vaporization on the donor layer. It, therefore, generates the formation of a high pressure bubble followed by dispensing bioink onto the substrate [22,26–28].

1.1.2 Scaffold-free Bioprinting

In scaffold-free bioprinting, cells assemble themselves to cell aggregates such as tissue spheroids [19,29–32], honeycombs [33,34] and tissue strands [35]. It has many advantages, including the cells' ability to secrete their extracellular matrix with an effective communication between them in a native-like microenvironment [19].

In contrast to traditional two-dimensional (2D) cultures, spheroids offer many advantages, such as the ability of the cell aggregates to secrete their extracellular matrix (ECM), increasing effective communication between all cells in a particular microenvironment. Some physical characteristics like cell interaction and function change or are completely eliminated when the cells are limited to grow in 2D and fail to provide a good model for complex structures found in nature and native tissue [36,37]. The use of tissue spheroids as building blocks can be used in toxicity assays and scale-up tissue biofabrication. As a result, this will enable us to get closer to developing physiologically correct models for use in tissue engineering and pharmaceuticals. The ability of spheroids to mimic *in vivo* cell interactions makes them excellent candidates to fabricate reproducible, complex tissue structures such as cartilage and cardiac tissues. Furthermore, the dense cellular environment and inherent ECM secretion within spheroids could allow for tissue constructs that support vascular networks which distribute oxygen evenly among the tissue [38].

There are several spheroid fabrication methods such as hanging drop [39], micro-wells [32], liquid overlay [40,41], magnetic assembly [42], microfluidic [43], and spinner flask [44–46] techniques for altering or controlling the geometries of the tissue (Figure 1.2).

In the hanging-drop method, gravity is the main force that drives the self-assembly of the cell culture. With this, a small amount of cell suspensions can be dropped onto a tissue culture. The plate is then inverted, and the droplet holds itself up because of surface tension. The cells then collect at the bottom of the droplet. At the bottom of droplet, cells can form into spheroids because

of cell-cell and cell-ECM interactions. This method is relatively labor-intensive with a low-throughput biofabrication of spheroids [32].

Spheroids can also be formed in micro-wells using micro-molding processes. Micro-molds can be produced as cylindrical or ring pins, and then non-adhesive hydrogels such as agarose can be cast on these molds to form micro-wells, into which cells get seeded. Since the surface is non-adhesive, cells can be assembled into aggregates. This method offers high-throughput biofabrication of spheroids that have uniform size, shape, and cellular distribution. Besides, new drug candidates or media supplements can be tested during the culture.

In the liquid overlay method, cells are seeded on a low-adhesive surface where they are able to form multicellular spheroids since the adhesive forces from the plate are lower than the intracellular forces. Cells then start to adhere to each other and form spheroids over time [40,41].

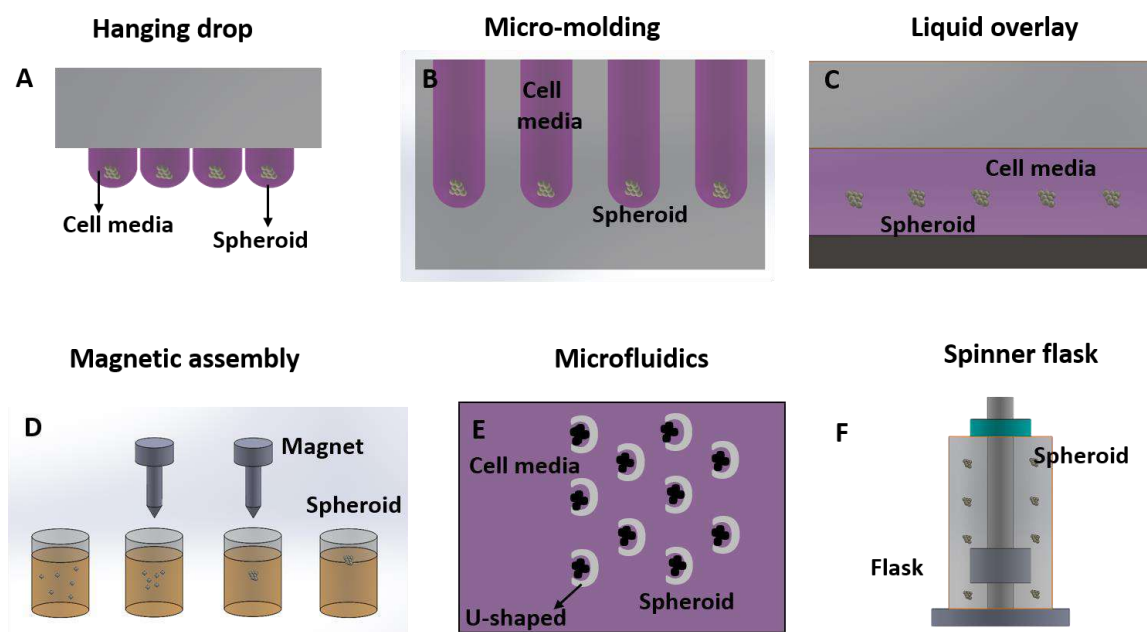


Figure 1.2: Spheroid fabrication method. (A) Hanging drop technique (B) Micro-molding technique (C) Liquid overlay technique (D) Magnetic assembly technique (E) Microfluidic

technique where cells were trapped into U-shaped hydrogel microstructure (F) Spinner flask technique.

In order to fabricate multicellular spheroids, external forces such as magnetic [42] or acoustic power [47] can be used. In the magnetic assembly method, patterning agents such as iron oxide nanoparticles are induced into cells, and then cells can be patterned with a magnet. However, patterning agents for fabricating spheroids decreases cell viability over time [42].

In the microfluidic-based spheroid fabrication method, U-shaped microfluidic chips are utilized [43]. A combination of fluid flow and gravitational forces trap cells in the pockets of the chip, which leads to compact structures with high cell densities. The microstructure of the U-shape controls the relative size of the spheroids, prevents shear stress, and allows for diffusion. A perfusion system around the microstructure helps keep the cells in place and form the spheroids. The downside of this method is that the spheroids created are very difficult to retrieve from the microfluidic channels [43].

The spinner flask method is used in the larger scale production of spheroids [44–46]. The rotation of the fluid within the flask is controlled by convective forces. These forces are generated by an impeller of a magnetic bar at the bottom of the flask. The flask needs to rotate at an appropriate speed so as not to damage the cells, yet while also preventing adherence to the flask

1.3 Background and Current Problems

Bioprinting of spheroids has gained noteworthy attention in the tissue engineering field due to its ability to generate anatomically-correct, physiologically-relevant, functional, and vascularized tissue and organ models [22,48]. However, only a limited number of approaches including Extrusion-based bioprinting, droplet-based bioprinting, Kenzan, and bio-gripper have

been able to demonstrate spheroid bioprinting. In extrusion-based spheroid bioprinting, loading tissue spheroids into a nozzle, which is generally a glass pipette [49], is quite challenging. Besides, spheroids quickly fuse to each other, which triggers their aggregation inside the nozzle tip before bioprinting [14]. During bioprinting, there is also a risk that spheroids may not be bioprinted at the predetermined positions since their flow within the pipette is varied. When the spheroid size is too small compared to the pipette, then bioprinting of spheroids cannot be controlled. The Kenzan method utilizes an array of needles on which a robotic arm skewers spheroids, but this approach requires identical size spheroids since bioprinted spheroids have to fit properly in the needle array. All the existing techniques used in the literature suffer from the positional accuracy and precision of spheroid bioprinting; substantial damage to biological, structural, and mechanical properties of spheroids; and limitations in the use of same-size spheroids for successful bioprinting. Therefore, efficient and high-precision methods of spheroid bioprinting should be investigated [15]. No study in the literature has demonstrated high-precision bioprinting of organ-specific spheroids thus far.

1.4 Research Objectives

The ability to biomanufacture design-specific and functional human tissues using spheroids would enable numerous applications, including drug delivery, *in vitro* disease modeling, drug development, tissue engineering, and bioprinting. This dissertation has centered on understanding, designing, prototyping, and applying a new hybrid bioprinting process, Aspiration-assisted Bioprinting (AAB), which enables the user to pick and precisely bioprint a wide of range of biologics, with dimensions in an order-of-magnitude range (80-800 μm), into/onto a hydrogel substrate with minimal cellular damage. In Chapter 2, prototyping, a mathematical model, and bioprinting capabilities of AAB are presented where the effects of distance on angiogenic sprouting behavior of spheroids is explored. In Chapter 3, scaffold-free bioprinting osteogenic using AAB is

demonstrated. In Chapter 4, aspiration-assisted bioprinting and characterization of osteochondral interface are investigated. Finally, the summary and future directions are provided in Chapter 5.



Chapter 2

Aspiration-assisted Bioprinting for Precise Positioning of Biologics^{*†}

2.1 Chapter Summary

Three-dimensional (3D) bioprinting is an additive manufacturing process to fabricate design-specific tissue construct on demand. Nevertheless, bioprinting of biologics with high precision and accuracy is the major bottleneck in Biofabrication. In this chapter, a new 3D bioprinting process called “Aspiration-assisted Bioprinting (AAB)” was explored and physical mechanism was studied in order to understand the interactions between physical governing forces and aspirated tissue building blocks. As applications, the utilization of AAB in both scaffold-based and scaffold-free configurations was demonstrated including the self-assembly of spheroids for large-scale tissue biofabrication and development of physiologically relevant culture environments for studying angiogenic sprouting.

2.2 Introduction

Aggregated cells have been formed into spheroids [19,48,50], honeycombs [33], and strands [35] from a variety of different cell types and their co-cultures. They have many advantages including the cellular capability to secrete extracellular matrix (ECM) components with an effective

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[†] Dong Nyoung Heo assisted with tissue characterization, Madhuri Dey assisted with angiogenic sprouting part, Adomas Povilianskas assisted with development of AAB, and Zhifeng Zhang assisted with theoretical part of this chapter.

communication between cells in a native-like microenvironment [19]. When cells are grown in an isolated fashion (i.e., cells in monolayers or cell-laden hydrogels), they usually do not facilitate native-like tissue microenvironment because of limited cell-cell and cell-ECM interactions [36]. Three-dimensional (3D) cell aggregates, particularly tissue spheroids, are excellent candidates to mimic *in vivo* tissue microenvironments, which can be reconstituted to form reproducible complex tissues [such as bone [51] and pancreas [52]] and or tissue models [cancer [53]]. Furthermore, co-cultured aggregates can be used as building blocks for fabricating scalable tissue complexes [48]. Spheroids loaded with endothelial cells can also facilitate a denser tissue microenvironment, inherent ECM secretion, and prevascularized network [22]. As a result, large-scale vascularized tissue complexes can be biofabricated using prevascularized cell aggregates as fundamental building blocks [51,54], which allow more accurate representations of native tissues. Moreover, these tissue complexes stand to develop physiologically correct models for drug screening, disease modeling (i.e., cancer), and high-throughput screening [55]. Therefore, various advantages of tissue spheroids make them a great candidate as building blocks for 3D bioprinting.

Despite these advantages, only a few methods including extrusion-based bioprinting [48,54,56,57], droplet-based bioprinting [58], KENZAN [59], and biogripper approaches [33,60] have been demonstrated for 3D bioprinting of spheroids made of cells, such as but not limited to human articular or nasal chondrocytes, human umbilical vein endothelial cells (HUVECs), human umbilical vein smooth muscle cells, etc. Extrusion-based bioprinting technique dispenses similar-sized spheroids suspended in a hydrogel ink through a glass nozzle. During bioprinting, spheroids in the hydrogel ink may self-aggregate inside the nozzle, leading to clogging issues [48]. Although extrusion-based bioprinting of cellular aggregates in the form of strands can generate scalable tissues [35,50], their use in high-precision applications, such as organ-on-a-chip platforms or microphysiological systems, is quite challenging. In droplet-based bioprinting, a single spheroid is

loaded into a droplet during bioprinting, which enables the positioning of spheroids in 2D [58]. Kenzan method uses an array of needles on which spheroids are skewered by a robotic arm; however, this method requires same-size spheroids since bioprinted spheroids have to fit properly in the needle array and smaller spheroids would be prone to fragment during insertion on the needle [59]. Recently, the biogripper technique was introduced to manipulate defined micromolded tissue blocks. However, this technique enables bioprinting of only molded tissue blocks in the range of 600 μm to 3.4 mm, where submillimeter scale is quite large for typical tissue building blocks [33,60]. All the existing techniques used in the literature suffer from the positional accuracy and precision of spheroid bioprinting; may induce substantial damage to biological, structural, and mechanical properties of spheroids; and are limited to the use of same-size spheroids for successful bioprinting.

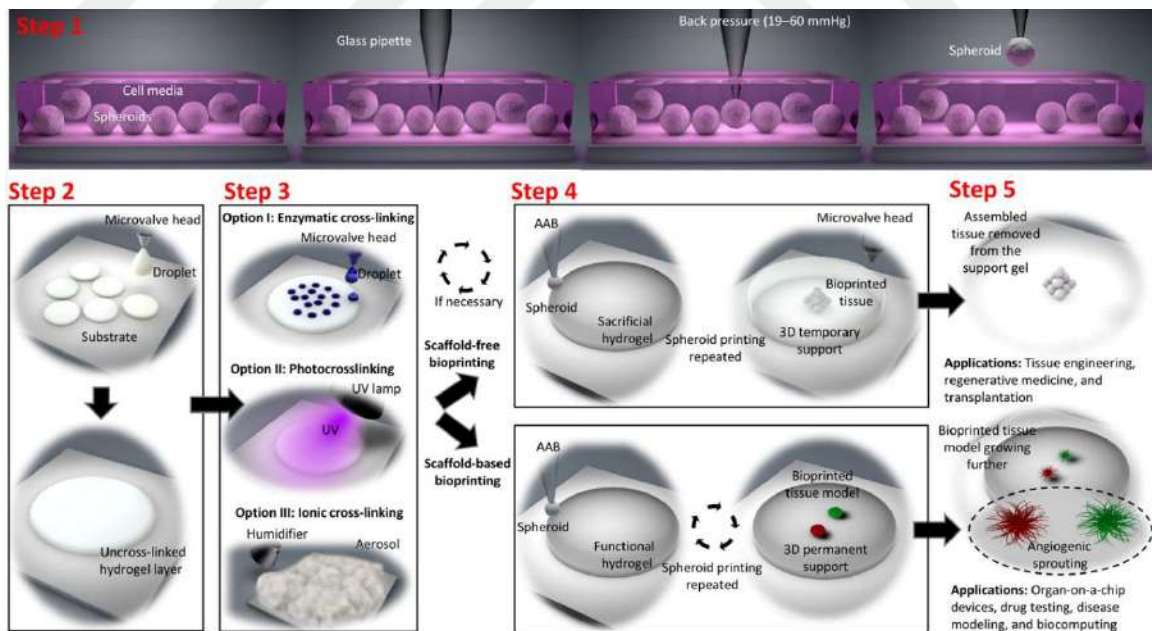


Figure 2.1: Step-by-step illustration of picking and bioprinting of spheroids. In step 1, spheroids are picked from the cell media by a glass pipette, where required back pressure is set to lift spheroids. Afterward, spheroids can be bioprinted into sacrificial hydrogels (scaffold-free

bioprinting) or functional hydrogels (scaffold-based bioprinting). In this regard, in step 2, microvalve bioprinting is used to bioprint a gel substrate, which can then be partially cross-linked using various different cross-linking schemes—such as but not limited to enzymatic, photo, and ionic cross-linking—as highlighted in step 3. Next, in step 4, spheroids are bioprinted precisely into designed positions, and spheroid bioprinting is repeated as many times as needed. Steps 2 to 4 can be repeated as needed. In step 5, bioprinted tissues are isolated from the support hydrogel (for scaffold-free bioprinting) or further grown in the functional hydrogel (for scaffold-based bioprinting). UV, ultraviolet.

Here, we present a new hybrid bioprinting approach through harnessing the power of aspiration forces, which enables us to pick and precisely bioprint a wide of range of biologics, with dimensions in an order-of-magnitude range (80 to 800 μm), into/onto a gel substrate with minimal cellular damage. To better understand the response of biologics to the bioprinting process, we unveil the underlying mechanism explaining the physical behavior of viscoelastic spheroids and their interactions with physical governing forces during aspiration, lifting, and bioprinting. Compared to the abovementioned methods, the presented approach facilitated the bioprinting of spheroids in higher positional precision and accuracy, ~ 11 and $\sim 15\%$ with respect to the spheroid size, respectively.

In addition, it enabled bioprinting in 3D with flexibility of bioprinting of nonuniform, any-size spheroids into (i) a functional gel as a scaffold-based approach or (ii) a sacrificial gel as a scaffold-free approach (Figure 2.1). The newly developed aspiration-assisted bioprinting (AAB) platform, modified from a MakerBot Replicator 1 ($< \$1000$) (see Figure 2.2), operates a custom-made glass pipette, which is used to “pick up” biologics and “3D bioprint” them into or onto a gel substrate. AAB was coupled with microvalves for droplet-based bioprinting of functional or sacrificial hydrogels. To bioprint spheroids into a functional hydrogel (i.e., fibrin), fibrinogen and

thrombin layers were printed via microvalve bioprinting to obtain fibrin constructs. Spheroids were then bioprinted into desired positions before the constructs were fully cross-linked. To bioprint spheroids onto a sacrificial hydrogel (i.e., alginate), microvalve bioprinting and aerosol cross-linking processes were used. The first step included the generation of sodium alginate droplets on a glass substrate. Second, calcium chloride (CaCl_2) was fumed for instant cross-linking [61]. Spheroids were then picked and 3D bioprinted onto the partially cross-linked alginate. Next, the print area was overlaid with sodium alginate droplets. Then, aerosol form of CaCl_2 was applied again. The process was then repeated as many times as needed for building other layers. Next, bioprinted spheroids were maintained in the support gel temporally until partial fusion was realized. Last, the support gel was gently removed through decross-linking using a lyase solution.

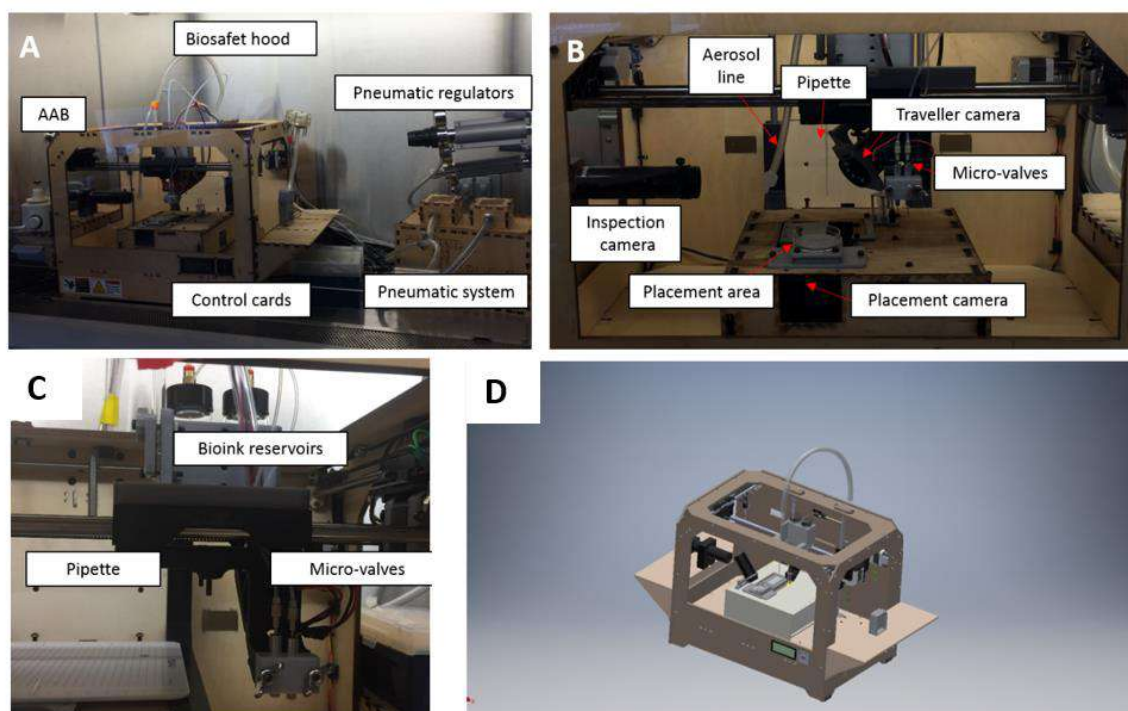


Figure 2.2: Aspiration-assisted bioprinter. (A) The bioprinter setup in a biosafety hood (Nuair, Plymouth, MN). (B) A closer view of the AAB platform, where micro-valves and a customized pipette head were integrated on the print head. (C) A photograph showing the bioprinter head

loaded with a pipette, micro-valve holder, and bioink reservoirs. (D) A computer-aided design (CAD) model of the AAB platform developed using Autodesk Inventor (Autodesk, San Rafael, CA).

2.3 Materials and Methods

2.3.1 Materials

Sodium alginate solution was made by dissolving 1% (w/v) sodium alginate (Sigma-Aldrich, UK) in deionized (DI) water. Calcium chloride solution was prepared by dissolving 4% (w/v) calcium chloride (CaCl_2) (Sigma-Aldrich, St. Louis, MO) in DI water. For constructing fibrin scaffolds, fibrinogen (6 mg/ml) (Sigma-Aldrich) and thrombin (2.4 U/ml) (Sigma-Aldrich) were prepared for microvalve bioprinting. For bioprinting of electrocytes, agarose (A20070-100, Research Products International, IL) was dissolved in DI water to obtain a solution of 1% (w/v) agarose at 90°C.

Collagen type-I (COL I) was extracted from rat tails according to our recently published study [62]. Briefly, extracted collagen fibers from rat tail tendons were dissolved in 0.02 N acetic acid (Sigma Aldrich) to prepare collagen solution, and then freeze-dried to obtain collagen sponges. The collagen sponges were again dissolved in acetic acid at a desired concentration, centrifuged to remove insoluble supernatants, and then sterilized using Spectra/Por 1 Dialysis Tubing (6–8 kDa MWCO) (Spectrum Labs, Rancho Dominguez, CA). Collagen (1 mg/ml) was crosslinked by neutralizing its pH using 1N sodium hydroxide (NaOH) and incubating at 37 °C for 30 min.

Gelatin-methacryloyl (GelMA) was synthesized according to a published protocol [63]. Briefly, porcine gelatin (Sigma Aldrich) was dissolved in Dulbecco's phosphate-buffered saline (DPBS) at 50 °C for 4 h to obtain 10 % (w/v) homogenous solution. The solution was then transferred to a 12–14 kDa cut-off dialysis membrane and dialyzed against distilled water at 50 °C

for a week. Afterwards, the solution was freeze-dried, lyophilized, and kept at -80°C for further use. The material in powder form was mixed in DPBS with 0.05% (w/v) photo-initiator (2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, Sigma Aldrich) and stirred at 80°C . GelMA was then exposed to UV light (320-500 nm, Omnicure S1500, Ontario, Canada) for 30 sec at 2.5 mW/cm^2 for partial crosslinking followed by another 30 sec exposure to UV under same conditions post bioprinting.

2.3.2 Cell preparation

The mouse fibroblast cell line, 3T3, and the mouse mammary carcinoma line, 4T1, were obtained from the laboratory of A. Mastro, PSU (State College, PA). Culture media for 3T3 fibroblasts composed of Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA), supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY) and 1% penicillin-streptomycin (Corning). 3T3 cells were used at passages 22 through 27. 4T1 cells were grown in RPMI (Corning), 10% FBS, 1% penicillin-streptomycin. Passages 7 through 12 were used for 4T1 cells. HUVECs were purchased from Lonza and cultured in MCDB 131 base media (Corning) supplemented with 10% FBS (Corning), 1% glutamine (Gibco, Life Technologies), 0.5% bovine brain extract (Lonza, Walkersville, MD), heparin (10 U/ml) (Sigma-Aldrich), endothelial cell growth supplement (3 mg/ml) (Sigma-Aldrich), and 1% penicillin-streptomycin (Corning). HUVECs were used at passages 3 through 8. HUVECs were also transduced in house with EF1 tdTomato lentivector (Vectalys, Toulouse, France) to ease cell visualization for fluorescence microscopy according to the manufacturer's instructions. GFP⁺ HUVECs were purchased from Angio-Proteomie (cAP-0001GFP; Boston, MA) and were used at passages 2 through 5. Human dermal fibroblasts (HDFs), obtained from N. Zavazava's laboratory at The University of Iowa (Iowa City, IA), were cultured in DMEM supplemented with 10% FBS (Corning), 1% glutamine (Gibco), 1% sodium pyruvate (Gibco), and 1% penicillin-streptomycin (Corning). HDFs were used at passages 7 through 12. GFP⁺ HDFs were purchased from Angio-Proteomie (cAP-0008-adGFP;

Boston, MA) and were used at passages 2 through 6. Human mesenchymal stem cells (MSCs) were obtained from Lonza (Walkersville, MD) and RoosterBio (Frederick, MD) and cultured in SU-005 RoosterBasal-MSC (RoosterBio). Passages 4 through 8 were used for MSCs. GFP⁺ MSCs were purchased from Cyagen, cultured in SU-005 RoosterBasal-MSC (RoosterBio), and used at passages 2 through 6.

All cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. Cell culture medium was changed every 2 to 3 days. Subconfluent cultures were detached from the flasks using a 0.25% trypsin–0.1% EDTA solution (Life Technologies) and split to maintain cell growth.

2.3.3 Fabrication of tissue spheroids

Adherent cultures of each cell type were detached from the culture vessels with 0.25% trypsin-EDTA (Life Technologies, Grand Island, NY) solution. Trypsin was neutralized with appropriate GM, and cells were counted by a hemocytometer. Each cell type was then diluted to a concentration of 2500, 5000, and 10,000 cells in 200 µl of appropriate GM. The cell suspension (200 µl) was then pipetted into a single well of a U-bottom 96-well microplate with a cell-repellent surface (Greiner Bio-One, Monroe, NC). For fabrication of MSC/HUVEC spheroids made of 50,000 cells, MSCs and HUVECs were combined at a ratio of 92:8. The microplates were then incubated at 37°C in a 5% CO₂ humidified atmosphere. Spheroid formation was monitored daily on an EVOS FL cell imaging system (Life Technologies). For fabrication of GFP⁺ HDF/MSC coculture spheroids, 5000 cells were used in total, and GFP⁺ HDFs and MSCs were cocultured in ratios of 2:1 and 1:1 for 1 day. During the fabrication and culture of HUVECs spheroids, EGM-2MV medium (Lonza) was used.

2.3.4 Analyzing the compactness and integrity of spheroids during the first 24 h of spheroid culture

Spheroids made of 2.5k cells were monitored closely for the first 24 h and imaged every 4 h on the EVOS FL Cell Imaging system. Spheroid integrity was also inspected every 4 h by aspirating and flushing one time with a pipette. Spheroids broke apart during the process were considered not compact to withstand aspiration forces, which was marked as 'X.' Otherwise, they were considered compact and marked as '✓'.

2.3.5 Electrocyte dissociation

Brachyhypopomus gauderio were obtained from the laboratory of Dr. Jason Gallant, Michigan State University (East Lansing, MI). Fish were maintained and then euthanized in a humane manner according to the animal protocol (protocol #47870), which was approved and performed based on the guidelines established by Institutional Animal Care and Use Committee (IACUC) at PSU. Prior to dissociation, a saline solution composed of 114 mM NaCl (Research Products International, Mt. Prospect, IL), 2 mM KCl (Fisher Scientific, Fair Lawn, NJ), 4 mM CaCl₂ (Avantor, Center Valley, PA), 2 mM MgCl₂ (Fisher Scientific), 5 mM HEPES (Research Products International), and 6 mM Glucose (Research Products International), was prepared and titrated with NaOH (Sigma Aldrich) to 7.2 pH. The *B. Gauderio* was euthanatized with 0.1% (w/v) Tricaine-S (Western Chemical Inc., Ferndale, WA). Subsequently, a section with a 4-5 cm length was cut from the tip of the tail and placed into saline. The skin was removed from the section. To weaken the connective tissue around electrocytes, the section was cut into 1 cm pieces and placed into conical tubes containing saline with 2% (w/v) collagenase type IV (Worthington Biochemical, Lakewood, NJ). Next, tubes were placed on a shaker table (Southwest science, Trenton, NJ) running at 200 rpm for 2 h inside an incubator. The pieces were further broken down using a pipettor with a wide cut tip (larger than 3 mm). The collagenase solution containing dissociated electrocytes was placed under a microscope and electrocytes were removed from the solution using

a pipettor, which had a tip at least two times larger than the diameter of electrocytes. Once removed, electrocytes were rinsed with saline solution and placed into a 1.5 ml centrifuge tube for bioprinting.

2.3.6 Scanning electron microscopy (SEM)

Field-emission SEM (FEI Nova NanoSEM 630) was used to investigate the surface topography of fabricated spheroids and dissociated electrocytes. Spheroids were harvested after 2 days of culture. Spheroids and electrocytes were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight; samples were then carefully washed in phosphate-buffered saline (PBS) and dehydrated using graded ethanol solutions (25 to 100%). To ensure complete removal of water, samples were further dried in a critical point dryer (CPD300, Leica EM, Wetzlar, Germany). On complete dehydration, they were sputter-coated with iridium (Leica) and imaged at an accelerating voltage of 3 to 5 keV.

2.3.7 Hydroxyproline assay

A total of 1000 2-day cultured spheroids of each type (with ~2500 cells per spheroid) were collected and homogenized in 100 μ l of distilled water. Then, 100 μ l of cell homogenate was transferred to a 2-ml pressure-tight vial, added with 100 μ l of 12 M hydrochloric acid, and hydrolyzed at 120°C for 3 hours. The total collagen amount per spheroid type was quantified using a hydroxyproline colorimetric assay kit (BioVision Inc., CA) according to the manufacturer's instructions. Collagen amount expressed by each spheroid type was determined using a PowerWave X-340 spectrophotometer (BioTek, Winooski, VT) at 560 nm, and the results were normalized to the collagen amount expressed by HUVEC spheroids at day 2. Experiments were repeated four times.

2.3.8 Preparation of pipettes

The customized tapered micropipettes were prepared from borosilicate pasteur pipettes (vWR, 14673-043, Radnor, PA) on a P1000 Flaming/Brown micropipette puller (Sutter Instrument,

Novato, CA). Parameters for heat, pull, velocity, time, and pressure were set at 575, 0, 5, 100, and 50, respectively, in order to produce pipettes in $\sim 80 \mu\text{m}$ diameter. The use of straight as well as tapered pipettes in cell culture and tissue engineering applications have been studied in the literature (19, 24); however, the tapered pipette has numerous advantages over the straight one. One advantage of using the tapered pipette for spheroid lifting is that it can provide larger forces at the same pressure than a straight pipette with the same diameter and height advancement. Owing to its advantages, a tapered pipette was used as the aspiration tool for the AAB system.

2.3.9 Surface tension measurement

Spheroids were collected into a petri dish from U-bottom 96-well plates, and then, their surface tension was measured using a micropipette aspiration technique, as explained in our recent work [52]. Aspirated spheroids were monitored via an STC-MC33USB monochromatic camera (Sentech, Japan) equipped with 1-61448 and 1-61449 adaptor tubes (Navitar, Rochester, NY).

2.3.10 Dynamic contact angle measurements

Dynamic contact angle of each spheroid type was measured using a Basler ACE acA2000-165 μm NIR camera (Basler, Germany) equipped with 1-61448 and 1-61449 adaptor tubes (Navitar, Rochester, NY, USA). Spheroids were captured inside an appropriate cell media for each cell type and were lifted by a tapered glass pipette. Frames of spheroid lifting process were saved and dynamic contact angles were measured by ImageJ (National Institute of Health (NIH), USA).

2.3.11 Surface tension measurement of cell media

To measure the surface tension coefficient of cell media (σ_m), we designed a bubble tensiometer according to the Young-Laplace equation under the assumption that the bubble radius of the end cap is much larger than that of the front cap:

$$\sigma_m = \frac{\Delta P}{2/R_p} \quad (1)$$

In this measurement, the equilibrium aspiration pressure (ΔP) was measured when the front cap of bubble outside the pipette reached the pipette tip radius (R_p), after which the bubble busted into the cell media. The surface tension coefficient was averaged from three measurements.

2.3.12 Development of the aspiration-assisted bioprinter

Bioprinting of spheroids was used with a MakerBot Replicator 1 3D printer (MakerBot, NY). The extrusion head was removed, and a holder for a pipette and two microvalve heads was 3D-printed using an Ultimaker 2 (Ultimaker) 3D printer. To control the 3D motion stage, a smoothie board (Uberclock, OR) was integrated.

For fabrication of support constructs, microvalves (INKX0517500A, Lee Company, Bashville, TN) with 250- μ m nozzles (INZA3100914K, Lee Company) were integrated on the bioprinter head. To operate microvalves, a control board (IECX0501350A, Lee Company) was used, which was controlled using Arduino Uno (Arduino, Italy). To cross-link sodium alginate, a portable ultrasonic humidifier (CZHD20, Comfort Zone, China) was used to generate the aerosol form of CaCl_2 .

Computer interface software was developed using Python 3 (Python Software Foundation, DE). Software interface was the central location where sensor input data was processed and G-Code commands were generated to control the motion controller system.

In order to observe spheroids during the process, a travelling USB camera with 800x magnification (4331891986, eBotrade, China), a bottom USB camera with 250x magnification (USB2-MICRO-250X, Plugable), and a STC-MC33USB monochromatic camera (Sentech, Japan) equipped with 1-61448 and 1-61449 adaptor tubes (Navitar, Rochester, NY) were integrated to the system. For lighting the monochromatic camera, LED arrays were fed with pulse width modulation (PWM) signal generated from custom-made software. The pneumatic system combined vacuum and pressure airflow in order to maintain required pressure within the system. A vacuum regulator (Megasan, Turkey), a multiple pressure regulator (KC LH-54S-3, Kegco, CA), and 1/4" NPT

12VDC normally-closed solenoid valves (USS2-00051, U.S. Solid, OH) were used together to maintain the required pressure. A barometric pressure sensor (BMP180, Sparkfun) monitored the pressure within the pneumatic line

2.3.13 Determination of precision and accuracy of AAB process

In order to determine the positional precision and accuracy of AAB process, we used empty pipettes ($n=24$), agarose beads ($n=24$) and MSC/HUVEC spheroids ($\sim 300 \mu\text{m}$, $n=16$). Empty pipettes (in $\sim 80 \mu\text{m}$ diameter) were made as explained before and agarose beads (G-AMB-0601-0010), in 250-300 μm diameter, were obtained from Bioscience Bead Division (West Warwick, RI). Empty pipettes were used as a control group in our experiments in order to rule out the error induced by the bioprinter itself. Agarose beads were also used as a control group, due to their near perfect spheroidal shape, in order to rule out the effect of imperfect spheroid shape.

Agarose beads and MSC/HUVEC spheroids we reprinted at a target position on agarose substrates in order to evaluate the bioprinting positional precision and accuracy. In this regard, 1% (w/v) agarose substrates were made by dissolving agarose (1004512, Research Products International Corp., Mount Prospect, IL) in DI water and gently spreading it on a microscope calibration slide with coordinate planes (Motic, China). The slide was then placed on the bioprinter table. Similarly, empty pipettes were transferred to the target position. After positioning pipettes, beads and spheroids, images were taken by a placement camera, then processed in ImageJ.

Accuracy was represented as the root mean square error (RMSE), which was calculated using the below equation:

$$\text{RMSE} = \left[\frac{\sum_{i=1}^n (X_{\text{target}} - X_i)^2 + (Y_{\text{target}} - Y_i)^2}{n} \right]^{1/2} \quad (2)$$

where X_{target} and Y_{target} are X and Y coordinates of the target position, respectively, X_i and Y_i are the position of the measured values in X- and Y-axis, respectively, and n is the sample number. Precision was represented as the square root of standard deviation.

2.3.14 Immunostaining and imaging

Spheroids fixed in 4% paraformaldehyde (Sigma-Aldrich) and rinsed in Dulbecco's PBS (DPBS) were stained with NucBlue ReadyProbes reagent (Life Technologies) to visualize the cell nuclei; actin cytoskeletal fibers were stained with ActinGreen 488 ReadyProbes reagent according to the manufacturer's instructions. Imaging and 3D reconstruction of a heterogeneous pyramid structure was performed using a Zeiss confocal microscope (LSM 880, Carl Zeiss AG, Oberkochen, Germany) using a 5× lens. The 3D reconstruction was obtained using Zen blue software (Carl Zeiss AG). A diamond structure was first imaged through Zeiss Axiozoom (Carl Zeiss AG, Oberkochen, Germany) to capture the entire 3D structure and then further imaged on a confocal microscope (Olympus FV1000, Olympus America Inc., Center Valley, PA) to closely image the DAPI (4',6-diamidino-2-phenylindole), F-actin, and CD31 staining.

2.3.15 Cell viability

Spheroids were removed from the U-bottom well plates and subjected to the bioprinting process. Spheroids directly taken from plates were used as a positive control group. Viability was assessed using a LIVE/DEAD viability assay kit (Life Technologies, Grand Island, NY). Briefly, spheroids were washed twice in DPBS and then placed in the dye solution consisting of 1 μ M calcein acetoxymethyl and 1.6 μ M ethidium homodimer-1 in PBS. Live cells were able to take up and retain the calcein dye, resulting in bright green fluorescence of their cytoplasm. The ethidium homodimer could only enter dead cells where it binds to nucleic acids, producing a bright red fluorescence. Spheroids were imaged at 20- μ m z-stack step size on the Olympus FV1000 confocal microscope with a 60- μ m aperture setting resulting in approximately five to seven images per spheroid. Each image was then analyzed using ImageJ software (National Institutes of Health, USA).

2.3.16 Self-assembly of spheroids

Spheroids made of 3T3 cells were collected from U-bottom 96-well plates and then washed with DPBS twice. CellTracker Orange CMTMR [5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine] (Thermo Fisher Scientific) and CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) (Thermo Fisher Scientific, MA) were used according to the manufacturer's instructions. Spheroids were bioprinted with close proximity, and then, images were captured at 0, 24, 48, and 72 hours using EVOS FL Cell Imaging System. The contact length and contact angle of two spheroids were measured every 4 hours up to 24 hours.

2.3.17 Imaging and quantification of endothelial sprouting

Images of sprouting GFP⁺ and tdTomato⁺ HUVEC spheroids at day 7 were taken on the Zeiss Axiozoom microscope at a magnification of 20x to capture the spheroids bioprinted at various distances. Images of sprouts at days 2 and 5 were taken using the EVOS FL Cell Imaging System. Sprouting behavior of spheroids was analyzed using AngioTool [64]. To obtain the cross-sectional view of the capillary-like structures formed by GFP⁺ and tdTomato⁺ HUVEC spheroids at day 7, samples were imaged using a 40× oil lens on the Olympus FV1000 confocal microscope. For the study with GFP⁺ HDF/MSC coculture and HUVEC spheroids, samples were imaged on the EVOS microscope using fluorescent (4×) channels at days 2 and 5. Images of those samples on day 7 were taken using fluorescent (16× and 40×) and phase (16×) channels by the Axiozoom microscope.

2.3.18 Directionality analysis of angiogenic sprouts

The line tool in ImageJ was used to trace out a line following the length of each sprout. The line was traced starting from the spheroid edge to the end of the sprout. The angle made by this line with the horizontal line (0/180°) on a polar plot was recorded as the angle made by the sprout towards the direction of the neighboring HUVEC or HDF/MSC spheroid. Sprouts growing towards the other spheroid was of interest, and therefore, the Angle of Interest (AOI) was defined for the angle span[60°, -60°] for GFP⁺ HUVEC and [120°, 240°] for tdTomato⁺ HUVEC spheroids.

The angle span outside AOI was defined as 'Other.' To better represent the distribution of sprouts at AOI, AOI was depicted by green and red bars depending on the fluorescent labeling of HUVEC spheroids. The sprouts formed in other regions were depicted by grey bars for both spheroids. The number of sprouts in 'AOI' and 'Other' were determined, and then normalized with respect to the total angle (°) span in that zone, and the percentage (%) of normalized number of sprouts for each zone was calculated accordingly.

For GFP⁺HDF/MSC co-culture experiments, AOI was determined to be [120°, 240°] for tdTomato⁺ HUVEC spheroids and the percentage (%) of normalized number of sprouts for 'AOI' and 'Other' were determined as explained above.

2.3.19 Statistical analysis

All data were presented as means $\pm$ SD and analyzed by Minitab 17.3 (Minitab Inc., State College, PA, USA) using one-way analysis of variance (ANOVA) to test for significance when comparing the data. Post hoc Tukey's multiple-comparison test was used to determine the individual differences among the groups. Differences were considered significant at *P < 0.05, **P < 0.01, and ***P < 0.001. For directionality analysis, t test was used to compare the results between AOI and Other (where differences were considered significant at *P < 0.05, **P < 0.01, and ***P < 0.001), and ANOVA (with Tukey's multiple comparison test) was used to compare the results among different groups (where differences were considered significant at #P < 0.05, ##P < 0.01, and ###P < 0.001). Repeated-measures ANOVA (with Tukey's multiple-comparison test) was conducted to compare the results among different days of same groups, and differences were considered significant at *P < 0.05, **P < 0.01, and ***P < 0.001.

2.4 Results and Discussion

2.4.1 Spheroid fabrication

In order to demonstrate picking and lifting of spheroids, a wide range of spheroid types with different viscoelastic and surface tension properties were fabricated using HUVECs, 3T3, 4T1, HDFs, co-culture of MSCs and HUVECs, and MSCs. Various kinds of spheroids in a diameter range of 200–600 μm were obtained using cell-repellent 96-well plates. Spheroids were fabricated at a cell density from 2.5K to 10K/spheroid and cultured for 1 to 3 days. During spheroid fabrication, Spheroid formation was monitored daily on an EVOS FL Cell Imaging System (Life Technologies). It was observed that the relative size of spheroids depends on their cell number, culture time and cell types (Figure 2.3). The average diameter of HUVEC spheroids increased significantly with higher cell concentrations. Spheroids made of 5k cells resulted in a ~43% increase in their diameter while spheroids made of 10k cells were ~88% larger when compared to spheroids made of 2.5k cells on Day 1. No significant changes were observed in the average diameter of HUVEC-only spheroids over the 3-day culture period.

The average diameter of 3T3 spheroids made of 2.5k cells decreased by ~19% on Day 2 and ~27% on Day 3. The average diameter of 3T3 spheroids, made of 5k cells, displayed a decrease in their diameter by ~16% on Day 2 and ~24% on Day 3, while the average diameter of 3T3 spheroids made of 10k cells was declined by ~19% on Day 2 and ~27% on Day 3. A significant increase in the average diameter of 3T3 spheroids was observed when the cell number increased. Doubling the number of cells from 2.5k to 5k resulted in a ~31% increase in spheroid diameter while spheroids made of 10k cell number were ~67% larger when compared to spheroids made of 2.5k cells on Day 1.

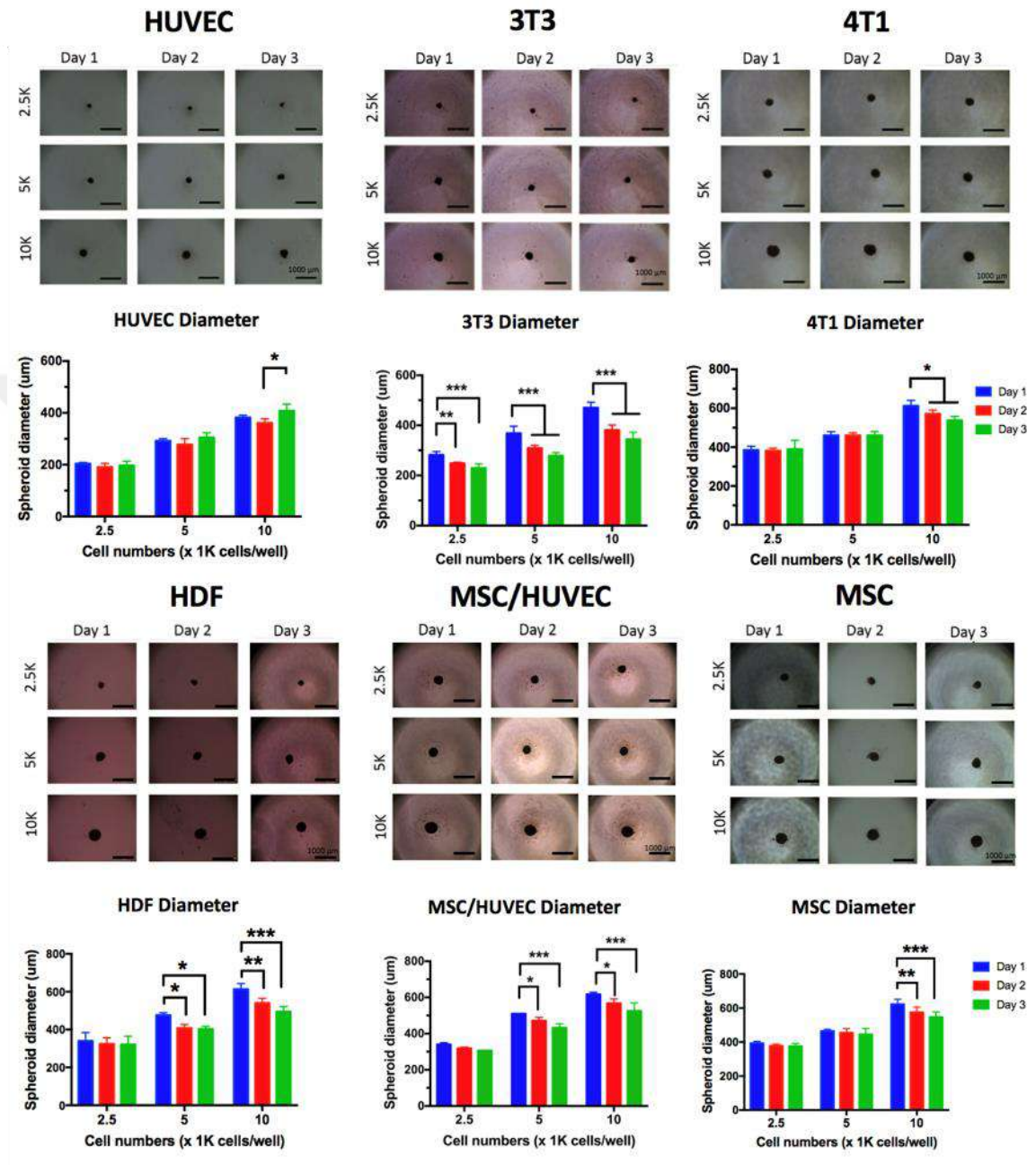


Figure 2.3: Dimensional measurements. (A) Fabrication of HUVEC, 3T3, 4T1, HDF, MSC/HUVEC, and MSC spheroids made of 2.5k, 5k, and 10k cells on Days 1, 2 and 3, and related diameter measurements. ($n=5$; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

An increase in the average diameter of 4T1 spheroids was observed when the cell number increased. Spheroids made of 5k cells resulted in a ~20% increase in spheroid diameter and spheroids made of 10k cells were ~59% larger when compared to spheroids made of 2.5k cells. A significant decrease in 4T1 spheroid diameter due to prolonged culture time was observed in spheroids made of 10k cells, decreased by ~7 % on Day 2 and ~12% on Day 3, while spheroids made of 2.5k and 5k cells did not change significantly during the 3-day culture period. 4T1 spheroids maintained their spherical structure when made with high cell concentrations (5k and 10k) resulting in larger diameters when compared to 3T3 and HUVEC spheroids with the same cell number and culture time.

During HDF spheroid fabrication, the average diameter of HDF spheroids made of 5K cells decreased by 14.5% from Day 1 to 2. However, no significant difference was observed from Day 2 and 3. Increasing the HDF number by four folds (from ~2.5k to ~10k) resulted in ~80, 67, 54% increase in the spheroid diameter at Days 1, 2, and 3, respectively.

The average diameter of MSC/HUVEC spheroids made of 2.5k cells decreased by ~7% on Day 2 and ~9.8% on Day 3. The average diameter of MSC/HUVEC spheroids, made of 5k cells, displayed a decrease in their diameter by ~7% on Day 2 and 15% on Day 3, while the average diameter of MSC/HUVEC spheroids made of ~10k cells diameter was declined by ~8% on Day 2 and ~15% on Day 3. No significant changes were observed in the average diameter of MSC/HUVEC spheroids made of 2.5k over time.

An increase was observed in the average diameter of MSC spheroids when the cell number was increased. The average diameter of MSC spheroids, made of 10k cells, exhibited shrinkage in their diameter by ~7.6% on Day 2 and ~5% on Day 3. No significant changes were observed over the 3-days culture in the average diameter of MSC spheroids made of 2.5k and 5k cells.

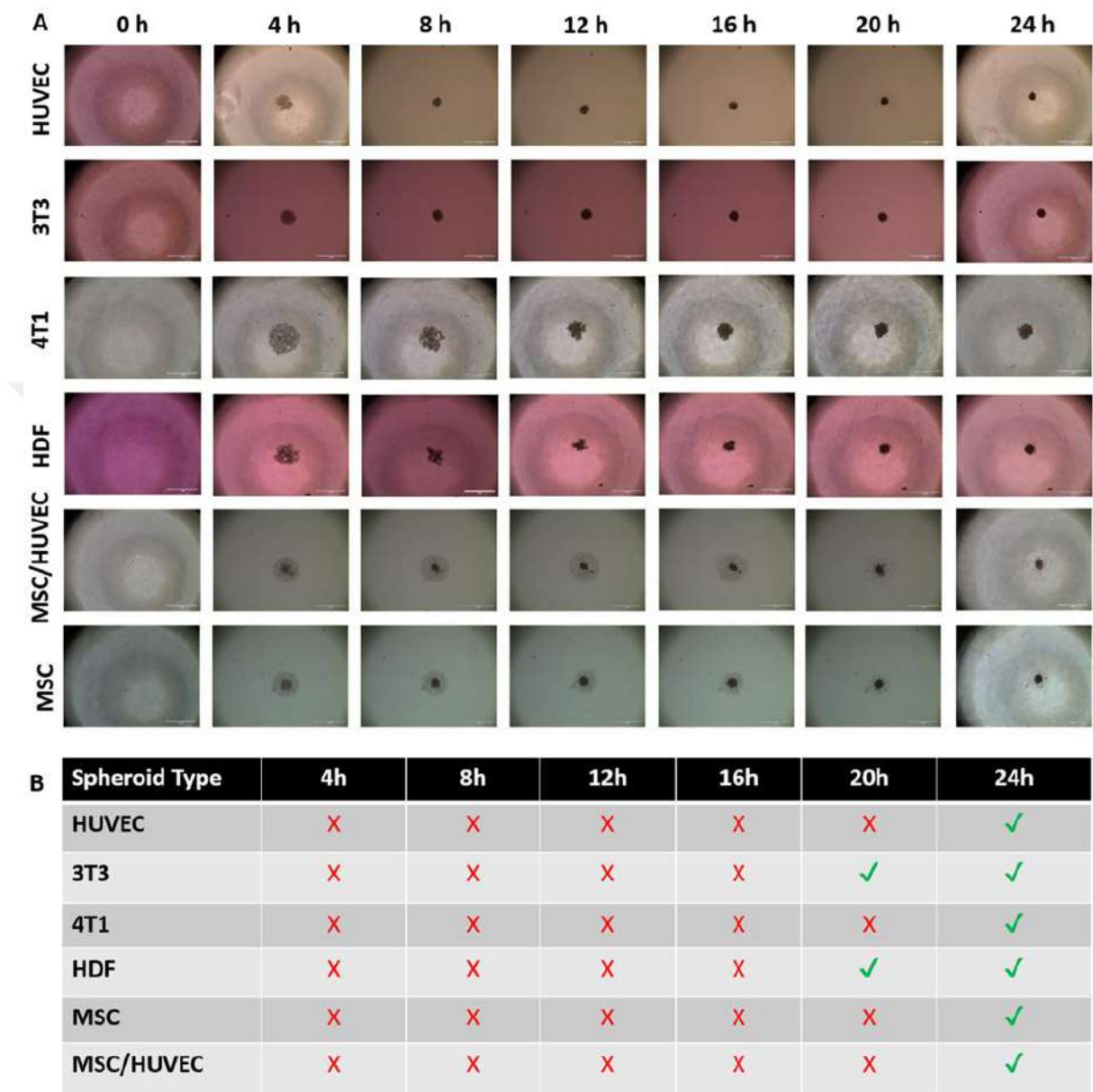


Figure 2.4: Compactness of different spheroid types over time. (A) Optical images from 0 to 24 h (in 4h intervals) demonstrating cell aggregation for HUVEC, 3T3, 4T1, HDF, MSC/HUVEC, and MSC spheroids (made of 2,500 cells) and (B) a table showing compactness of spheroids at these time points.

GFP⁺ HDF/MSC spheroids were made of 5k cells and cultured for one day only, yielding ~350 μm size.

During the first 24 h, we closely monitored spheroid formation and observed that 3T3 and HDF spheroids became compact and withstood aspiration forces at the end of 20 h while others became compact at 24 h implying that these spheroids could be lifted anytime thereafter (Figure 2.4).

2.4.2 Working mechanism of AAB

In AAB process, the first step is to pick a spheroid and then lift it and drag it rapidly outside the culture media by aspiration forces as shown in Figure 2.5A. The spheroid should be captured with a minimum aspiration force in order to transfer it but do not break or damage it. During aspiration under an applied stress (σ), a spheroid with viscoelastic properties exhibit both viscous and elastic properties [65]. It exhibits elastic properties like a solid at times shorter than relaxation time τ and like a fluid for $t > \tau$ (Figure 2.10).

During this process, two major impediments were experienced. First, spheroids were prone to fragment due to low cellular cohesivity and elasticity even under very low pressure levels. Second, detachment of spheroids from the pipette tip at the three-phase contact (air-liquid-tissue) was observed during lifting. The former can be mitigated by engineering of spheroids to attain better elastic properties (such as culturing them longer [66] or incorporating cells expressing higher cell-cell and cell-matrix adhesion or ECM proteins [67]) and the latter can be addressed by determining the minimum aspiration pressure (critical lifting pressure). A spheroid is lifted by pipette aspiration after overcoming gravity, buoyance force, hydraulic drag, and thermodynamic barrier at the interface. The main difficulty in lifting a spheroid is the binding energy caused by the surface tension at the interface between the culture media and air. The spheroid contributes to the lifting barrier in the form of a contact angle at the three-phase (tissue, air, and media) contact line. The energy barrier in order to lift a spheroid into air can be expressed as:

$$E = A_s \sigma_{1,2} \cos^2 \frac{\theta_d}{2} \quad (3)$$

where A_s is the surface area of a spheroid, $\sigma_{1,2}$ is the surface tension between the media and air, and θ_d is the contact angle at the three-phase contact line. During lifting, the three-phase contact angle (dynamic contact angle) is greater than the static contact angle due to dynamics effect [68]. The dynamic contact angle of spheroids made of several types of cells were enumerated in Table 2.1. By comparison, the thermodynamics barrier is about 500 times of gravity, 100 times of Stokes' drag, and five times of Young-Dupre critical pressure. During lifting, the energy barrier can be estimated by adding a geometrical correction factor (m) as a result of slight changes in the spheroid geometry due to the aspiration of a tongue (see Figure 2.5B). The initial contact length of the tongue ($h_{t=0}$) of a spheroid ranges between R_p to $2R_p$. Thus, m can be defined as follows:

$$m = 1 - \frac{4\pi R_p^2}{4\pi R_s^2 - 4\pi R_p^2} = 1 - \frac{R_p^2}{R_s^2 - R_p^2} \approx 1 - \left(\frac{R_p}{R_s}\right)^2 \quad (4)$$

In Equation (4), R_p is the pipette tip radius and R_s is the spheroid radius. m can be approximated to 1 when the spheroid radius is much larger than the radius of the pipette tip. However, when lifting a small spheroid, or when the radius of pipette is comparable to that of spheroid, m becomes much smaller. Consequently, the critical lifting pressure of a spheroid (P_C) from the media can be expressed as follows [68]:

$$P_C = m F_{max} / A_h = 2m R_s \sigma_{1,2} \cos^2 \frac{\theta_d}{2} / (\pi R_p^2) \quad (5)$$

The critical lifting pressure is proportional to the surface tension coefficient of the media-air interface, the radius of the spheroid, the dynamic contact angle at the three-phase contact line, and the tapered angle of the pipette (influences R_p , but this parameter was fixed). Surface tension coefficient of different types of cell media utilized in our experiments were enumerated in Table 2.2.

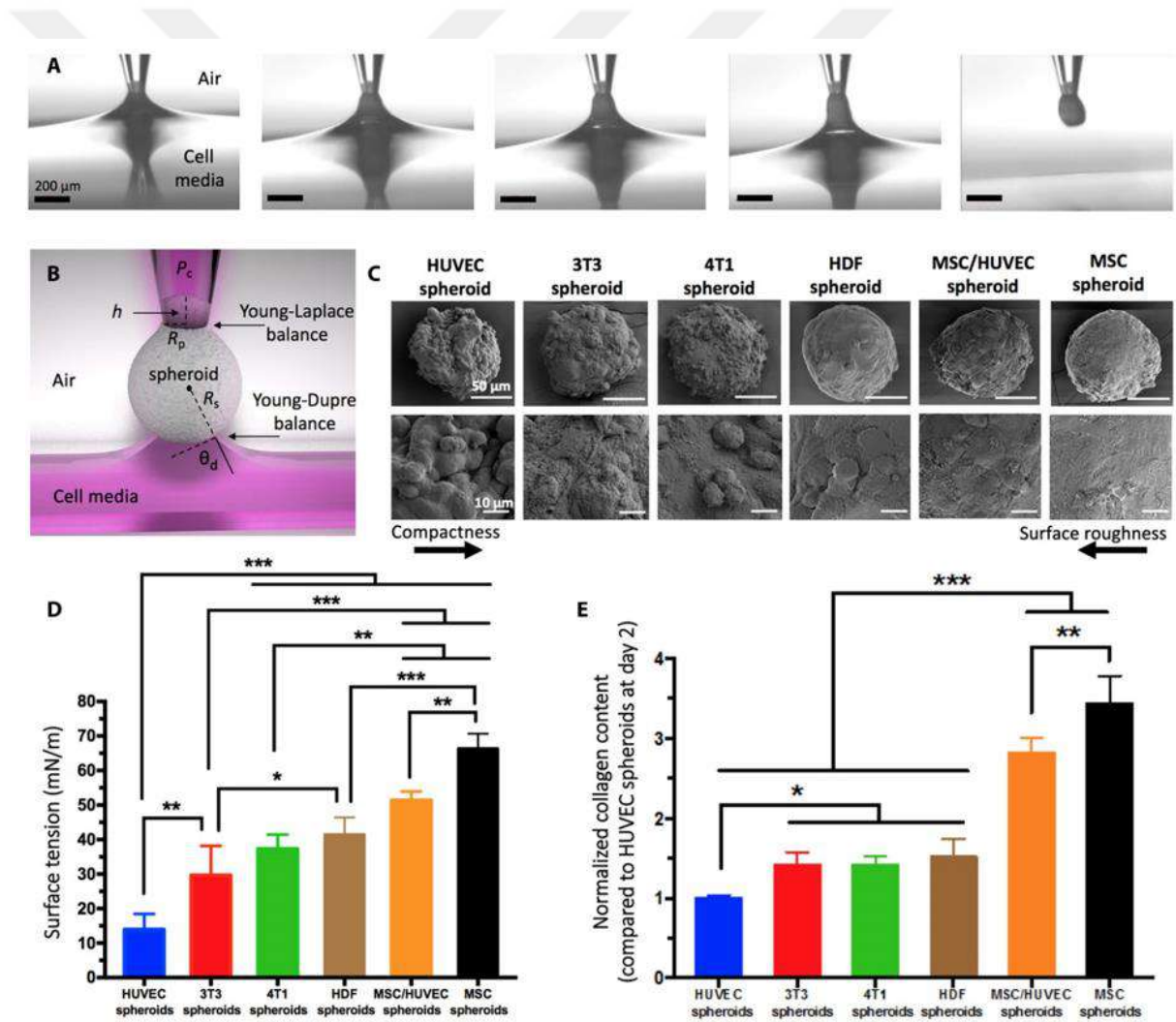


Figure 2.5: Picking and lifting spheroids. (A) Time-lapse images during spheroid lifting process (at the interface of cell media and air). (B) A schematic showing physical parameters involved in lifting

of a spheroid from the cell media. (C) SEM images, (D) surface tension ($n = 5$), and (E) the normalized collagen content of HUVEC, 3T3, 4T1, HDF, MSC/HUVEC, and MSC spheroids (compared to HUVEC spheroids) at day 2 ($n = 4$; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

Table 2.1: Dynamic contact angle of spheroids at lifting speed of 5 mm/s

Spheroid type	Dynamic contact angle (°)
HUVEC	76.0±4.0
3T3	78.2±6.4
4T1	65.0±9.4
HDF	78.4±5.0
MSC: HUVEC (92:8)	76.38±1.4
MSC	81.8±2.6

Table 2.2: Surface tension coefficient of different types of cell media utilized in our experiments

Cell media type	Surface tension coefficient (mN/m)
HUVEC media	51.5±0.4
3T3 and HDF cell media)	62.9±0.3
4T1 cell media	57.4±0.3
MSC: HUVEC (92:8) cell media	62.6±0.4
MSC growth media	63.1±0.4

In order to investigate the ultrastructure of spheroids, spheroids were analyzed by SEM and SEM images demonstrated clear differences in surface morphology and compactness of different spheroids cultured for 2 days (Figure 2.5C). The surface topology of MSC spheroids displayed a substantial amount of secreted ECM with smoother and more compact structure due to tight cell-cell and cell-ECM interactions. On the other hand, the surface topology of HUVEC spheroids displayed lesser ECM compound, which resulted in a pitted surface and tiny gaps between cells. In order to lift spheroids, spheroids were required to be compact and captured in the cell media, and withstand aspiration forces. Spheroids also possess surface tension, with each cell being analogous to a liquid molecule [69,70]. Thus, surface tension of spheroids were investigated by a micropipette aspiration technique [65,71] according to the Young-Laplace equation, which gave the relationship between the internal pressure of spheroids and cell media across a curved interface. According to the Young-Laplace equation, the surface tension coefficient can be estimated as $\sigma = \frac{\Delta P}{2(1/R_p - 1/R_s)}$, where ΔP is the equilibrium aspiration pressure when the advancement of the spheroid inside the pipette was the same as the radius of the pipette tip. As shown in Figure 2.5D, the surface tension of HUVEC, 3T3, 4T1, HDF, MSC/HUVEC and MSC spheroids at 2 days of culture were measured to be ~14, 30, 37, 41, 51 and 66 mN/m, respectively. Surface tension of MSC-only spheroids was approximately five times higher than that of HUVEC spheroids. It is worth mentioning that Norotte *et al.* used a different method to measure the surface tension of spheroids and obtained a similar result for HUVEC spheroids [72].

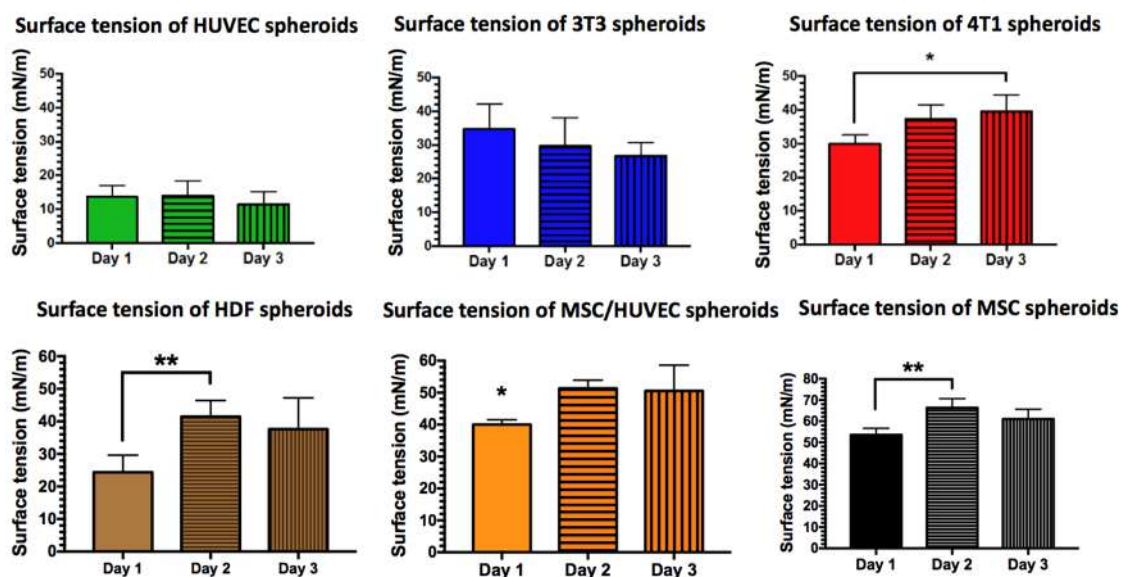


Figure 2.6: Surface tension of HUVEC, 3T3, 4T1, HDF, MSC/HUVEC and MSC spheroids on Days 1, 2 and 3. ($n=5$; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

While the surface tension of 4T1, HDF and MSC/HUVEC spheroids increased over time, surface tension of spheroids made from other cell types maintained similar properties during the 3-day culture (Figure 2.6). This time frame, on the other hand, can vary for spheroids prepared using other techniques, such as but not limited to hanging drop, microfluidic, rotator flask, and liquid overlay methods [29]. Our results indicate that there is a positive correlation between surface tension of spheroids and their compactness confirming previous findings by Foty and Steinberg [73]. Additionally, the total collagen amount of spheroids was investigated using hydroxyproline colorimetric assay kit since collagen is one of the major ECM components of spheroids [50]. The measured collagen amount for 3T3, 4T1, HDF, MSC/HUVEC, and MSC spheroids at Day 2 was ~1.4-, 1.4-, 1.5-, 2.8-, 3.4-fold higher compared to that for HUVEC spheroids, respectively (Figure 2.5E). The normalized collagen content in different spheroids showed a similar trend as compared to the compactness of spheroids; however, we did not observe any significant differences among

the normalized collagen content for 3T3, 4T1 and HDF spheroids. Indeed, a recent study reported a positive correlation between compactness of cell aggregates and their collagen expression [74].

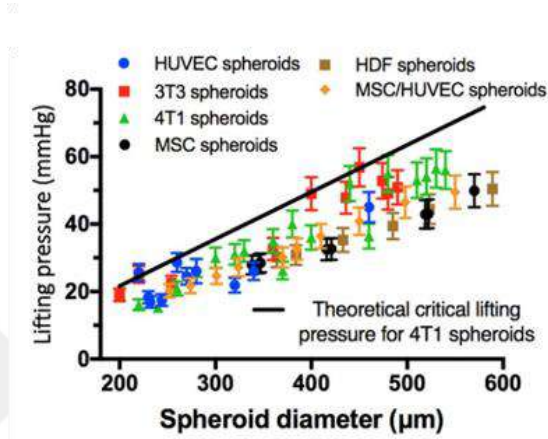


Figure 2.7: Calibration curve of AAB. Critical lifting pressure to lift spheroids (in the range of 200 to 600 μm in diameter) ($n = 5$). The experimental data spread under the theoretical curve, which was determined using the experimental data for 4T1 spheroids with parameters ($\theta_d = 64^\circ$ and $s_{1,2} = 57.4 \text{ mN/m}$). Spheroids made of other cell types had lower theoretical critical lifting pressure values (data are not shown in the dissertation). For instance, the theoretical critical lifting pressure for HUVEC spheroids was 20% smaller than that of 4T1 spheroids.

Figure 2.7 denotes a linear relationship between the critical lifting pressure and diameter of spheroids (in the range of 200-600 μm). When the diameter of spheroids increased, the critical lifting pressure also increased. The theoretical critical lifting pressure for each spheroid types at different diameters were determined using Equation (5), where the maximum theoretical value belonged to 4T1 spheroids, which could be used as a baseline for new spheroid types to be tested during aspiration. Additionally, the minimum critical lifting pressure among all spheroids types (from 200 to 600 μm) was determined to be $\sim 19 \text{ mmHg}$.

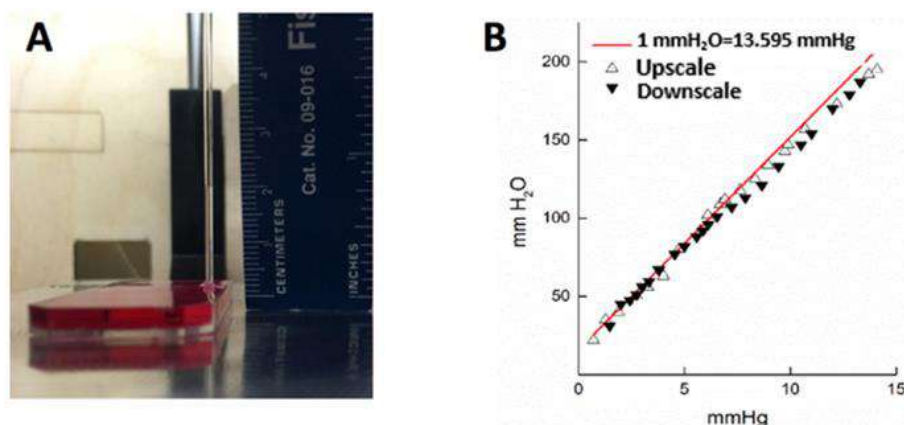


Figure 2.8: Calibration of the pneumatic system and positional precision and accuracy for AAB. (A) Vacuum system calibration by a glass pipette with a diameter of $\sim 80 \mu\text{m}$. (B) Calibration results by the comparison of digital reading (x-axis) and liquid height (y-axis) showing $\sim 10\%$ error ($1 \text{ mmH}_2\text{O} = 13.595 \text{ mmHg}$). Calibration was conducted by upscaling and downscaling measurements of the water column height and pressure. The pressure difference could be caused by the vacuum pressure drop and leakage in the pipeline (another possible factor). The pressure difference between the sensor and pipette tip was within 10% error.

The difference between the theoretical and experimental value of the critical lifting pressure was due to i) the irregular shape of spheroids (elliptic or pancake-like) influencing the maximum energy barrier [75,76], and ii) the measurement inaccuracy of the pressure sensor (which was around 10% , see Figures 2.8A-B). Performing calibration for the vacuum system was an important step towards the development of the AAB platform since it determines the accuracy of measurements taken. As shown in Fig. S5A, we calibrated the pressure sensor by a straight vertical pipette with an inner diameter of $\sim 80 \mu\text{m}$. We compared the pressure from the digital indicator with the converted pressure from water column (we used the following classic conversion of units: $1 \text{ mmHg} = 13.595 \text{ mmH}_2\text{O}$). As shown in Figure 2.8B, the red solid line was the conversion relation

between the water column height and pressure sensor reading. After dipping the pipette into the cell media, there was a capillary action along the inner wall of the pipette. For 4T1 cell media, the capillary action in the pipette was 16 mmH₂O, which was the intercept of linear relation at zero aspiration pressure.

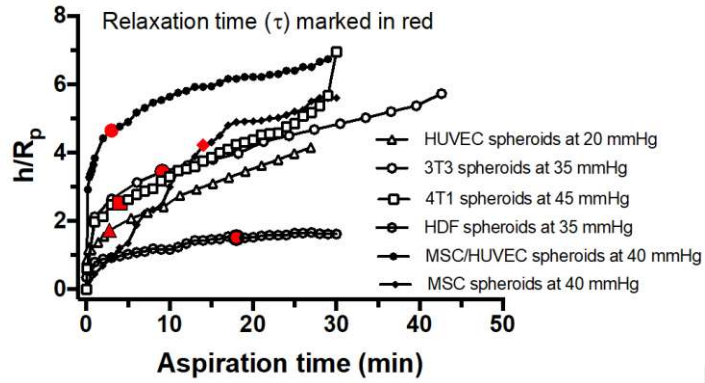


Figure 2.9: Viscoelastic behavior of spheroids under aspiration ($n = 3$). Here, h denotes the advancement of spheroids inside a glass pipette. The aspiration experiment used a similar pipette as that in bioprinting. The aspiration pressure was determined according to the size of spheroids satisfying the condition that spheroids could be lifted from the cell media.

Another vital factor for successful bioprinting was to determine viscoelastic properties of spheroids, which were reflected by the relationship between stress and strain as a function of time. During AAB process, spheroids were kept within a solid elastic regime. The operation time of AAB was defined by the elapsed time between spheroid picking and bioprinting, which was commonly less than 30 sec. Therefore, spheroids were successfully bioprinted while their relaxation time (τ) was longer than the bioprinting operation time. For this reason, we plotted the aspiration curves of various spheroids (Figure 2.9). At the early stage of aspiration, spheroids deformed very quickly

within the elastic regime. Then, after a relaxation time, accumulated stress was gradually released and spheroids experienced a ‘relaxation’ process and return to their original shapes. As shown in Figure 2.9, the relaxation time for HUVEC, 4T1 and MSC/HUVEC spheroids were around 3-5 min. The relaxation time for HDF spheroids could reach up to 18 min. Overall, these relaxation times were sufficient to assure successful bioprinting without permanent deformation to spheroids.

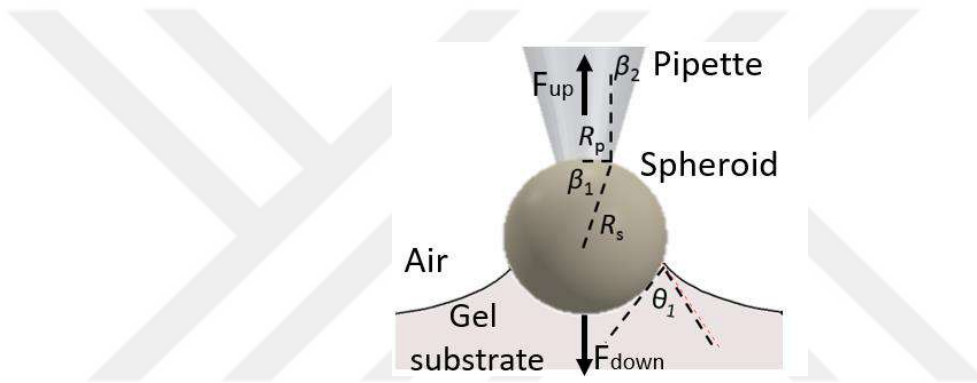


Figure 2.10: Patterning and bioprinting of a spheroid. (A) A schematic showing critical parameters during bioprinting.

After lifting spheroids from the cell media and transferring them to the bioprinting stage, the back pressure was released and the spheroids were fully recovered, or in other words, they returned to their original shapes as bioprinting time was less than the relaxation time. In order to bioprint a spheroid, the spheroid was partially submerged into a pre-crosslinked or partially-crosslinked gel-substrate as going deeper could result in their breakage due to penetration of the pipette tip into the spheroid. When the nozzle moved up, the spheroid was stuck to the gel due to adherence between the spheroid and gel (Figure 2.10) and released from the pipette and deposited into the gel. In AAB, successful bioprinting was determined by a constant adherence at the pipette tip (upwards, F_{up}) and a variable bioprinting force at the interface of gel (downwards, F_{down}). In

order to determine F_{up} , two angles were defined: $\beta_1 = \arccos \frac{R_p}{R_s}$, and β_2 : the pipette taper angle. F_{up} can be expressed as $F_{up} = 2\pi R_p \gamma_s \cos(\beta_1 + \beta_2)$, where γ_s is the surface tension of the spheroid. At the bottom of the spheroid, the maximum force pulling the spheroid downwards due to the surface tension of the gel substrate, i.e. the maximum bioprinting force provided by the gel, can be expressed as $F_{down} = 2\pi R_s \gamma_g \cos^2(\frac{\theta_1}{2})$. Here, γ_g is the surface tension of the gel, θ_1 is the effective angle of wetting. For successful bioprinting, the maximum force pulling the spheroid downwards needs to overcome the surface tension adherence at the tip of the pipette. Thus, successful printing needs to satisfy the condition of $F_{down} > F_{up}$. However, if a spheroid is not fully recovered (when the spheroid is submerged rapidly into the gel), there can be an extra stress (F_{ext}) at the pipette tip. The extra stress may increase the difficulty of bioprinting since F_{down} needs to overcome $F_{ext} + F_{up}$.

In order to verify the presented theoretical approach, β_1, β_2 and θ_1 angles were measured and the surface tension of pre-crosslinked gel (1% (w/v) alginate) was obtained from the literature [77]. The results indicated that F_{down} was ~32, 14, 12, 11, 9, 7 times greater than F_{up} for HUVEC, 3T3, HDF, MSC/HUVEC and MSC spheroids, respectively, as enumerated in Table 2.3. Surface tension of gel solutions can vary from gel to gel. In some gels (such as alginate [77]), surface tension is dependent on concentration; however, there are hydrogels where surface tension is not depended on concentration, such as polyacrylamide [78]. Therefore, we cannot make a general statement on the correlation between concentration and surface tension. In addition, concentration of the gel should also be compatible with micro-valve bioprinting as higher concentrations of precursor solutions are in general bioprintable as such concentrations can support droplet formation [25]. For bioprinting into a gel substrate, the gel precursor solution should be flowable as well so that the solution can overlay the bioprinted spheroid quickly; therefore, we performed bioprinting on partially-crosslinked or uncrosslinked gel precursor.

Table 2.3: Determination of F_{up} and F_{down} for bioprinting of various spheroids in 1% (w/v) alginate.

Spheroid type	F_{up} (N)	F_{down} (N)	F_{down}/F_{up}
HUVEC	7.7×10^{-7}	2.5×10^{-5}	~32.4
3T3	1.9×10^{-6}	2.5×10^{-5}	~13.6
4T1	2.1×10^{-6}	2.5×10^{-5}	~12.1
HDF	2.4×10^{-6}	2.5×10^{-5}	~10.5
MSC: HUVEC (92:8)	3×10^{-6}	2.6×10^{-5}	~8.6
MSC	3.8×10^{-6}	2.5×10^{-5}	~6.6

2.4.3 Development of AAB System

Auxiliary control, position control, motion controller, and pneumatic system were the main parts of the AAB platform (Figure 2.11). Software interface was the central location where sensor inputs (i.e., pneumatic pressure sensor) and cameras were processed and G-Code commands were generated to control the motion controller system. There were three cameras within the AAB system. Traveler camera was used to observe spheroid capturing and inspection. Placement camera was located under the Petri dish holder. Placement camera was used to observe spheroid deposition. Computer vision algorithms were applied to the real-time camera video stream in order to measure and identify spheroids and other objects within the AAB system. The diameter of captured spheroids was used to determine the minimum pressure required to overcome the surface tension

in order to successfully capture spheroid while minimizing the tissue exposure to mechanical stress during aspiration.

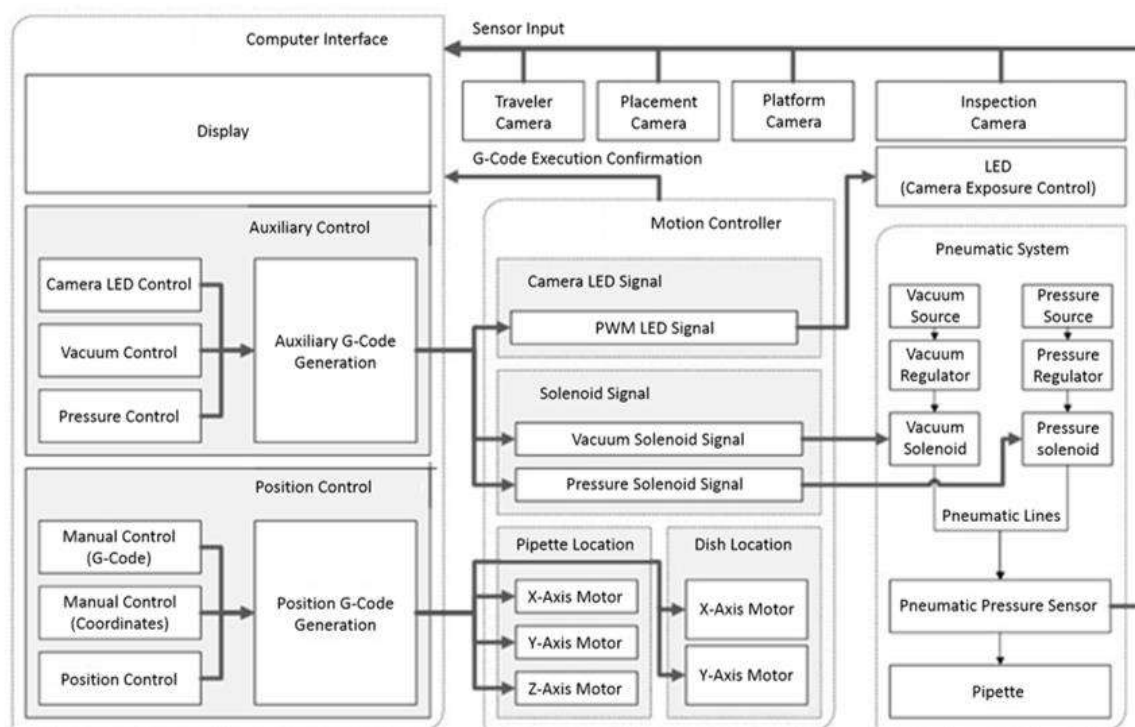


Figure 2.11: Block diagram of AAB

During bioprinting, the diameter of the captured spheroids was used to determine the optimal distance between substrate and pipette in order to minimize the mechanical compression of biologics. Placement camera was in charge of inspection and monitoring of the bioprinted tissue constructs. The distance between the individually placed spheroids was measured and monitored automatically to ensure that the dimensional accuracy was maintained within the required tolerances.

The AAB System can be operated in semi-automatic and fully automatic modes. System was fully programmable by the user to perform a desired task such as patterning of arbitrary shapes

and performing complex sequence of actions efficiently. Computer vision algorithms were utilized on camera sensor input streams to identify and dimension spheroids as well as surroundings in real time. This information complemented the data obtained from the pressure sensor and other subsystems of the bioprinter in order to reduce the mechanical stress experienced by the tissue as well as increase the speed, accuracy and efficiency of the bioprinting process. During spheroid capturing, the system monitored the pipette tip until a spheroid of a desired size and shape was detected. Dimensions obtained during the capturing process were used to determine the minimum back pressure required to overcome surface tension forces and lift spheroids from the cell media. In order to ensure that spheroids were successfully captured without any damage, spheroids were identified and dimensioned after lifting. Only the spheroids of correct type, shape and size were used for bioprinting while spheroids outside the required tolerance range was identified, discarded and the capturing procedure was repeated until a suitable spheroid was successfully picked and dimensioned. Spheroids were bioprinted into or onto a gel substrate one by one according to a pattern defined by the user. In order to further reduce the mechanical stress exerted on spheroids during bioprinting, the dimensions obtained during the capturing process were used to calculate the minimum required offset distance between the substrate surface and pipette tip. This enables us to place the spheroid into or onto a gel substrate resulting in minimal mechanical stresses and increased placement accuracy. Once the spheroid was bioprinted on top of the gel, the placement microscope camera was used to ensure that precision of placement meets the tolerances required by the tissue construct design constraints.

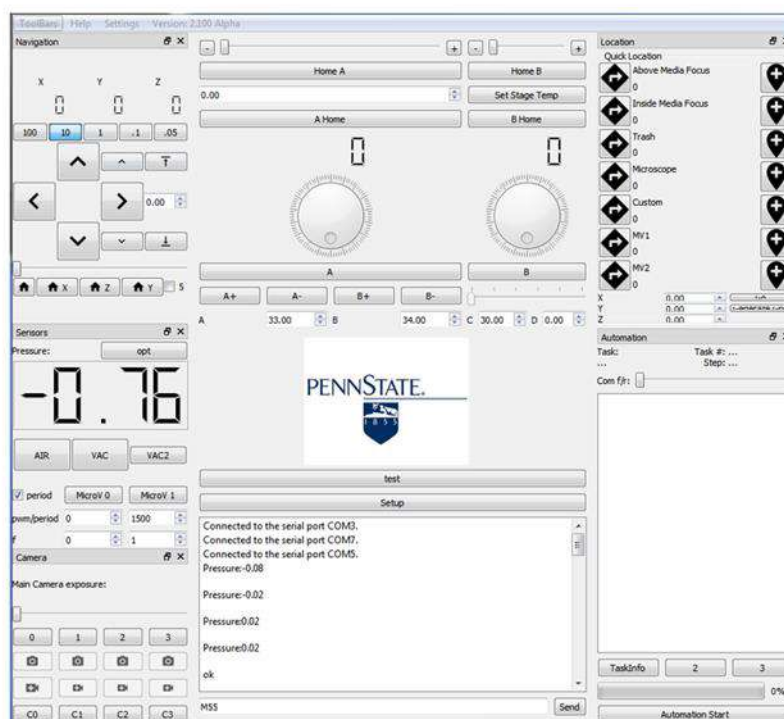


Figure 2.12: Computer interface of AAB

The interface enables the control of bioprinting parameters and observing sensor input on a single interface (Figure 2.12). G-code was generated by the computer interface software, which controls the motion stage and other peripherals mounted on the bioprinter setup. Position control system consisted of a manual and positioning control subsystem, which generated G-code for the 3D motion. G-code was interpreted by the motion controller (Smoothie) managing stepper motor signals controlling three independent axes of the 3D printer stage (Makerbot). Auxiliary control system-generated G-code was used to control the camera, LED, PWM control, and pneumatic systems vacuum/pressure solenoid valves signaling. G-code was compiled and streamed to the bioprinter or generated in real-time based on the sensor input data and specific requirements for different tissue constructs.

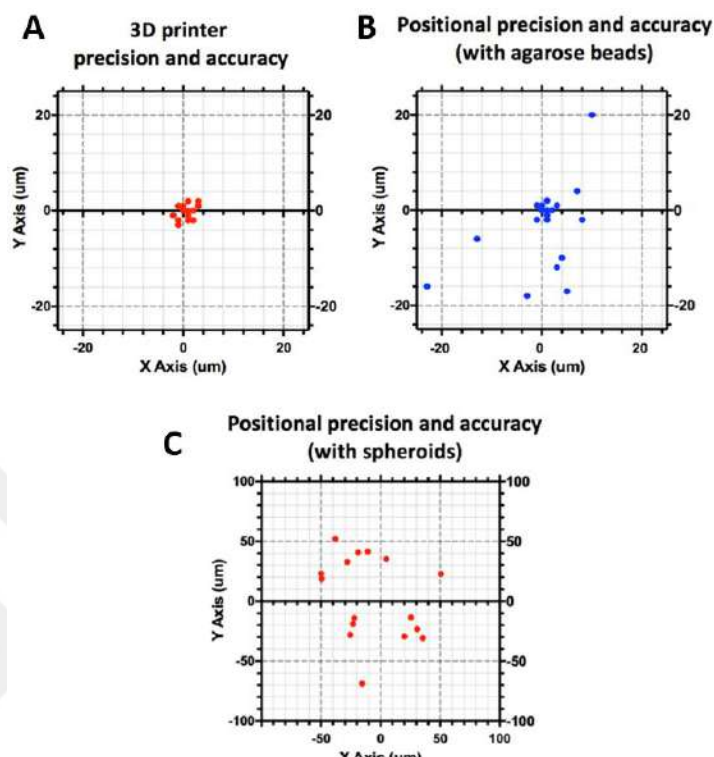


Figure 2.13: Positional precision and accuracy for AAB. Positional precision and accuracy of (A) the Makerbot-modified bioprinter, (B) printing of agarose beads and (C) MSC/HUVEC spheroids (with a diameter of $\sim 300 \mu\text{m}$).

2.4.4 Capabilities of AAB in patterning and 3D bioprinting of biologics

Before performing the patterning and 3D bioprinting of spheroids, we first inspected the quality of positioning during bioprinting. In this regard, agarose beads (with near perfect sphericity) and the motion stage of the 3D bioprinter were used as control groups. The positional precision and accuracy for the motion stage (empty pipettes), spherical agarose beads (ranging from 250 to 300 μm in diameter) and 2-day cultured MSC/HUVEC spheroids ($\sim 300 \mu\text{m}$ in diameter) were determined to be ~ 2 and 2.1 μm , 14.4 and 10.4 μm , and 34.7 and 45.9 μm , respectively (Figure 2.13). The results indicate that the bioprinter motion stage had a small effect on the positional error

during bioprinting and bioprinting of spheroids was about 2- and 4-fold less precise and accurate compared to printing of agarose beads, respectively. This could be due to the viscoelastic nature, lesser sphericity, and slightly higher average diameter of tissue spheroids compared to more rigid agarose beads.

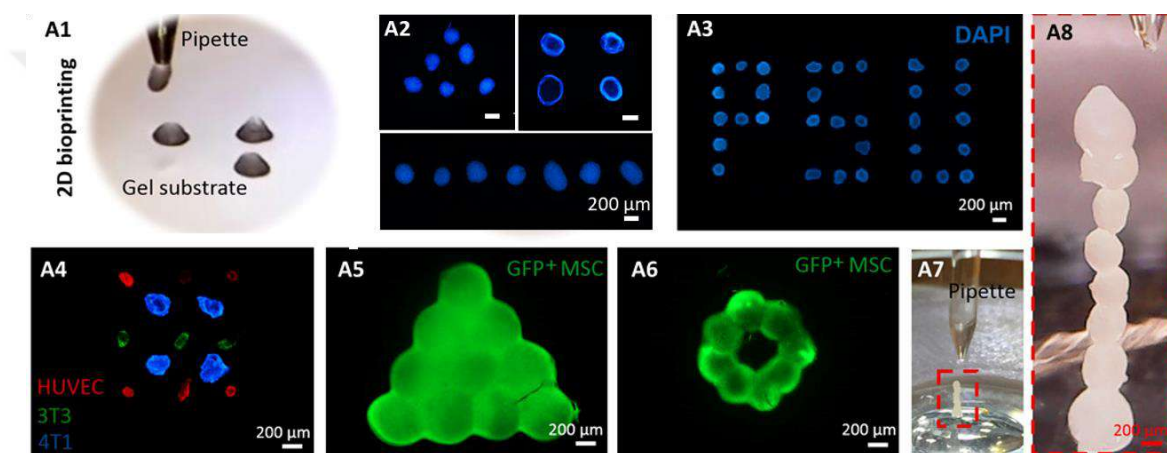


Figure 2.14: Patterning of spheroids. (A1) An image from the traveling camera showing spheroid placement onto a gel substrate. Fluorescent images showing (A2) a triangle, square, line, (A3) PSU and (A4) matrix patterns. GFP⁺ MSC spheroids were patterned onto (A5) COL I and (A6) gelatin-methacryloyl (GelMA). (A7 and A8) Images showing that eight fixed spheroids were bioprinted on top of each other in air without any gel support during bioprinting. DAPI, 4',6-diamidino-2-phenylindole.

We demonstrated the ability to bioprint spheroids precisely (~11% with respect to the average spheroid size) onto alginate through patterning of various shapes (Figures. 2.14A1-A2), initials of Penn State University (PSU) (Figure 2.14A3), and a matrix of spheroids of various shapes and dimensions (including irregular shapes) in the diameter range of 80–200 μm (made of HUVEC,

3T3 and 4T1 cells) (Figure 2.14A4). Moreover, we also demonstrated the bioprinting of green fluorescent protein (GFP)-labeled MSC spheroids in the shape of triangle and ring into other gels, including collagen type-I and gelatin-methacryloyl (GelMA), as shown in Figure 2.14A5 and Figure 2.14A6, respectively. In order to highlight the unprecedented precision of AAB, we bioprinted eight different-sized MSC spheroids on top of each other without any gel support (Figures 2.14A7-A8).

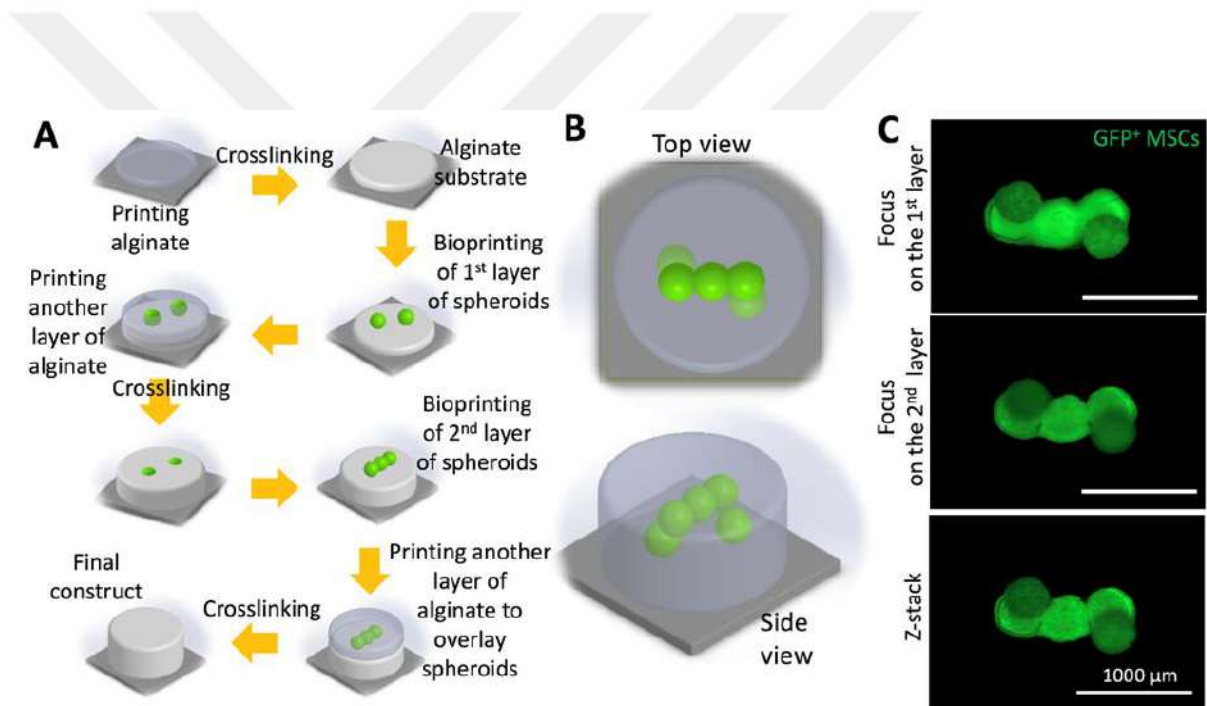


Figure 2.15: Bioprinting of a representative 3D hollow structure. (A) A step-by-step demonstration and (B) top and side views of the final construct. (C) Fluorescent images of the bioprinted construct made of GFP⁺ MSC spheroids. In the 2nd layer, the middle spheroid was only supported by the sacrificial alginate substrate. On the other hand, the other two spheroids were both supported by the gel and spheroids in the 1st layer.

Moreover, we demonstrated another example of a hollow bridge configuration, where the middle spheroid in the 2nd layer was supported by the underlying gel without any spheroid support underneath (Figure 2.15). This was accomplished by bioprinting the gel and spheroids alternately at each layer, which necessitated the precise control of gel thickness; however, the gel thickness was dependent on several factors, such as the droplet resolution, crosslinking time and spreading behavior of the droplet, which were difficult to control at the same time. In order to overcome this fundamental limit, self-healing Bingham-plastic support gels [79] could be used to freely move the spheroids inside the gel, which may bring more flexibility in self-assembly of highly complex geometries including hollowed-out structures.

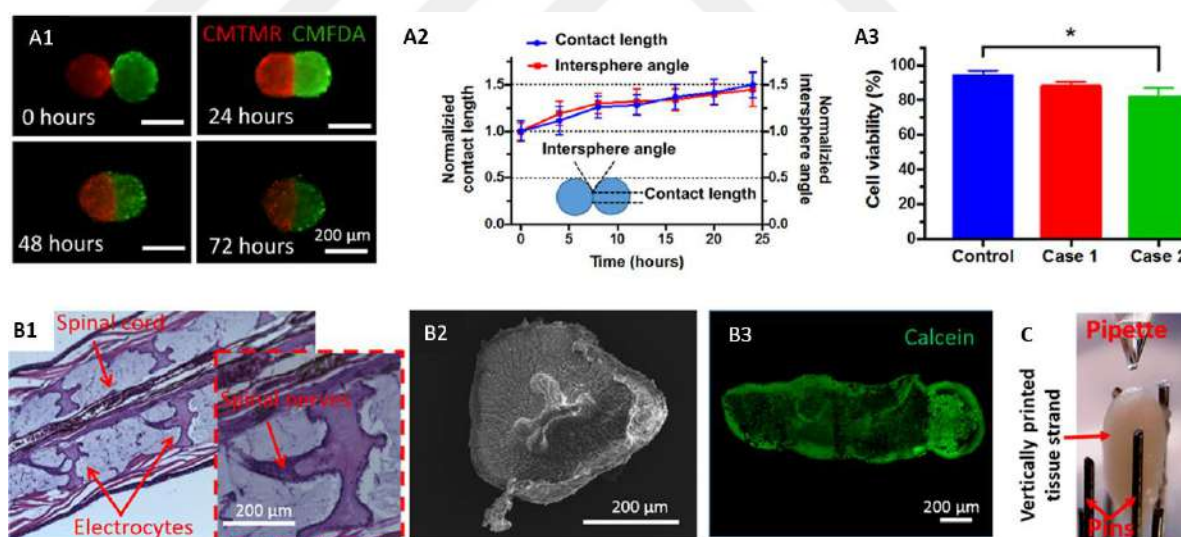


Figure 2.16: Bioprinting of a wide range of biologics. (A1) Time-lapse images of self-assembly process after bioprinting of 1-day cultured 3T3 spheroids at 0, 24, 48, and 72 hours (A2) and the normalized contact length and intersphere angle of fusing spheroids up to 24 hours. (A3) Cell viability of 3T3 spheroids that were not treated with bioprinting (control), after bioprinting inside the cell media (case 1), and after bioprinting into a gel substrate (case 2) ($n = 3$; $*P < 0.05$).

CMTMR, 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine; CMFDA, 5-chloromethylfluorescein diacetate. (B1) Hematoxylin and eosin staining from the sagittal plane of a tail segment of electric fish showing stacked electrocytes in series. (B2) The SEM image of a single electrocyte. (B3) Calcein staining of bioprinted electrocytes. (C) A bioprinted cartilage tissue strand between pins.

Additionally, self-assembly of spheroids was investigated at predetermined time points. As shown in Fig. 3B1, 3T3 spheroids were bioprinted into fibrin with close proximity, and then cultured for 72 h. The two individual 3T3 spheroids bioprinted next to each other gradually fused to form a larger spheroid in order to minimize their surface energy as described in a previous study [67]. The normalized contact length and intersphere angle increased by 50% over the first 24 h of culture (Figure 2.16A1-A2). In order to evaluate the role of bioprinting on viability of spheroids, two cases were evaluated. In Case 1, viability of spheroids was determined immediately after lifting spheroids and depositing them into another cell media reservoir. In Case 2, viability of spheroids was evaluated after bioprinting them into the gel substrate. Spheroids, which were not subjected to the bioprinting process, were used as a positive control group. Viability of spheroids in the positive control group and Case 1 were determined to be ~94 and ~88%, respectively (Figure 2.16A3). In Case 2, cell viability was measured to be ~82%. The decrease in cell viability could be due to the aspiration force or dehydration during the rapid transfer or cell damage when spheroids were submerged into the hydrogel substrate; however, viability levels over 80% for bioprinting of spheroids could be still considered moderate as viability of fabricated cells aggregates even without bioprinting could be in that range [80].

In addition to bioprinting of spheroids, AAB system also enabled the bioprinting of other biologics. For example, we demonstrated the bioprinting of electrocytes isolated from electrogenic

organs of electric fish. Due to the asymmetric functionality of their anatomy and their spatial arrangements in series configuration in an electric organ (similar to batteries connected in series, Figure 2.16B1), electrocytes produce a significant amount of electricity [81]. In this study, five electrocytes ($\sim 400\ \mu\text{m}$ in diameter, Figure 2.16B2) from weakly electric fish (*Brachyhypopomus gauderio*) were obtained according to the animal protocol (Institutional Animal Care and Use Committee (IACUC) #47870) and patterned next to each other onto agarose using AAB with a back-pressure of $\sim 20\text{--}25\ \text{mmHg}$ (Figure 2.1673). To the best of our knowledge, this is the first time of bioprinting of electrocytes, which can be used in biofabrication of biological batteries for various applications, such as pacemakers, cochlear implants, and brain chips in the future [82]. In addition, such cells could be arranged in the form of biological circuits for biocomputing or as bioelectric interfaces for use in treating limb loss, musculoskeletal disorders and body augmentation as well as cyborg organs interfacing electrical devices and biology [83]. The presented new approach showed the possibilities of bioprinting not only spheroids or single cells, but also irregular-shape aggregates. For example, Figure 2.16C shows vertical bioprinting of tissue strands (described in our previous work [35]) into the space between pins, which can be used for fabrication of scalable tissues of muscle, fat, cartilage, nerves, blood vessels, etc.

In order to demonstrate other unique capabilities of the presented approach, heterogeneous tissue complexes were 3D bioprinted using spheroids of different sizes and types including tdTomato⁺ HUVEC spheroids ($\sim 155\ \mu\text{m}$) and GFP⁺ MSC spheroids ($\sim 390\ \mu\text{m}$) (Figure 2.17A-B). Spheroids were arranged into a pyramid structure to clearly visualize the heterogeneous architecture using confocal imaging, where alginate was used as a sacrificial gel, and the 1st, 2nd, and 3rd layer was constituted of HUVEC, MSC and HUVEC spheroids, respectively. This example clearly demonstrates the unique deposition ability of the AAB technique as the last HUVEC spheroid was accurately placed into the small and confined space between three adjacent MSC

spheroids (~2.5-fold of HUVEC spheroid size) on the 3rd layer. Alginate preserved the structural integrity of the bioprinted pattern during the initial stage of culture. Moreover, it enabled the bioprinted entities to stick in their precise locations, which were defined before the bioprinting process.

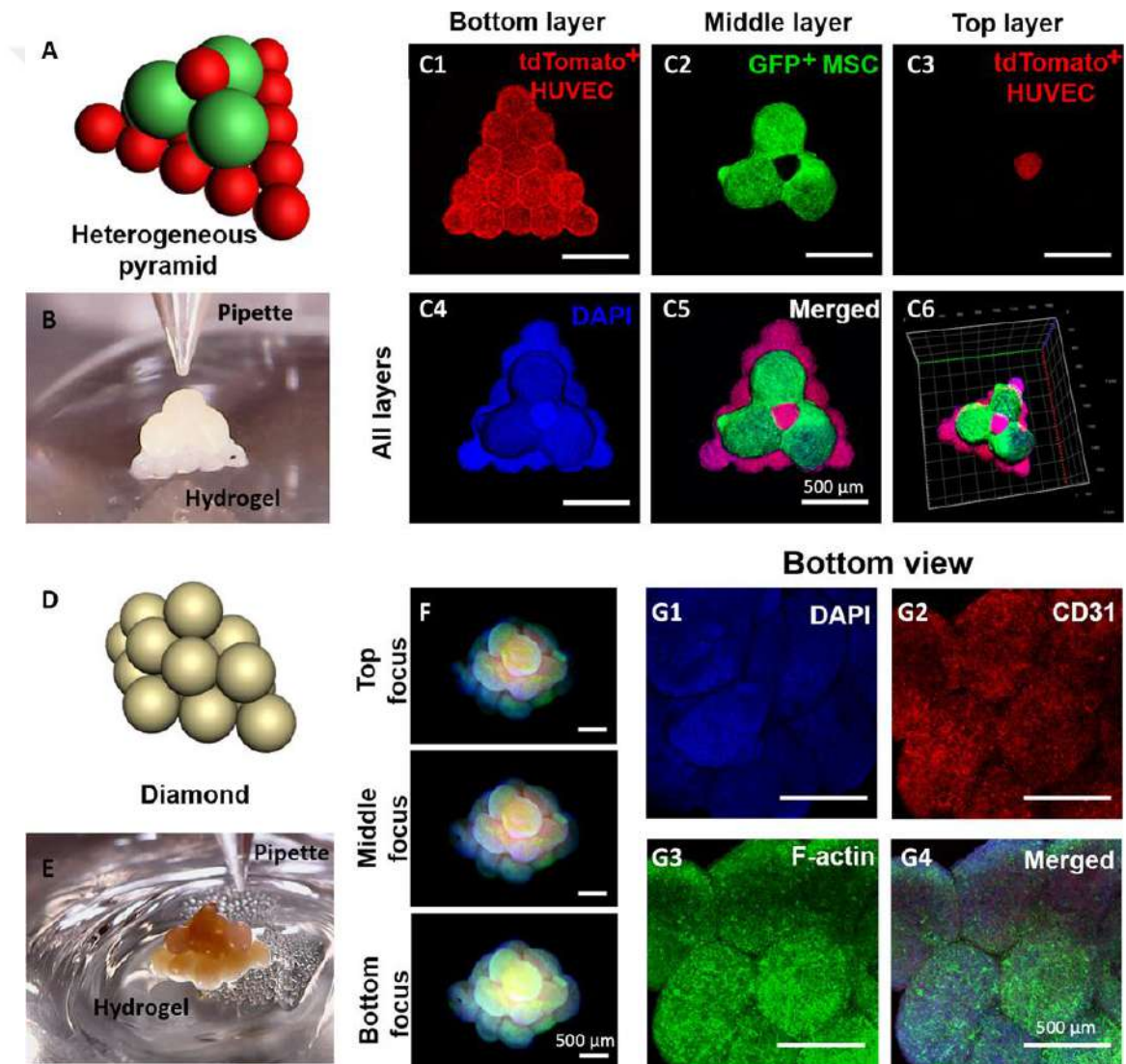


Figure 2.17: 3D bioprinting of spheroids. (A) A schematic illustration of 3D bioprinting of a heterogeneous pyramid construct using different sizes and types of spheroids. (B) A photograph of

the bioprinted three-layer heterogeneous pyramid. (C1 to C6) 3D reconstruction of confocal images of bioprinted pyramid of tdTomato⁺ HUVEC spheroids (first and third layers) and GFP⁺ MSC spheroids (second layer). (D) A schematic illustration of a diamond construct made of MSC/HUVEC spheroids. (E) A photograph showing 3D bioprinted diamond from the side camera. (F) Fluorescent images of the bioprinted diamond, which were stained with DAPI, CD31, and F-actin. (G1 to G4) Confocal images of the diamond construct (bottom view) (note that 1-day cultured HUVEC and 2-day cultured MSCs were used in these experiments).

In order to print the sacrificial alginate, a unipolar wave pulse with a dwell voltage (amplitude) of 5 V and a dwell time (valve opening duration) of 1,000 μ s was used for actuating the micro-valve dispenser, where a positive back pressure of $\sim$ 103 kPa was used for driving the flow of sodium alginate inside the tubing. The optimization of dwell time and droplet volume can be found in Figure 2.18.

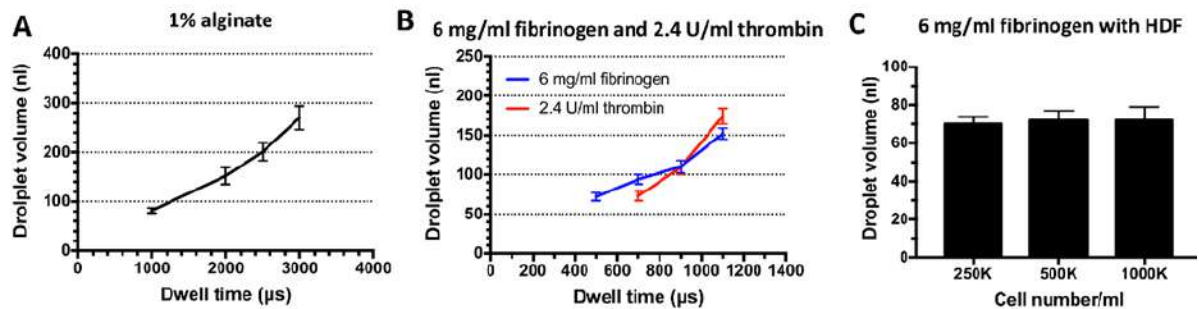


Figure 2.18: Printing parameters for micro-valve-based bioprinting. (A) Droplet volume vs dwell time for 1% alginate ($n=5$). (B) Droplet volume vs dwell time of 6 mg/ml fibrinogen and 2.4 U/ml thrombin ($n=5$). (C) Effect of the cell number on droplet volume ($n=5$).

Afterwards, sodium alginate layer was partially-crosslinked with aerosol form of CaCl_2 for 30 s, and spheroids were bioprinted as explained in Figure 1. Patterned spheroids were partially fused and maintained their initial position after 48 h incubation. After two days of culture, alginate was gently dissolved (using an alginate lyase as explained in our earlier work [35]) and the bioprinted constructs maintained their integrity even after the sacrificial alginate was removed. As evident from the confocal images (Figures 2.17C1-C6), spheroids were well connected in 3D. We also demonstrated more complex pattern such as a 3D diamond pattern with MSC/HUVEC spheroids (Figures 2.17D-G). Similar arrangement was also presented with MSC spheroids of three different dimensions, where spheroids got larger as more layers were laid down (Figures 2.19A-C). To the best of our knowledge, this is the first time of bioprinting tissue spheroids with such a positional accuracy, ~15% with respect to the spheroid size, yielding highly intricate geometries and heterogeneous structures in 3D.

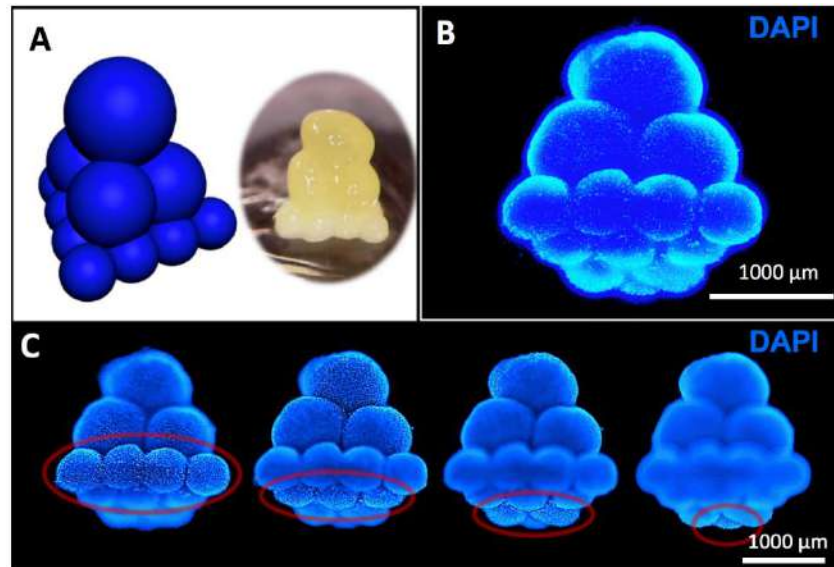


Figure 2.19: A heterogeneous pyramid construct. (A) A 3D CAD model and a photograph of its physically-bioprinted version made of different size MSC spheroids. (B) A stacked fluorescent

image of the bioprinted pyramid, where MSC spheroids on the 1st, 2nd, and 3rd layer were ~ 367 , 567 , and $733 \mu\text{m}$, respectively. (C) Fluorescent images of the same construct with focuses at different sites on the 1st layer.

2.4.5 Bioprinting of HUVEC spheroids to mediate angiogenic sprouting

In order to demonstrate the application of AAB in scaffold-based fabrication of physiologically-relevant culture environments, HUVEC spheroids were bioprinted into fibrin hydrogel with a predetermined distance apart and their collective angiogenic sprouting behavior was studied. This importantly signifies that angiogenic sprouting behavior can be tailored by varying the proximity of spheroids hence directly affecting cell-cell signaling. To study the effect of distance on angiogenic sprouting, droplets of 6 mg/ml fibrinogen (loaded with 0.5 million/ml HDFs) and 2.4 U/ml thrombin were deposited layer by layer (in 1:1 volume) to fabricate fibrin constructs as such concentration ranges support angiogenesis of HUVECs [84]. A positive back pressure of $\sim 103 \text{ kPa}$ was used for driving the flow of fibrinogen and thrombin solutions inside the tubing and dispenser. In order to dispense a ratio of 1:1 of fibrinogen and thrombin, dwell time of 500 and $700 \mu\text{s}$ were used for obtaining the same droplet volumes at the micro-valve dispensers, respectively. The reader is referred to Figure. 2.18B-C for the optimization of droplet generation for fibrinogen and thrombin solutions. HUVEC spheroids were then bioprinted into fibrin prior to complete gelation. The distances between the spheroids were maintained at 400 ± 10 , 800 ± 13 , and $3,000 \pm 16 \mu\text{m}$ (isolated spheroids used as a control group) (Fig. 2.20A1). Bioprinted constructs were cultured for a period of seven days. Angiogenic sprouting from HUVEC spheroids at Days 2, 5 and 7 was quantified using Angiotool software [64].

Spheroids with close proximity to each other were seen to have higher total vessel length as compared to isolated spheroids right from the initial stages of culture. The combined length of sprouts was 13 and 11 mm for 400 and 800 μm , respectively, as compared to the control group, which were around 6 mm (Fig. 2.21A2). A similar trend was also observed for the total vessels area. There was approximately 3- and 2.5- fold increase in the vessels area for 400 and 800 μm distance on Day 7 as compared to the control group, respectively.

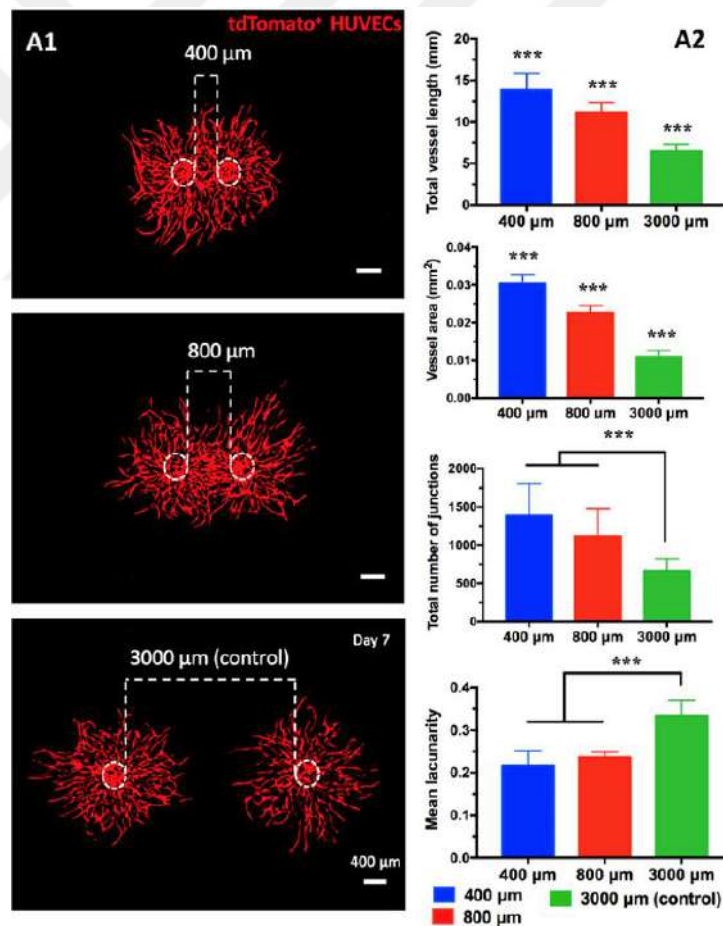


Figure 2.20: Bioprinting of physiologically relevant culture environments to study the angiogenic sprouting behavior of HUVEC spheroids. (A1) Epifluorescent images of bioprinted tdTomato⁺ HUVEC spheroids with varying distances (400 to 3000 μm) apart on day 7. (A2) Graphical

representation of various sprouting properties—namely, total vessels length, total number of junctions formed, vessel area, and mean lacunarity—obtained at day 7 for bioprinted HUVEC spheroids ($n = 3$; *** $P < 0.001$).

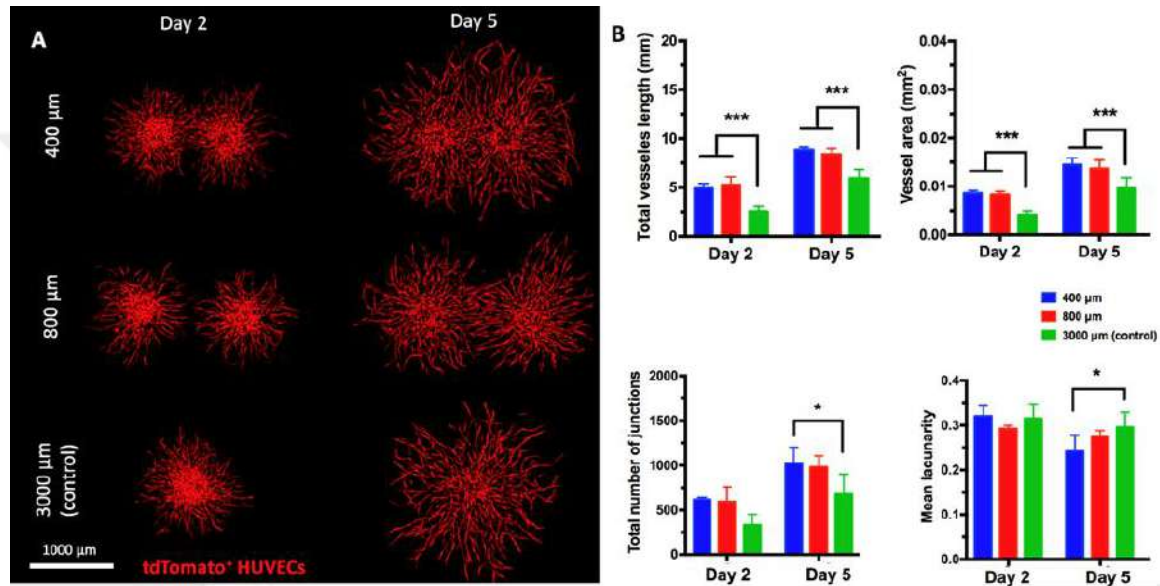


Figure 2.21: Effect of distance on angiogenic sprouting. (A) Epifluorescent images of bioprinted HUVEC spheroids with varying distances apart. The distance was varied from 400 to 3,000 μm . (B) Graphical representation of various sprouting properties, namely total vessels length, total number of junctions formed, vessel area and mean lacunarity, obtained at Day 2 and 5 for bioprinted HUVEC spheroids ($n=3$; * $p < 0.05$, *** $p < 0.001$).

Although the total number of branching points or junctions were not significantly different among different groups at Day 2, the sprouts branched more when spheroids were bioprinted close to each other as compared to the control group (Figure 2.21). On Day 7, the number of junctions for 400 and 800 μm distances were 2.1 and 1.7-fold greater than those for the control group,

respectively. The mean lacunarity refers to the amount of free space around the sprouted capillaries and an increase in angiogenic sprouting results in a decrease in lacunarity [85]. This yields a measurement of the sprouting activity and the mean lacunarity was the highest for the control group compared to other two groups.

In our study, HUVEC spheroids bioprinted with 400 μm and 800 μm distance apart were considered to be in close proximity. On the other hand, HUVEC spheroids bioprinted with 3,000 μm distance apart were far enough to be considered ‘isolated spheroids’ (control group). Proximity between cells triggers a cascade of events that in turn influence cellular behavior, and in order to study these events, it is important to control localization under in vitro conditions [86]. The presented approach offers the capability of controlling the distance between spheroids and thus aims to better understand this signaling between spheroids. Sprouting properties such as vessel length, junction formation, and vessel area were all enhanced when spheroids were close to each other as compared to when they were placed far apart. The presence of HDFs in the matrix surrounding HUVEC spheroids also reinforced the formation of stable sprouts. It is known that fibroblasts act as supporting cells and surround the capillary like structures, which gradually develop by self-organization of endothelial cells and simultaneous degradation of fibrin matrix [87]. Moreover, fibroblasts secrete various soluble growth factors such as vascular endothelial growth factor (VEGF), angiopoietin-1, platelet-derived growth factor (PDGF) [88,89], which enhance and modulate the growth of angiogenic sprouts [90]. Bioprinted HUVEC spheroids close to each other probably upregulated the combined secretion of these growth factors in the vicinity of the spheroids, which led to enhanced sprouting properties. The sprouts from closely-placed spheroids extended towards each other and gradually fused to form a capillary network.

To better understand if the positioning of spheroids affected their collective angiogenic sprouting behavior, GFP⁺ and tdTomato⁺ HUVEC spheroids were bioprinted into fibrin gel (with

same properties as discussed before) at 400, 800 and 3000 μm distances apart, and cultured over a period of seven days (Figure 2.22A1). A directionality analysis was then performed in order to investigate the role of the presence of a nearby spheroid on the directionality of angiogenic sprouting. Thus, the angle made by each sprout with the horizontal line ($0/180^\circ$) was measured for all spheroids. The angle span $[60^\circ, -60^\circ]$ for GFP⁺ HUVEC and $[120^\circ, 240^\circ]$ for tdTomato⁺ HUVEC spheroids, where sprouts primarily grew towards the other spheroid, were considered the Angle of Interest (AOI) as highlighted in Figure 2.22A2.

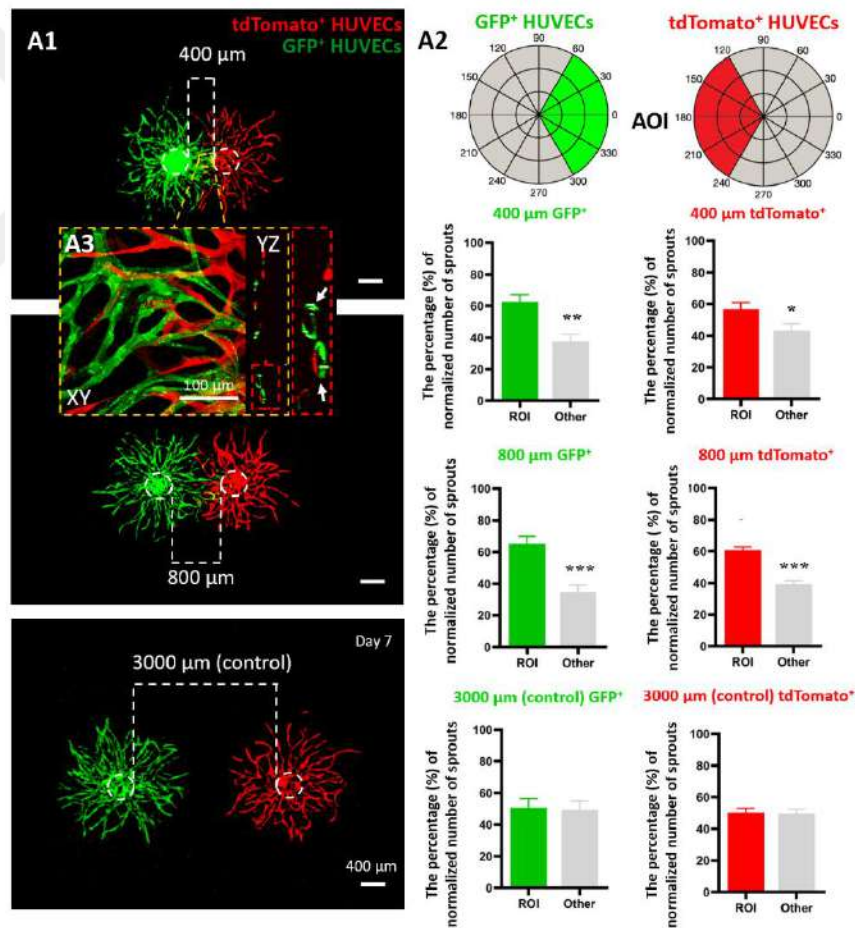


Figure 2.22: (A1) Epifluorescent images of bioprinted GFP⁺ and tdTomato⁺ HUVEC spheroids with varying distances (400 to 3000 μm) apart on day 7 along with higher-magnification confocal images of the interface region in XY and YZ planes showing capillaries formed by both GFP⁺ and

tdTomato⁺ HUVECs (indicated by white arrows). (A2) Directionality analysis demonstrating the direction and percentage of normalized number of sprouts on day 7 ($n = 3$; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) [note that green and red bars demonstrate the angles of interest (AOIs), which are $[60^\circ, -60^\circ]$ for GFP⁺ HUVECs and $[120^\circ, 240^\circ]$ for tdTomato⁺ HUVECs]. (A3) Confocal images at the interface of two spheroids showing capillaries formed by both GFP⁺ and tdTomato⁺ HUVECs.

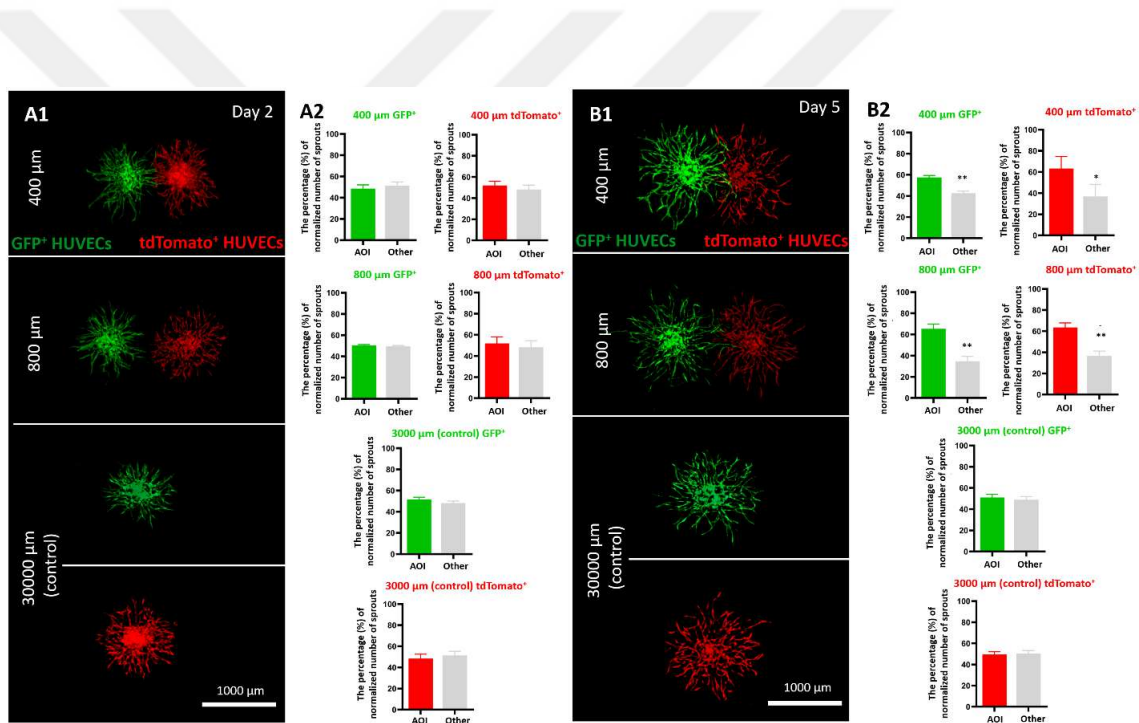


Figure 2.23: Effect of distance on the directionality of angiogenic sprouting. Epifluorescent images of bioprinted GFP⁺ and tdTomato⁺ HUVEC spheroids with varying distances (400 to 3,000 μm) apart on (A1) Day 2 and (B1) Day 5, and the corresponding directionality analysis demonstrating the direction and percentage of normalized number of sprouts on (A2) Day 2 and (B2) Day 5 ($n=3$; * $p < 0.05$, ** $p < 0.01$).

On Day 7, the percentage of normalized number of sprouts (where the total number of sprouts were normalized with respect to the total angle span) in AOI was significantly higher than that of the other angle span (indicated with 'Other') for 400 and 800 μm distances, indicating a dominating directionality of angiogenic sprouting towards the other spheroid. The percentage of normalized number of sprouts for AOI was ~63 and 65 for GFP⁺, and ~59 and 61 for tdTomato⁺ HUVEC spheroids, for 400 and 800 μm distances, respectively.

Even though the directionality of sprouts for all cases may not be very apparent on Day 2 (initial stages of culture, Figures 2.23A1-A2), on Day 5 (later stages of culture, Figures 2.23B1-B2, Figure 2.24), mature sprouts were more concentrated within AOI. Additionally, for sprouts formed in directions falling outside the AOI (depicted in grey), we observed more random growth of sprouts over time. The sprouts for spheroids that were 3,000 μm apart were random and did not indicate any directionality from Day 2 to 7 (Figure 2.22A2, bottom; Figure 2.24C). For 400 μm distance, it was also observed that the GFP⁺ HUVECs suppressed the sprouting ability of tdTomato⁺ HUVECs Day 2 onwards, where higher number of GFP⁺ HUVEC sprouts were observed at the interface (see fluorescent images in Figures 2.23B1).

Domination of GFP⁺ HUVEC sprouts could be due to the fact that GFP⁺ HUVECs in this experiment were from an earlier passage (passage 2 to 5) with respect to tdTomato⁺ HUVECs (passage 5 to 7) [91]. In addition, we investigated whether sprouts from both spheroids interacted and thus performed confocal imaging at the interface closed to the tdTomato⁺ HUVEC spheroid (Figure 2.22A3). We noticed that some capillaries were formed by both GFP⁺ and tdTomato⁺ HUVECs, as shown by arrows, which could be due to vascular anastomosis, or the migration of GFP⁺ HUVECs to the other side of the interface and their contribution to the capillary formation by tdTomato⁺ HUVECs spheroids. Overall, the results showed that HUVEC spheroids bioprinted

close to each other influence each other's sprouting behavior, which led to a well-organized network formation between the sprouting bodies.

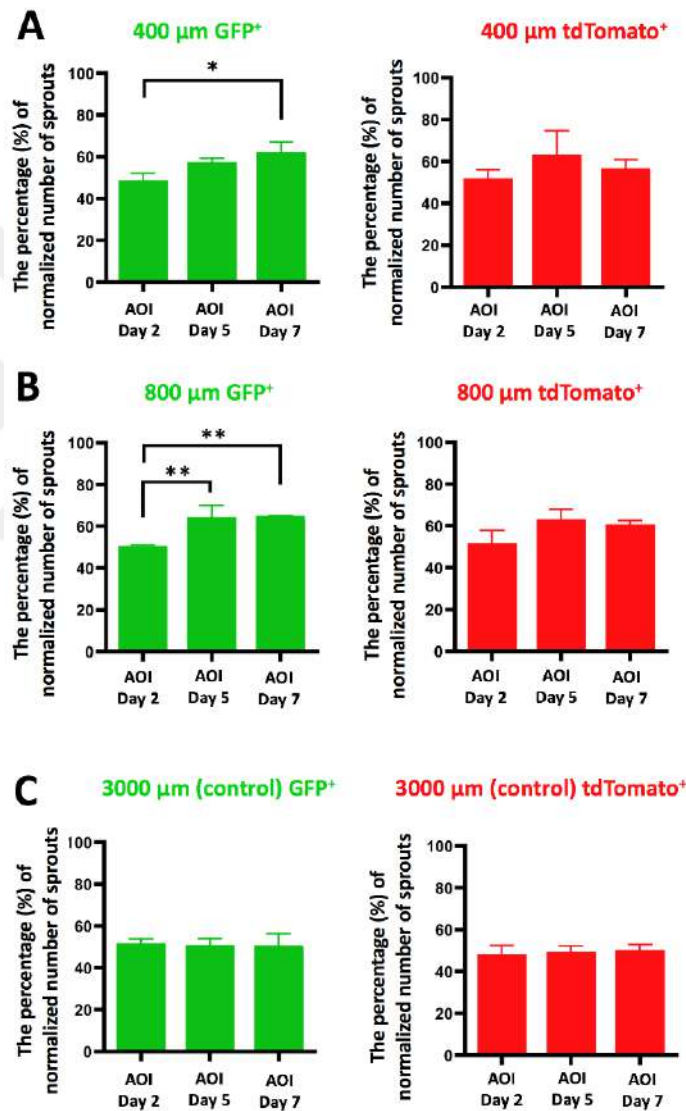


Figure 2.24: Directionality analysis of angiogenic sprouting over time. Graphs showing the percentage of normalized number of sprouts within AOI on Days 2, 5, and 7 for (A) 400, (B) 800, and (C) 3,000 μm ($n=3$; * $p < 0.05$, ** $p < 0.01$).

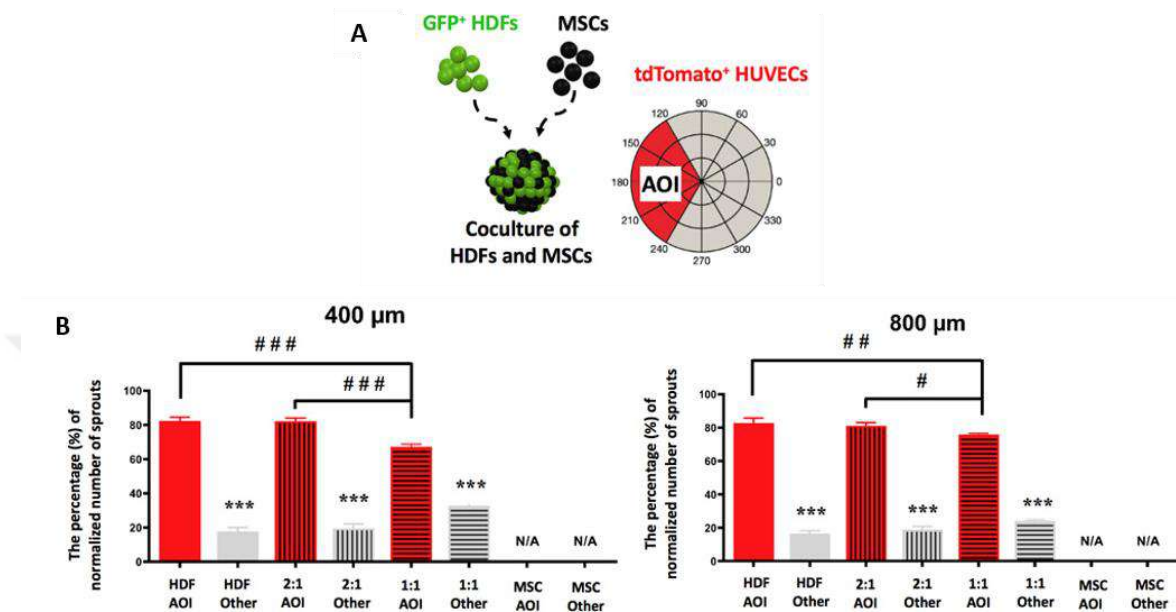


Figure 2.25: (A) A schematic illustration of the directionality of sprouts from a HUVEC spheroid toward a spheroid of GFP⁺ HDF and MSC co-cultures. (B) The directionality analysis for different mixing ratios of HDF:MSC, including HDF (control), 2:1, 1:1, and MSC (control), on day 7 ($n = 3$; *** $P < 0.001$ shows significance between AOI and “Other” for each group, and # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ show significance among AOIs of different groups) [note that N/A represents the none applicability of the directionality analysis, as no sprouts were observed in the MSC-only group; no directionality analysis was performed for the 3000- μm distance, as sprouting was not observed and HUVECs exhibited spreading only; 1-day cultured HUVECs were used in all experiments; and the critical lifting pressure for coculture HDF/MSC spheroids (2:1 and 1:1 ratio) was determined to be 28.7 and 29.1 mmHg, respectively, through interpolation of the critical lifting pressure values for HDF and MSC spheroids presented in Figure 2.7].

In abovementioned experiments, we investigated the role of multiple HUVEC spheroids on their collective sprouting behavior, where HDFs were bioprinted as single cell suspension in fibrin, had direct contact with HUVEC spheroids, acted as supporting cells, and aided in forming stable sprouts. In order to explore if HDFs could still influence the sprouting behavior of HUVECs even when they were not in direct contact with HUVECs, we reconfigured the design of the above-presented experiments. In this regard, a HUVEC spheroid was bioprinted along with a co-culture spheroid of GFP⁺ HDFs and MSCs (with controlled distance apart), which enabled us to change the concentration of HDFs and study the differential effect of co-culture composition on HUVEC sprouting. GFP⁺ HDF/MSCs spheroids, in 2:1 and 1:1 ratios, were bioprinted next to tdTomato⁺ HUVEC spheroids in fibrin with the same properties as discussed before (Figure 2.26A). HDF and MSC (homocellular) spheroids were used as control groups. The distance between these spheroids were varied from 400-3,000 μm as performed before. For 400 and 800 μm cases, HUVECs were attracted towards spheroids of HDF, 2:1 and 1:1 groups, and seen arranging into sprouts over a period of seven days (Figure 2.26). The directionality analysis on Day 7 revealed that the percentage of normalized number of sprouts within AOI for HDF, 2:1 and 1:1 groups for 400 μm distance were ~82, 80, and 67, respectively (Figure 2.25B).

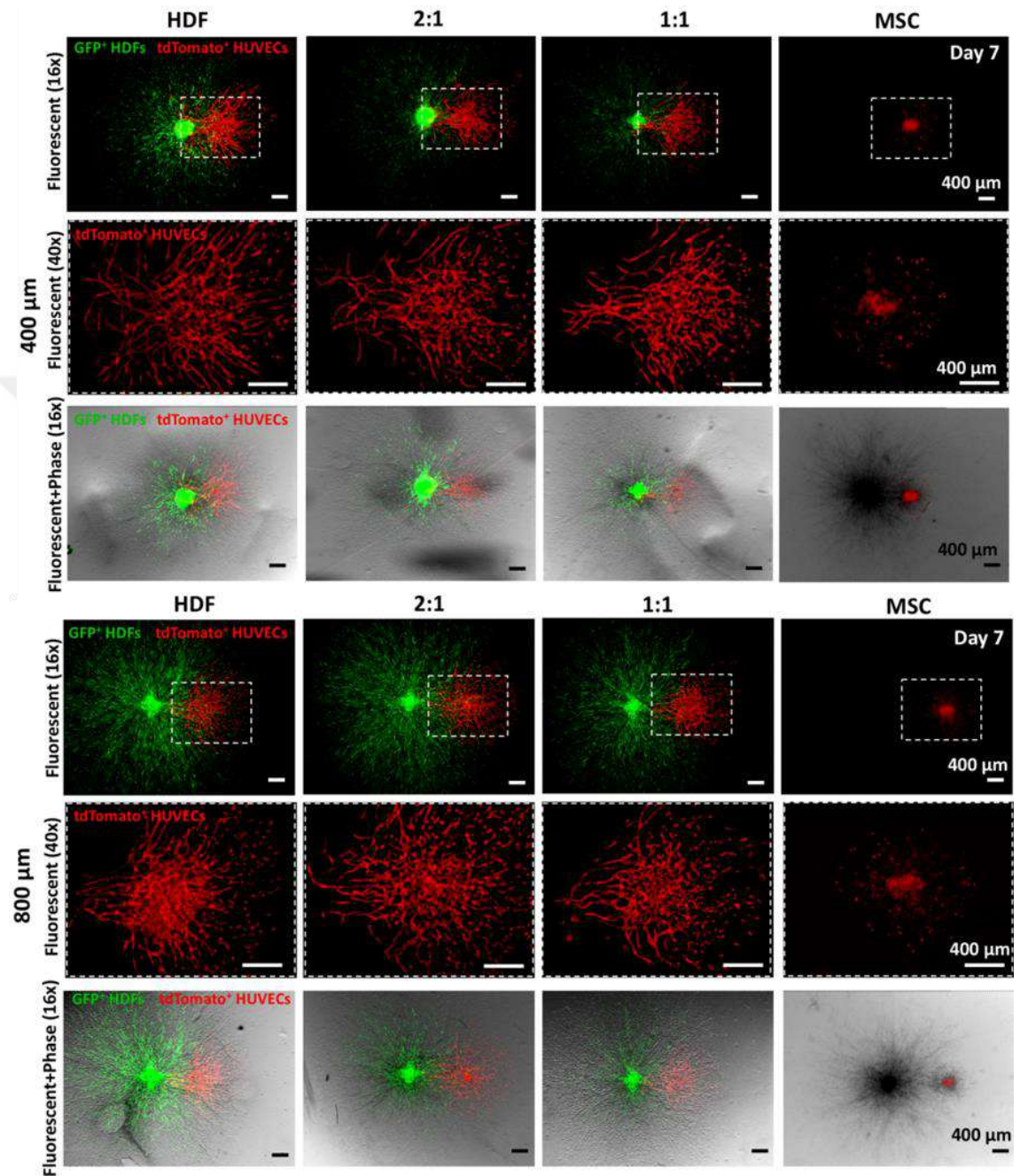


Figure 2.26: Fluorescent and fluorescent/phase images showing angiogenic sprouts from a tdTomato⁺ HUVEC spheroid towards a spheroid of GFP⁺ HDFs and MSCs co-culture in different mixing ratios, including HDF (control), 2:1, 1:1, and MSC (control), on Day 7. Spheroids were bioprinted with 400 and 800 μm apart (Note: No fluorescent labeling was used for MSCs and phase

images were included to demonstrate the spreading MSCs; only tdTomato⁺ HUVECs were shown in the middle row).

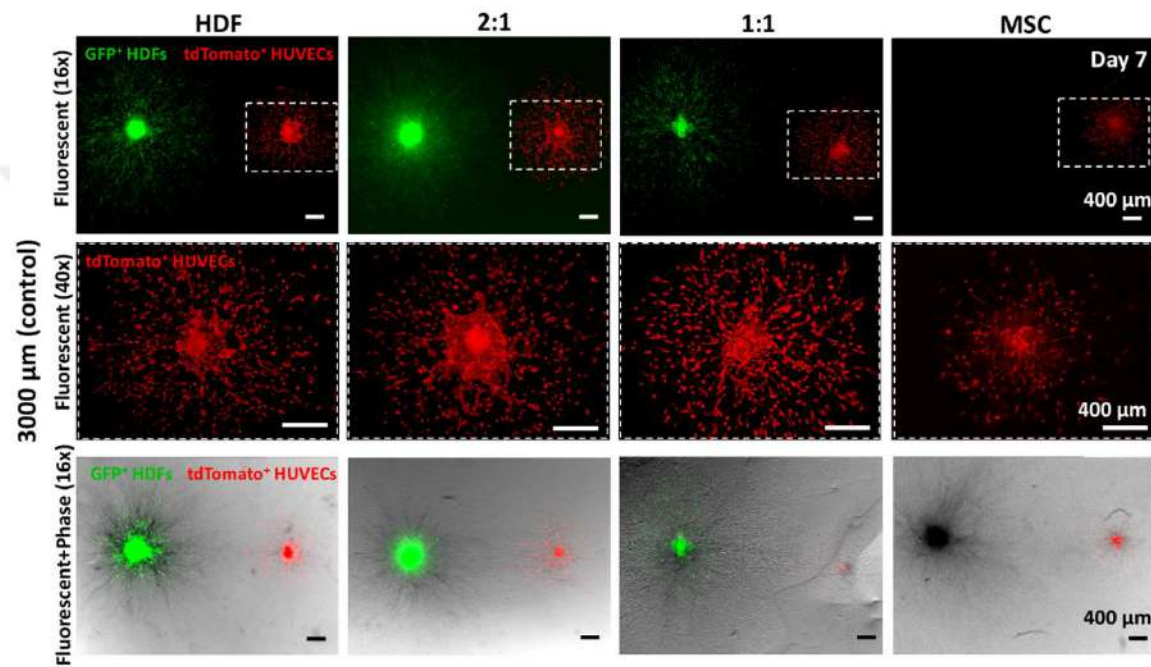


Figure 2.27: Fluorescent and fluorescent/phase images showing angiogenic sprouts from a tdTomato⁺ HUVEC spheroid towards a spheroid of GFP⁺ HDFs and MSCs co-culture in different mixing ratios, including HDF (control), 2:1, 1:1, and MSC (control), on Day 7. Spheroids were bioprinted with 3,000 μm apart (Note: No fluorescent labeling was used for MSCs and phase images were included to demonstrate the spreading MSCs; only tdTomato⁺ HUVECs were shown in the middle row).

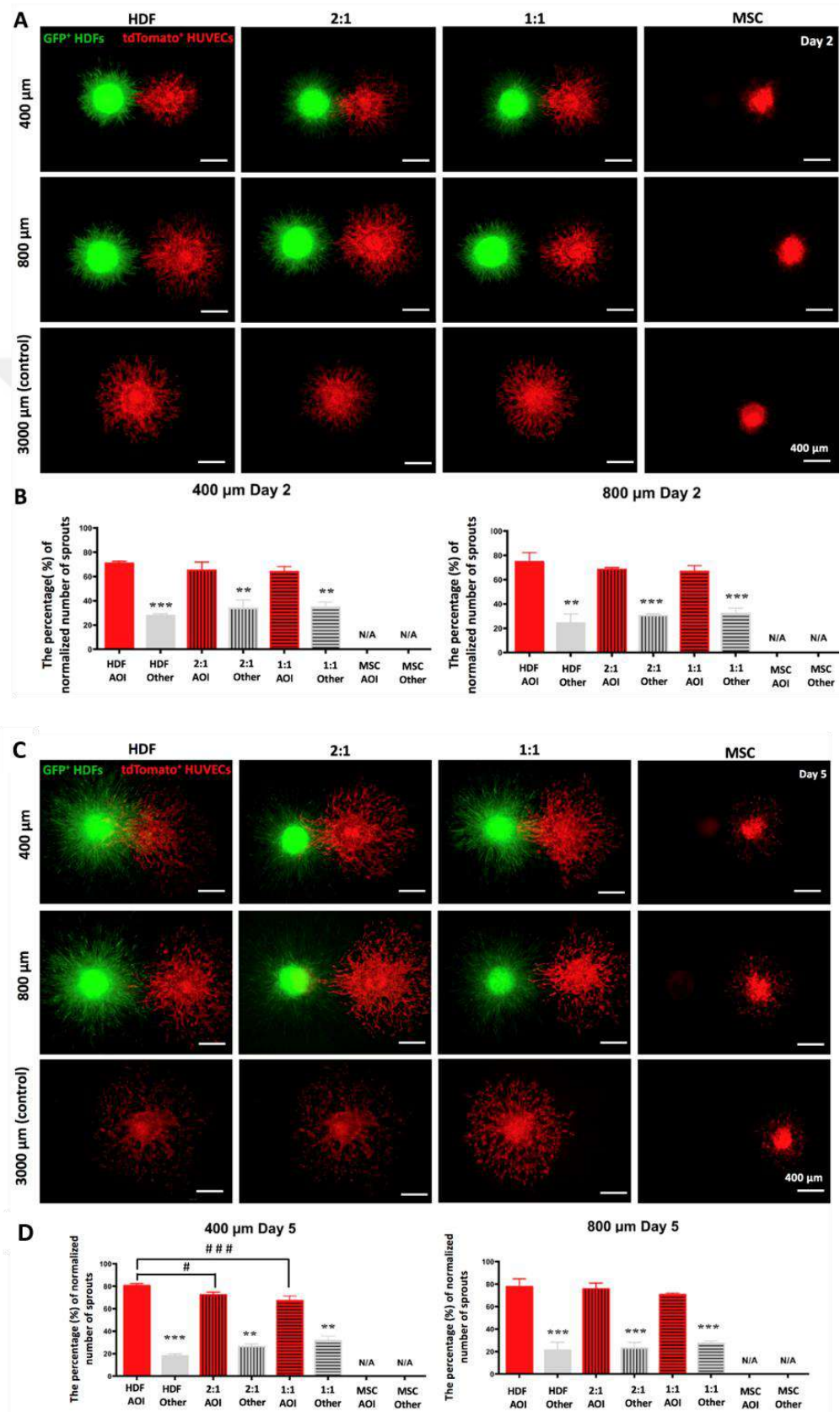


Figure 2.28: Directionality analysis of angiogenic sprouts towards GFP⁺ HDF and MSC co-culture spheroids on Day 2 and Day 5. (A) Fluorescent images showing angiogenic sprouts from a bioprinted tdTomato⁺ HUVEC spheroids towards a spheroid of GFP⁺ HDFs and MSCs co-culture on Day 2 (including HDF (control), 2:1, 1:1, and MSC (control)) with varying distances apart (400 to 3,000 μ m). (B) The directionality analysis of angiogenic sprouting ($n=3$; ** $p < 0.01$; *** $p < 0.001$ shows significance between AOI and other regions for each group on Day 2. (C) Fluorescent images showing angiogenic sprouts from a bioprinted tdTomato⁺ HUVEC spheroids towards a spheroid of GFP⁺ HDFs and MSCs co-culture on Day 5 (including HDF (control), 2:1, 1:1, and MSC (control)) with varying distances apart (400 to 3,000 μ m). (D) The directionality analysis of angiogenic sprouting ($n=3$; ** $p < 0.01$; *** $p < 0.001$ shows significance between AOI and other regions for each group, and # $p < 0.05$; ### $p < 0.001$ shows significance among AOIs of different groups on Day 5. Note: N/A represents the none applicability of the directionality analysis as no sprouts were observed in the MSC (control) group. In addition, no directionality analysis was performed for the 3,000 μ m distance as sprouting was not observed and HUVECs exhibited spreading only).

For 800 μ m distance, a similar directionality was observed, where the percentage of normalized number of sprouts within AOI for HDF, 2:1 and 1:1 groups were determined to be ~84, 81, and 76, respectively. On the other hand, spheroids in the MSC group (Figure 2.25B) or spheroids located apart with a 3,000 μ m distance (Figure 2.27) did not induce sprouting Day 2 onwards, rather, HUVECs exhibited spreading behavior. On Day 2 for the 800 μ m case, although the tip cells from HUVEC spheroids were not observed to have any physical contact with HDFs at the interface of both spheroids, sprout-like structures were already formed (Figure 2.28A). These structures were directed towards HDFs as the relevant directionality analysis revealed a significant

difference between AOI and ‘Other’ for HDF, 2:1 and 1:1 groups (Figure 2.28B). This could be due to the fact that HDFs communicated with HUVECs via paracrine signaling by the possible diffusion of secreted growth factors, such as VEGF, angiopoietin-1 and PDGF, as discussed before [88,89]. By Day 5 when HDFs substantially migrated out of the co-culture spheroids, HDFs interacted with HUVECs outside AOI, particularly within angle spans [60°-120°] and [240°-300°], and induced sprout formation in those directions (Figure 2.28C-D). Overall, more stable sprouts were observed in HDF-involved spheroids and the results revealed that better directionality of angiogenic sprouting could be attained when the density of HDFs increased in the co-culture spheroid. The presence of MSCs, on the other hand, had a negative impact on angiogenic sprouting of HUVECs, which could be due to the inhibitory effect of MSCs on the angiogenic potential of HUVECs via cell–cell contact through modulation of the VE-Cadherin/ β -catenin signaling pathway as described before [91].

2.5 Conclusion

In conclusion, we propose a most effective strategy of AAB system taking advantage of a simple-to-use, cost-effective (<\$1,000), and reproducible tissue bioprinting platform, which can be useful in a wide variety of applications, including but not limited to organ-on-a-chip devices, drug testing devices, microfluidics, in vitro human disease models, organoid engineering, biofabrication and tissue engineering, biocomputing, and biophysics. However, our AAB system requires further improvement in order to increase the time effectiveness in fabrication of scalable tissues. In the near future, our AAB platform could be advanced to be able bioprint an array of spheroids simultaneously for scale-up tissue fabrication in a shorter time.

Chapter 3

3D Bioprinting of Co-cultured Osteogenic Spheroids for Bone Tissue Fabrication^{*†‡}

3.1 Chapter Summary

Conventional top-down approaches in tissue engineering involving cell seeding on scaffolds have been widely used in bone engineering applications. However, scaffold-based bone tissue constructs have had limited clinical translation due to constraints in supporting scaffolds, minimal flexibility in tuning scaffold degradation, and low achievable cell seeding density as compared with native bone tissue. Here, we demonstrate a pragmatic and scalable bottom-up method, inspired from embryonic developmental biology, to build three-dimensional (3D) scaffold-free constructs using spheroids as building blocks. Human umbilical vein endothelial cells (HUVECs) were introduced to human mesenchymal stem cells (hMSCs) (hMSC/HUVEC) and spheroids were fabricated by an aggregate culture system. Bone tissue was generated by induction of osteogenic differentiation in hMSC/HUVEC spheroids for 10 days, with enhanced osteogenic differentiation and cell viability in the core of the spheroids compared to hMSC-only spheroids. Aspiration-assisted bioprinting (AAB) is a new bioprinting technique which allows precise positioning of spheroids (11% with respect to the spheroid diameter) by employing aspiration to

^{*} ^{*}A part of this content was published in *Science Advances* entitled as “Aspiration-assisted bioprinting for precise positioning of biologics” with minor changes. (Ayan, Bugra, et al. "Aspiration-assisted bioprinting for precise positioning of biologics." *Science Advances* 6.10 (2020): eaaw5111)

[†] A part of this content is under submission entitled as “3D bioprinting of co-cultured osteogenic spheroids for bone tissue fabrication” by (Heo, D.N.^{*}, Ayan, B.^{*}, Dey, M., Banerje, D., Wee, H., Lewis, Ozbolat, I.T.)(^{*}Equal contribution)(Submitted).

[‡] Dong Nyoung Heo assisted with tissue characterization, Madhuri Dey assisted with imaging, Hwabok Wee and Gregory Lewis assisted with μ CT imaging, Dishary Banerjee help with data analysis.

lift individual spheroids and bioprint them onto a hydrogel. AAB facilitated bioprinting of scaffold-free bone tissue constructs using the pre-differentiated hMSC/HUVEC spheroids. These constructs demonstrated negligible changes in their shape for two days after bioprinting owing to the reduced proliferative potential of differentiated stem cells. Bioprinted bone tissues showed interconnectivity with actin-filament formation and high expression of osteogenic and endothelial-specific gene factors. This study thus presents a viable approach for 3D bioprinting of complex-shaped geometries using spheroids as building blocks, which can be used for various applications including but not limited to, tissue engineering, organ-on-a-chip and microfluidic devices, drug screening and, disease modeling.

3.2 Introduction

Bone is a highly vascularized dynamic tissue, observed in a variety of shapes and sizes in the body. The skeleton serves crucial roles such as supporting the framework for the body and protecting vital organs [92,93]. Large bone defects cannot self-regenerate, and can be due to trauma, fracture nonunion, infection, bone tumor resections, and removal/revision of joint replacements and other implant [94–96]. There is thus a substantial demand for engineered constructs to restore and regenerate the diseased/excised part of the bone tissue [95,97,98]. To-date, significant progress has been made using three-dimensional (3D) bioprinting of living cells and tissues to recapitulate the bone tissue [13]. Most of these bioprinting approaches rely on scaffold-based techniques where biodegradable hydrogels are seeded or combined with mature osteoblasts or osteogenically-committed stem-cells [97,99,100]. These scaffolds can provide mechanical support, serve as a template for cell attachment and facilitate a conducive environment for cellular activities [94,97]. However, degradation of scaffolds, limited cell density compared to native-

tissues and limited communication among cells are some of the major drawbacks in scaffold-based approaches [19].

Scaffold-free bone tissue engineering pose a promising alternative to 3D bioprinting approaches [30]. Stem-cell derived aggregates, in the form of spheroids, are considered as building blocks for scaffold-free bioprinting and mimic the complex morphology and physiology of native tissue by inducing cross-talk among cells and cell-extracellular cell matrix (ECM) interactions [29,51]. In addition, osteogenic differentiation is observed to increase due to stronger integrin-ECM interaction caused by the presence of both stroma and structure within spheroids [101]. However, non-uniform bone tissue regeneration owing to the non-homogeneous oxygen diffusion across the entire spheroid domain, especially to the core of spheroids [101–103] forms the major roadblock in successful usage of spheroids in 3D bioprinting applications. 2D co-culture systems with mature osteoblasts or stem cell derived osteogenic progenitor cells with cells from endothelial lineage have shown potential to address this limitation by enhanced secretion of endothelial cell-mediated paracrine factors secretion under hypoxia [104]. In this study, we intended to exploit this potential of endothelial cells and investigate their role in 3D on osteogenic differentiation. We achieved this by the co-culture of a minimal number of human umbilical vein endothelial cells (HUVEC) in human mesenchymal stem cells (hMSC) spheroids, and by induction of osteogenesis with bone regeneration across the entire domain of spheroids.

Spheroids have been utilized in successful biofabrication of functional bone tissue substitutes [48,50,105,106]. Although several approaches have been presented in the literature for bioprinting of spheroids, most of these suffer from poor spatial control in 3D, significant damages to spheroids with loss of viability and structural integrity, poor repeatability of the process while using spheroids that are non-uniform in size, inability to form complex 3D shapes, inability to maintain designed shape due to cell-mediated compaction post-bioprinting, and lack of scalability for translation from bench to bedside [48,50,105]. Aspiration-assisted bioprinting (AAB), a newly

developed technique by our group, bioprints spheroids in 3D space using the power of aspiration forces. AAB leverages the fact that spheroids can be formed from diverse cell types at high densities [106]. When using human stem cells, these spheroids can recapitulate aspects of embryonic development to self-assemble [48] into various organs. Using this technique, in this study, we have demonstrated that spheroids with viscoelastic properties can be lifted by aspiration forces, and then positioned precisely (11% with respect to the spheroid diameter) into a hydrogel substrate, circumventing the limitations of the other spheroid bioprinting techniques available.

Here, co-culture of hMSCs and HUVEC spheroids were utilized to enhance cellular function, osteogenic differentiation, calcium deposition, and cell viability. After biofabrication of spheroids, two different strategies were demonstrated for the bioprinting of bone tissue using hMSC/HUVEC and pre-differentiated hMSC/HUVEC spheroids. Compaction of spheroids leading to significant changes in geometry of tissue constructs compared to the desired has been a common issue post bioprinting, and hence, we utilize 3D bioprinting of pre-differentiated hMSC/HUVEC spheroid to minimize shape changes. Post-bioprinting, we showed that we were able to control the shape of the bioprinted construct, using pre-differentiated hMSC/HUVEC spheroids. In this study, thus, we attempted to address major limitations of spheroid bioprinting by bioprinting spheroids using AAB, fabricating complex-shaped bone tissue constructs, engineering the spheroids in a way to induce osteogenesis across the entire spheroid domain, and controlling osteogenic induction timelines before and after bioprinting in order to reduce spheroid compaction and increase retention of geometry of the tissue constructs.

3.3 Materials and Methods

3.3.1 Cell culture

hMSCs (Lonza, Walkersville, MD) and HUVECs (Lonza) were used to fabricate of 3D cellular spheroids. hMSCs were cultured in all-in-one ready-to-use hMSC growth medium (Cell Applications, INC., San Diego, CA). HUVECs or tdTomato⁺ HUVECs were cultured in MCDB

131 medium (Corning, New York, NY) supplemented with 10% fetal bovine serum (Corning), 2 mM glutamine (Thermo Fisher Scientific, Waltham, MA), 1% penicillin/streptomycin (Corning, New York, NY), 4.5 ug/mL bovine brain extract (Lonza, Walkersville, MD), 10 unit/mL heparin (Sigma-Aldrich, St. Louis, MO), and 1.86 mg/mL endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO). Cells passages from three through seven were used for both hMSCs and HUVECs. The cells were expanded at 37 °C with 5% CO₂ in a humidified sterile incubator.

3.3.2 Spheroid fabrication

hMSCs and HUVECs were harvested with trypsin and collected by centrifugation at 1600 rpm for 5 min for the fabrication of the spheroids. hMSCs were reconstituted to 2.5×10^5 cells per ml, with various mixing ratios of HUVECs (8, 15, and 25 %), referred as 92:8, 85:15 and 75:25 hMSC/HUVEC respectively. 200 µl of cell suspension was seeded into each well of 96-well plates with U-bottom, cell-repellent surface (Greiner bio-one, Frickenhausen, Germany) to achieve ~50,000 cells/spheroid. Cells were maintained in hybrid growth media (75% MSC and 25% HUVEC growth media) to allow compaction at 37 °C. HMSC-only spheroids were fabricated similarly and used as control to understand the functionality of HUVECs in the spheroids. Osteogenic differentiation was induced in hMSC-only spheroids and hMSC/HUVEC spheroids by cultured in human osteogenic differentiation medium (Cell Applications, INC., San Diego, CA) for 10 days. The cell medium was changed every three days.

3.3.3 Characterization of spheroids

The diameters of hMSC-only and hMSC/HUVEC spheroids were imaged and further quantified using EVOS FL cell imaging system (Thermo Fisher Scientific, Waltham, MA) and ImageJ (NIH Freeware) respectively from each of days, 1 to 5 post-seeding. Cell viability was confirmed using a calcein-AM/ethidium homodimer-1 (EthD-1) LIVE/DEAD assay kit (Invitrogen, Carlsbad, CA) [106]. Briefly, five days after incubation, spheroids were washed with

Dulbecco's phosphate-buffered saline (DPBS), stained with 2 μ M calcein AM and 4 μ M EthD-1 solutions for 40 min, and then imaged as described before.

To evaluate the cellular organization of hMSC-only and hMSC/HUVEC spheroids, spheroids were fixed in 10 % formalin, embedded in paraffin using a Leica TP 1020 automatic tissue processor (Leica, Wetzlar, Germany) and sectioned with 10- μ m thickness. Cross-sectioned samples were stained with hematoxylin and eosin (H&E) using a Leica Autostainer XL (Leica, Wetzlar, Germany) and then imaged using optical microscopy. In addition, the cytoskeletal organization was confirmed with actin staining. Cross-sectioned samples were permeabilized in 0.1% Triton X-100 for 30 min, blocked with 2.5% normal goat serum (NGS) for 60 min at room temperature and incubated with ActinGreen™ 488 (1:1000 in 2.5% NGS) and 4',6-Diamidino-2-Phenylindole (DAPI; 1:1000 in 2.5% NGS) for 60 min. Then, the stained samples were washed thrice with DPBS and observed under an optical microscope.

3.3.4 Surface tension measurement

The surface tension of hMSC-only and hMSC/HUVEC spheroids was measured using a micropipette aspiration technique, as previously described [52]. The customized micropipettes were prepared from borosilicate Pasteur pipettes (vWR, 14673-043, Radnor, PA) on a P1000 Flaming/Brown micropipette puller (Sutter Instrument, Novato, CA). Parameters for heat, pull, velocity, time, and pressure were set at 575, 0, 5, 100, and 50, respectively according to our recent study [106]. All spheroid types were aspirated by a customized glass pipette and monitored via an STC-MC33USB monochromatic camera (Sentech, Japan) equipped with 1-61448 and 1-61449 adaptor tubes (Navitar, Rochester, NY).

3.3.5 Quantitative real-time polymerase chain reaction (real-time PCR)

Real-time polymerase chain reaction (RT-qPCR) was performed after five days in growth media, and an additional 10 days in osteogenic media to evaluate the gene expression profiles of hMSC-only and hMSC/HUVEC spheroids. The total RNA of hMSC-only and hMSC/HUVEC

spheroids was isolated using a RNeasy Plus Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions and quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The primers of the measured mRNA genes were as follows: OCT4 (forward, GCA GCG ACT ATG CAC AAC GA and reverse, CCA GAG TGG TGA CGG AGA CA), PECAM-1 (forward, TAA TAC AAC ATC CAC GAG GGT CC and reverse, ACA AAA TTG CTT GCT AAA GAA GTG G), HIF1A (forward, CCA GTT AGG TTC CTT CGA TCA GT and reverse, TTT GAG GAC TTG CGC TTT CA), COL6A1 (forward, CCT GGA GGG CTA CAA GGA A and reverse, GTG CTT GGC CTC GTT CAC), COL1 (forward, ATG ACT ATG AGT ATG GGG AAG CA and reverse, TGG GTC CCT CTG TTA CAC TTT), ALP (forward, AGC TGA ACA GGA ACA ACG TGA and reverse, CTT CAT GGT GCC CGT GGT C), BSP (forward, AAC GAA GAA AGC GAA GCA GAA and reverse, TCT GCC TCT GTG CTG TTG). RT-qPCR was analyzed using SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA) and all values were normalized by a house-keeping gene GAPDH. Threshold cycle values were calculated using a comparative cycle threshold method. The fold-change of hMSC-only was set at 1-fold, and the ratio of the normalized fold-change was calculated based on the standard condition.

3.3.6 3D Bioprinting spheroids

AAB system was used to bioprint hMSC-only and hMSC/HUVEC spheroids as previously described [106]. AAB system was equipped with microvalves (INKX0517500A, Lee Company, Bashville, TN) with 250- μ m nozzles (INZA3100914K, Lee Company, Bashville, TN) and a portable ultrasonic humidifier (CZHD20, Comfort Zone, China). 1g of sodium alginate (Sigma-Aldrich, UK) was dissolved in 100 ml deionized (DI) water to prepare 1% (w/v) sodium alginate as a sacrificial material. In order to generate alginate droplets using the microvalve dispenser, dwell time of 700 μ s was utilized with positive back pressure of $\sim$ 103 kPa. Calcium chloride (CaCl_2) solution was prepared by dissolving 4% (w/v) (CaCl_2 (Sigma-Aldrich, St. Louis, MO) in DI water. Sodium alginate was dispensed on the glass substrate using microvalves, then the aerosol form of

CaCl₂ was utilized to crosslink sodium alginate partially. Spheroids were collected into 1.5 ml conical tubes and transferred to the bioprinting platform. Afterwards, the top portion of the conical tubes were cut by a scissor. A customized glass pipette (~80 µm in diameter) was dipped into a conical tube and air pressure of 25 mmHg was applied to lift spheroids. Critical lifting pressure was determined theoretically from the mean radius of spheroids, surface tension coefficient of cell media and air interface, and the dynamic contact angle at three phases (air, spheroid, and cell media) [106] to pick spheroids. hMSC-only and hMSC/HUVEC spheroids were lifted from cell media with backpressure of 43 and 52 mmHg, respectively. After the fabrication of complex-shaped arrangements, the bioprinted constructs were overlaid with alginate and crosslinked with the aerosol form. The whole construct was then transferred to a Petri dish and cultured with tissue-specific cell media. 4% (w/v) sodium citrate solution was added and agitated gently to remove alginate.

3.3.7 Characterization of osteogenic differentiation by immunocytochemistry and alizarin red S staining

To visualize the morphologies of osteogenically differentiating spheroids, spheroids cultured in osteogenic differentiation medium were stained with runt-related transcription factor 2 (RUNX2) and CD31. After osteogenic differentiation for 10 days, hMSC-only and hMSC/HUVEC spheroids were cross-sectioned, permeabilized in 0.2% Triton X-100 for 30 min and blocked with 2.5% NGS for 1 h. Then, the samples were incubated with mouse anti-RUNX2 primary antibody (1:100 in 2.5% NGS) and rabbit anti-CD31 primary antibody (1:100 in 2.5% NGS) for 60 min at room temperature, washed three times with DPBS, and incubated with goat anti-mouse Alexa Fluor 488 secondary antibody (1:250 in 2.5% NGS), goat anti-rabbit Alexa Fluor 647 secondary antibody (1:250 in 2.5% NGS), rhodamine-phalloidin (1:250 in 2.5% NGS) and DAPI (1:1000 in 2.5% NGS) for 60 min at 4 °C. The stained samples were then washed three times with DPBS and imaged using an Olympus FV10i-LIV Confocal Laser Scanning Microscope (Olympus America Inc., Melville,

NY). The calcium deposition was visualized by staining cross-sectioned slides with a 2 % alizarin red S staining solution for 10 min at room temperature. Stained samples were washed three times with DI water and imaged using optical microscopy.

To quantify the intensity of RUNX2 and CD31, areas of interest on confocal images were selected using ImageJ. Fifty regions of interest were determined at the surface of the assembled tissue, spheroid-spheroid interface, and core of spheroids on fluorescent images. Each box was used for quantification of the fluorescence intensity. The intensity of RUNX2 and CD31 was normalized by the intensity of DAPI. A representative heat map was generated for the RUNX2/DAPI intensity.

3.3.8 Micro-computed tomography (μ CT) measurement

μ CT scanner (VivaCT 40, Scanco Medical, Switzerland) was used with 10.5 μ m voxel resolution, 55 kV energy, 145 μ A intensity, 21.5mm diameter field-of-view, and 300 ms integration time to evaluate the mineralization of spheroids in bioprinted tissues. The samples were placed inside the μ CT scanner and scanned. DICOM files were processed in Avizo software (FEI Company, Hillsboro, OR). A hydroxyapatite (HA) phantom (Micro-CT HA, QRM, Germany) was included in each scan to generate a standard curve to convert Hounsfield units to mgHA/ccm [107]. Images were processed with a Gaussian smoothing filter (sigma 0.9) to reduce noise, and a threshold of 200 mgHA/ccm was used to visualize mineralized spheroids and quantify mineralized volume.

3.3.9 Statistical analysis

All values are presented as mean ($\pm$) standard deviation. Multiple comparisons were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The differences with p-values ($*p < 0.05$, $**p < 0.01$) were considered statistically significant. All statistical analysis was performed by Statistical Product and Service Solutions software (SPSS, IBM, Armonk, NY).

3.4 Results and Discussion

3.4.1 Fabrication and characterization of hMSC-only and hMSC/HUVEC spheroids

96-well plates with cell-repellent surface were used to form spheroids composed of hMSC-only and hMSC/HUVEC as described in our previous work [29]. As shown in Figure 3.1A, optical microscopy images demonstrated that cell suspension with a density of 50,000 cells per well was undergoing formation of a 3D cellular network, where cells coalesced into a single spheroid along the bottom surface of the well plate. Once aggregation was completed, spheroids slightly decreased in size, but progressively compacted over the course of culture period (Figure 3.1A). All spheroids demonstrated a spherical and uniform shape at day 5 of culture. The mean diameter of hMSC-only, and 92:8, 85:15, and 75:25 hMSC/HUVEC spheroids was determined to be ~ 694 , ~ 655 , ~ 649 , and ~ 592 μm , respectively (Figure 3.1B). Cell viability of spheroids at day 5 using LIVE/DEAD staining depicted ~ 86 , and $\sim 92\%$ for hMSC-only, and all hMSC/HUVEC spheroids respectively (Figure 3.1C). The cell viability of all the groups introduced with HUVECs was found to be significantly higher than that of hMSC-only group, which is in accordance to previous 2D co-culture reports [108]. In particular, cell viability was uniform across the entire spheroid domain for HUVEC-involved groups. H&E staining images (Figure 3.1A) of cross-sections of spheroids confirmed evenly distributed and well-connected cells with no significant difference in cell viability (Figure 3.1C) when comparing the number of HUVECs in hMSC spheroids. Also, immunofluorescent staining of F-actin corroborated the results from the H&E staining. hMSC/HUVEC spheroids exhibited denser and more uniform cytoskeletal organization as compared to hMSC-only spheroids, which exhibited lesser cellular density and empty space at the core. Moreover, hMSC/HUVEC spheroids had significantly higher RNA amount. As shown in Figure 3.1D, the RNA amount for hMSC-only, 92:8, 85:15, and 75:25 hMSC/HUVEC spheroids was ~ 79 , 126, 162, and 196 ng/spheroid, respectively. Interestingly, the RNA amount of

hMSC/HUVEC spheroids decreased with increase in HUVEC concentration. This showed that larger cellular spheroids needed to generate a higher amount of RNA for the maintenance of cellular activities [109]. Thus, the dead empty core with reduced cell viability and RNA amount in hMSC-only spheroid are a direct evidence to the role of HUVECs in enhancing the crosstalk amongst hMSCs in the spheroids. Moreover, visibly more compaction, also corroborated by the diameters of the spheroids, in the hMSC/HUVEC spheroids improved the diffusion of the nutrients into the core of the spheroids and facilitated the removal of waste products, enhancing the cell viability.

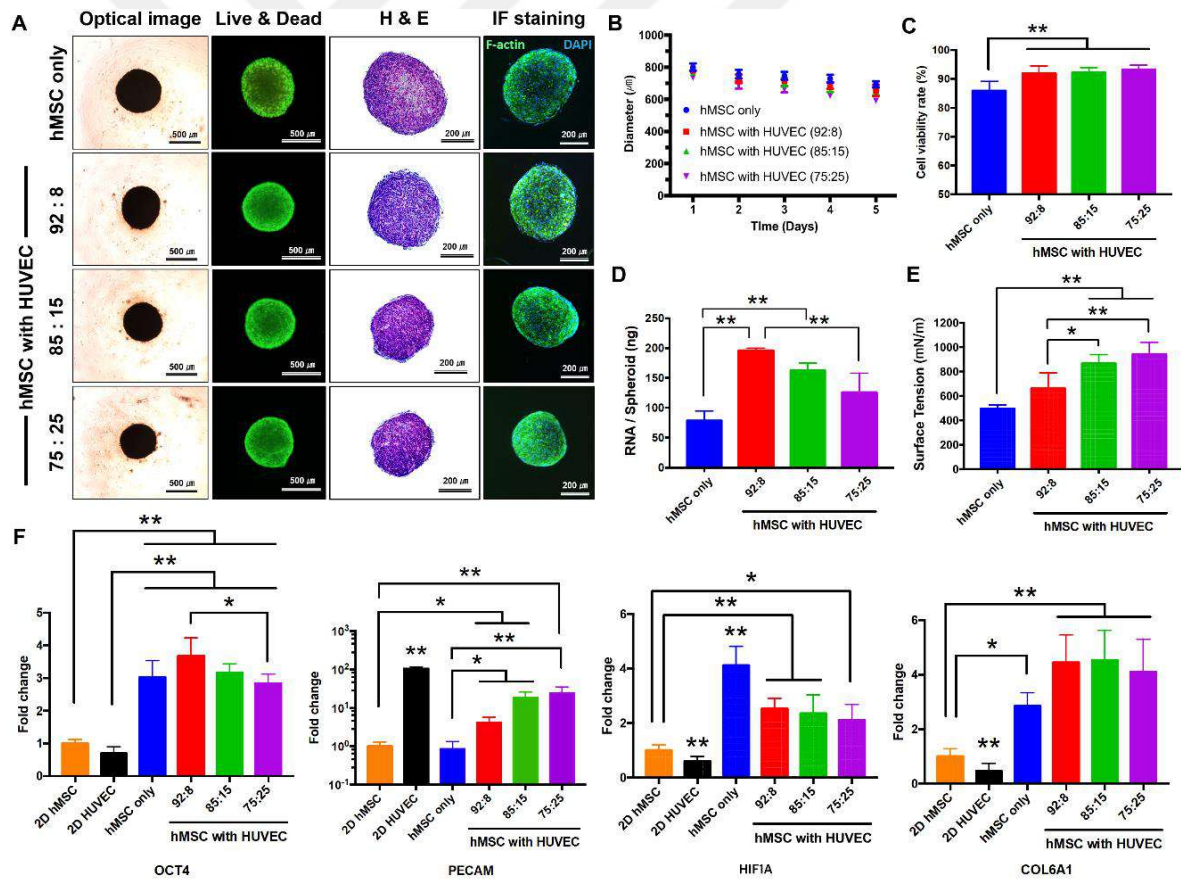


Figure 3.1: Formation of 3D spheroids and their characterization five days after incubation: (A) optical, LIVE/DEAD assay, histology, and F-actin staining images of hMSC-only and hMSC/HUVEC spheroids, (B) compaction of the spheroids over time, determined from the

reduction in their spheroid diameter. (C) cell viability, quantified from LIVE/DEAD assay (D) quantification of RNA concentration using Nanodrop (E) surface tension (F) gene expression measurements for pluripotent stem cell (OCT4), endothelial cell (PECAM), hypoxia (HIF1A), and ECM (COL6A1) related markers in 2D hMSC, hMSC-only and hMSC/HUVEC spheroids; * $p < 0.05$ and ** $p < 0.01$. We showed that introduction of only 8% HUVECs into the hMSCs is sufficient enough to enhance cell viability at the core of the spheroid with improved mechanical properties

The surface tension of spheroids was measured as 495.8 ± 30.2 mN/m for hMSC-only spheroids, 661.9 ± 127.1 mN/m, 865.7 ± 73.8 mN/m, and 940.6 ± 98.8 mN/m for 92:8, 85:15, and 75:25 hMSC/HUVEC spheroids respectively (Figure 3.1E). Notably, all hMSC/HUVEC groups showed increase in surface tension as compared to that of hMSC-only spheroid. The 75:25 group showed the highest surface tension amongst all groups, attributed to decrease in diameter of hMSC/HUVEC spheroid with increase in HUVEC concentration, as discussed earlier (Figure 3.1B). This shows that the compaction of the spheroids, depicted by the decrease in diameter, is a direct influencer of the surface tension of the spheroids. The increased compaction demonstrates that the hMSC/HUVEC spheroids are more in the viscoelastic spectrum, i.e. more elastic (solid) than viscous (liquid), and hence possess enhanced mechanical properties compared with hMSC-only spheroids.

In order to quantify the gene expression profiles of hMSC-only and hMSC/HUVEC spheroids, mRNA expression of OCT4, PECAM-1, HIF1A, and COL6A1 genes was investigated by RT-qPCR. As shown in Figure 3.1F, the mRNA expression of OCT4, represented as pluripotency-associated gene [110], significantly upregulated for hMSC-only and hMSC/HUVEC spheroids compared to conventional 2D culture condition of hMSC (2D hMSC) and HUVEC (2D HUVEC) after five days of culture in growth media. Among all groups, 92:8 hMSC/HUVEC

spheroids showed the highest expression level of OCT4 compared to that for other groups. Additionally, all hMSC/HUVEC spheroids exhibited higher PECAM-1 gene expression compared to 2D hMSC and hMSC-only spheroids. PECAM-1 expression level was significantly increased with increase in HUVEC concentration and 75:25 group exhibited the highest PECAM-1 expression. On the other hand, the expression of HIF1A, which is a hypoxia-responsive transcription factor [111], was significantly decreased with increase in HUVEC concentration. The highest expression level of HIF1A was observed in hMSC-only spheroid. COL6A1 is a major structural component of microfibrils in connective tissues and interact with other ECM components [112]. COL6A1 expression of hMSC-only and hMSC/HUVEC spheroids was significantly higher than that of 2D hMSC and 2D HUVEC. Overall, all hMSC/HUVEC groups showed increased mRNA expression of OCT4, PECAM, and COL6A1 and decreased HIF1A expression compared to hMSC-only spheroids.

In summary, 92:8 hMSC/HUVEC spheroids showed higher cell viability and mechanical properties (surface tension) compared to hMSC-only spheroids and demonstrated highest RNA content and pluripotency potential. Based on these results, we showed that introduction of only 8% HUVECs into the hMSCs is sufficient enough to enhance spheroid core cell viability with improved mechanical properties. Therefore, to accomplish our goal of building bone tissue and exploring the role of HUVECs in hMSCs spheroids on shape preservation of 3D constructs, we utilized 92:8 hMSC/HUVEC spheroids for the rest of the study, referred as hMSC/HUVEC spheroid henceforth.

3.4.2 Bioprinting of hMSC/HUVEC spheroids via AAB

In our previous study, we developed an AAB platform for biofabrication of 3D constructs with complex geometries [106]. This AAB system can precisely bioprint spheroids with a diverse range of sizes (diameter <80 μm – >800 μm) by picking up spheroids individually and placing them onto desired locations on hydrogel-coated substrates. In this study, alginate was printed onto glass

substrates using a micro-valve dispenser, and partially cross-linked using aerosol of CaCl_2 . hMSC/HUVEC spheroids were picked by a glass pipette and bioprinted at desired locations onto the gel, according to previously determined patterns (Figures 3.2A–C). This procedure was repeated for each of the spheroids and after completion, the spheroids were stacked up in three layers to fabricate each of the 3D complex-shaped constructs in mm scale, resembling a pyramid, hexagon, and diamond topology. Owing to the inherent adhesive nature of hMSC/HUVEC spheroids [106], they were well stacked up on the gel substrate and maintained the 3D complex topologies without structural collapse.

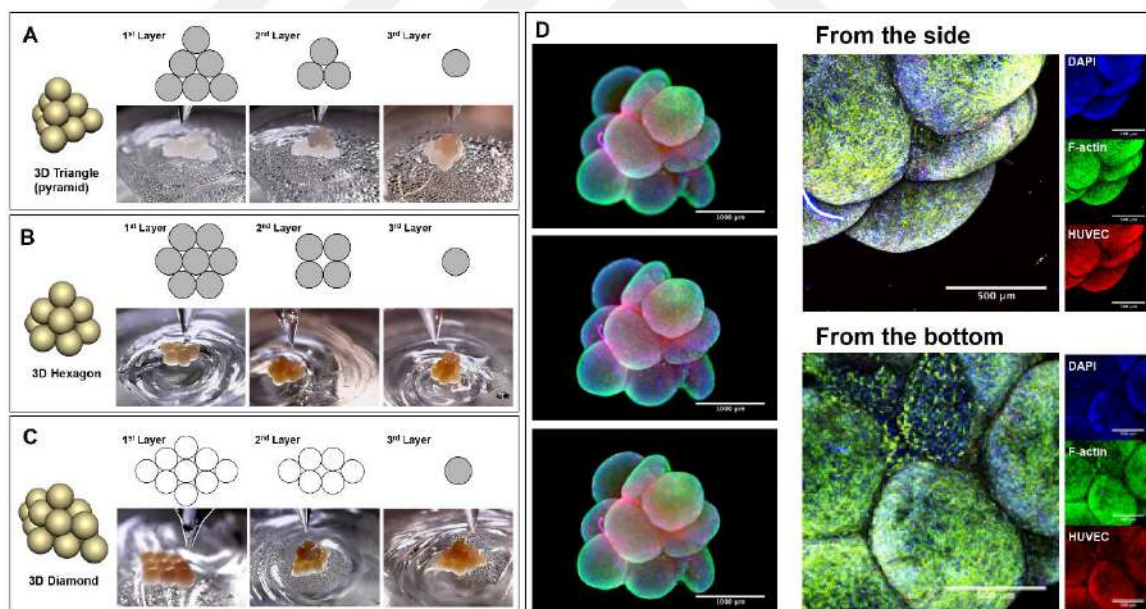


Figure 3.2: 3D Bioprinting of hMSC/HUVEC spheroids via Aspiration-assisted Bioprinting. Schematic diagrams and real-time photographs of a 3D pyramid (A), 3D hexagon (B), and 3D diamond (C) topographies using spheroids as building blocks. (D) Fluorescent images of bioprinted pyramid of hMSC/ tdTomato⁺ HUVEC spheroids, which were stained with DAPI and F-actin. Confocal images of pyramid constructs (side and bottom views).

After bioprinting, the constructs were overlaid with alginate using the micro-valve dispenser, crosslinked with the aerosols of CaCl_2 , and then incubated for two days to facilitate fusion amongst spheroids. As shown in Figure 3.2D, two days after bioprinting and incubation in alginate, the ~ 1 mm pyramid topography, assembled using hMSC/HUVEC spheroids, maintained its defined shape with evidence of organized cytoskeletal filaments. After removal of alginate and further incubation, the constructs were observed to undergo significant compaction, lost their designed configuration and turned into tissue balls (Figure 3.3), which is corroborated by previously published articles [51,106,113].

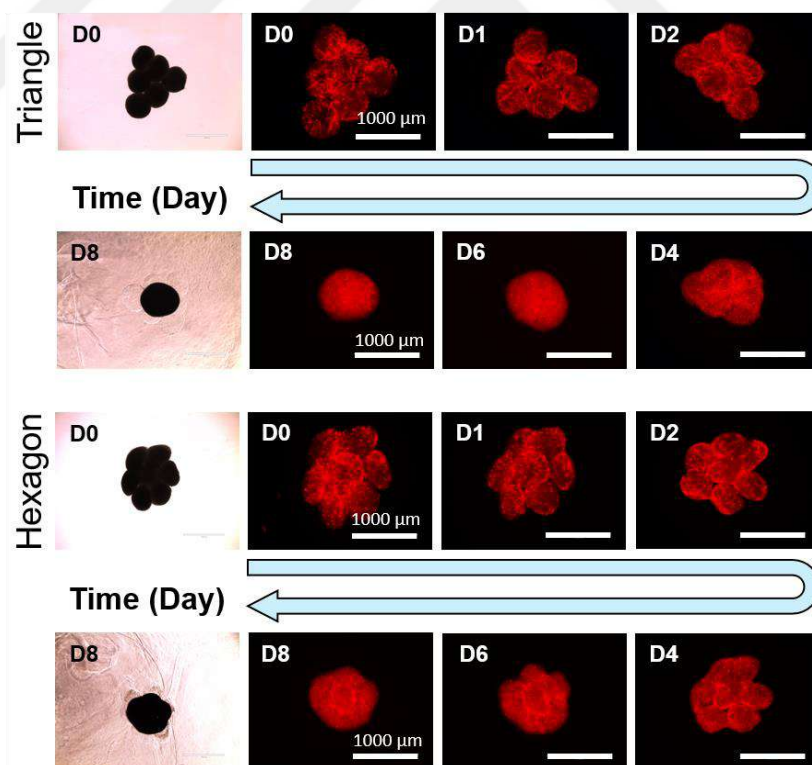


Figure 3.3: Post-bioprinting, hMSC/ tdTomato⁺ HUVEC tissue constructs were induced to osteogenic differentiation. The tissue constructs lost their shape and turned into tissue balls over a function of time (D: days).

In order to direct stem cell differentiation towards bone tissue formation, constructs composed of stem cells should be induced for about three weeks [51,102,114]. In this case, the bioprinted hMSC/HUVEC construct deformed into a tissue aggregate within four days of being guided into bone tissue differentiation. Hence, the concern with inherent compaction of spheroids leading to shape contortion hinders the whole objective of bioprinting spheroids in complex desired geometries. This led us to explore another strategy, by tuning the timelines of induction of osteogenic differentiation, for retaining the geometry of constructs while bioprinting using spheroids.

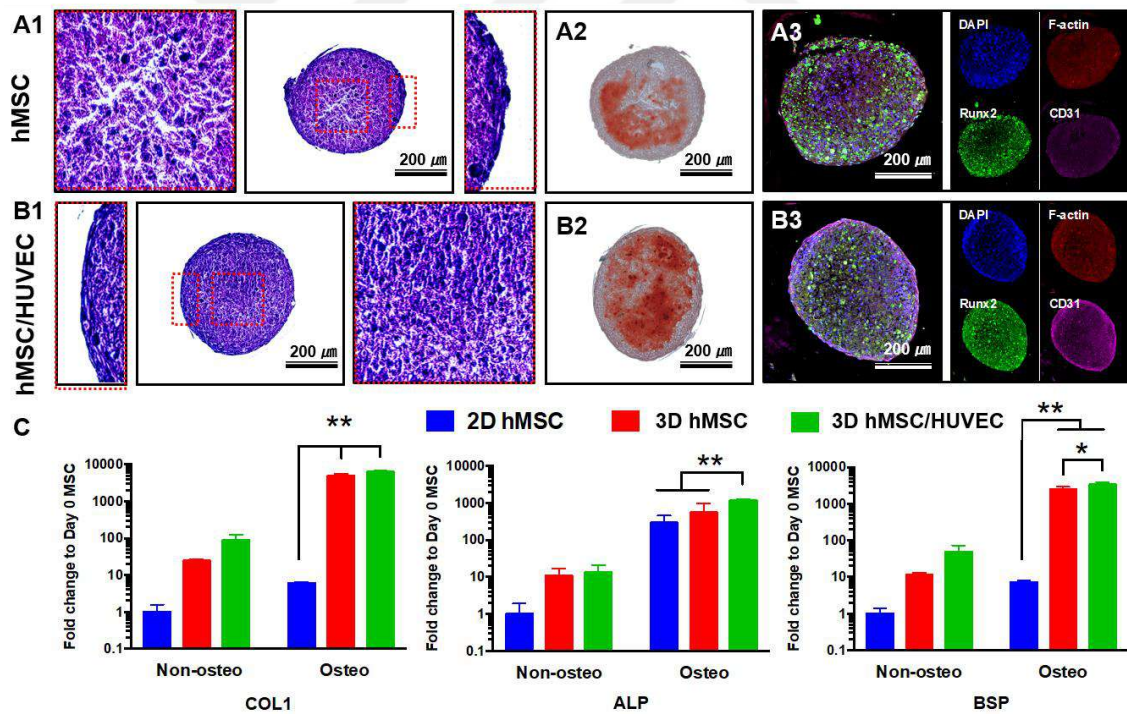


Figure 3.4: Characterization of osteogenically differentiated hMSC/HUVEC spheroids, cultured for five days in growth media and 10 days in osteogenic media: Representative images of (A1-B1) H&E staining, (A2-B2) ARS staining, and (A3-B3) IF staining with RUNX2 (Green), F-actin (Red), CD31 (Magenta), and cell nuclei (Blue) of hMSC-only and hMSC/HUVEC spheroids

respectively. (C) Osteogenic related gene expression levels of 2D hMSC, hMSC-only, and hMSC/HUVEC spheroids cultured in growth and osteogenic media; * $p < 0.05$ and ** $p < 0.01$. The results demonstrate the efficacy of HUVECs incorporation in hMSC spheroids to enhance osteogenic differentiation across the entire spheroid domain.

3.4.3 Osteogenic differentiation of hMSC/HUVEC spheroids

To induce osteogenesis, hMSC/HUVEC spheroids were cultured for five days in growth media and the following 10 days in osteogenic media. The core of the spheroids was visualized by H&E staining of the cross-sections. As shown in Figures 3.4A1-4B1, hMSC/HUVEC spheroids, after induction to osteogenic lineage, showed more compact and condensed structures with uniformly distributed cells compared to that in hMSC-only spheroids. Mainly, this phenomenon is more pronounced at the center of spheroids, with ring like structures at the periphery of the spheroids. This is due to the presence of HUVECs in cellular spheroids [102]. Osteogenic cells begin the deposition of mineral deposits when they differentiate into osteoblasts [115]. Calcium deposition of hMSC-only and hMSC/HUVEC spheroids was confirmed by alizarin red S (ARS) staining (Figures 3.4A2-B2). The deposited calcium amount of hMSC/HUVEC spheroid was substantially higher than that of hMSC-only spheroid with uniform calcium deposition across the entire cross-section, except for the periphery of the spheroid. The presence of the ring like structure (H&E) and absence of calcium deposition (ARS) at the periphery of the hMSC/HUVEC spheroids led us to characterize the localization of the hMSCs and HUVECs in the spheroids after osteogenic induction. RUNX2, an early osteogenic marker, was verified by immunocytochemical staining along with CD31 staining to confirm the cells of endothelial lineage. RUNX2 marker in hMSC/HUVEC spheroids was uniformly distributed across the cross-section of the spheroid while lesser expression of RUNX2 was observed in hMSC-only spheroid (Figures 3.4A3 -B3). Besides,

hMSC/HUVEC spheroids showed more endotheliogenic-specific marker than hMSC-only spheroids, as indicated by positive staining of CD31, with more localized CD31+ cells at the periphery of the spheroid, forming a ring like structure, which is in agreement with previous findings [116]. Thus, hMSC/HUVEC spheroids demonstrated enhanced proliferation and increased mineralization which is direct evidence of enhanced osteogenic potential due to a synergistic display between the two cell types [117,118]. Crosstalk between hMSCs and endothelial cells is not only dependent on microenvironmental diffusion factors but also, on gap junction alpha-1 protein [119] which demonstrates the necessity of direct cell-cell contact between hMSCs and HUVECs [120]. Besides this juxtacrine mechanisms by the gap junctional activity, endothelial cells mediated paracrine factors including vascular endothelial growth factor (VEGF) and the inflammatory mediator prostaglandin E₂ (PGE₂) augments the crosstalk between periodontal ligament stem cells (PDLSCs) and endothelial cells. This, in turn, enhances the osteogenic potential of PDLSCs under hypoxia regulated by mitogen-activated protein kinase (MAPK) kinase/extracellular signal-regulated kinase and p38 MAPK pathways [104]. As the core of the spheroids are deemed more hypoxic compared to the periphery, osteogenic differentiation of stem cells is enhanced by the PGE₂ and VEGF factors secreted by the HUVECs. HUVECs have also shown to express bone morphogenic protein 2, which induces osteogenic differentiation of stromal stem cells [121]

With considerable qualitative evidence demonstrating the effects of HUVECs on osteogenic potential of hMSCs, we also analyzed the mRNA expression of type I collagen (COL1), alkaline phosphatase (ALP), and bone sialoprotein (BSP) to quantify the same. All the experimental groups consisting of 2D hMSC, hMSC-only spheroids and hMSC/HUVEC spheroids were divided into two categories – cultured in either the maintenance media (labeled as “Non-osteo”) or osteogenic media (labeled as “Osteo”). As shown in Figure 3.4C, all groups cultured in osteogenic media exhibited higher expression of COL1, ALP, and BSP than those in maintenance media. The

early-stage osteogenic differentiation markers of COL1 and ALP increased in the order of 2D hMSC, hMSC-only, and hMSC/HUVEC in both types of culture media. COL1 expression for hMSC-only and hMSC/HUVEC spheroids was significantly higher than that for 2D hMSC. This demonstrates the efficacy of 3D spheroids to accurately demonstrate the influence of microenvironmental factors on osteogenic differentiation [106]. Also, hMSC/HUVEC spheroids cultured in osteogenic media exhibited significantly increased ALP expression compared to both 2D hMSC and hMSC-only spheroids cultured similarly. This is a direct evidence that the endothelial cells enhance the differentiation of hMSCs. Although, the precise role of HUVECs on osteogenic differentiation is debated, many 2D co-cultures with hMSCs and HUVECs have reported such observations [122,123]. The expression of BSP, a late osteogenic differentiation marker, significantly increased in the order of 2D hMSC, hMSC-only, and hMSC/HUVEC spheroids similar to the COL1 and ALP results. In particular, 3D hMSC/HUVEC showed the highest expression of BSP compared to other groups. Overall, it was found that hMSC/HUVEC spheroids exhibited enhanced early and late stage osteogenic differentiation, both in presence and absence of osteogenic media corroborating our earlier H&E, ARS and immunocytochemical staining and previous literature [122,123].

Therefore, hMSC/HUVEC spheroids were used for bioprinting of osteogenic tissues. Quiescence is inherently associated with niche resident stem cells. Previously studies have reported lower hMSCs proliferation rates in a co-culture system with HUVECs [116], suggesting the role of HUVECs in a retardation of hMSCs activity. Inducing the hMSC/HUVEC spheroids to differentiation media before bioprinting lowers the proliferative potential of hMSCs and allow the spheroids to be in their osteogenic differentiation pathway [124]. Hence, in this study, we utilized mid-term osteogenic hMSC/HUVEC spheroids for bioprinting scaffold-free bone-tissue constructs with an expectation of reduced deformation in the bioprinted geometry.

3.4.4 3D Bioprinting of osteogenic tissues

To demonstrate the ability of osteogenic hMSC/HUVEC spheroids to serve as building blocks in bioprinting, different topographies resembling triangle, hexagon, and diamond in a single layer were bioprinted using the AAB system. HMSC/HUVEC spheroids cultured for five days in maintenance media and differentiated for 10 days in osteogenic media were bioprinted on the gel substrate, overlaid with the sacrificial alginate, and then incubated for another two days in the osteogenic media. As shown in Figure 3.5A, hMSC/HUVEC spheroids were precisely arranged into a diamond shape and after incubation, fused into a single patch of tissue. We also demonstrated a diamond shaped topography in three layers with osteogenic hMSC/HUVEC spheroids (Figure 3.5B). Bioprinted single-layered osteogenic tissue construct exhibited high cell viability from the periphery to the core of the spheroid with a negligible number of dead cells, as indicated by LIVE/DEAD staining. In histological analysis, H&E-stained tissue sections revealed that osteogenic hMSC/HUVEC spheroids were tightly self-assembled to each other, and bioprinted tissues maintained their original shape without falling apart. The histological analysis of the cross-sections of the 3D diamond-shaped construct by H&E presented uniform cell distribution through the entire 3D structure with a very well-fused interface amongst the spheroids. Mineralization was confirmed by measuring mineralized volume and calibrated bone mineral density by μ CT analysis. The results showed that the single-layered bioprinted constructs were predominantly composed of mineralized matrix, which was indicated by values exceeding the threshold range of 200 mgHA/ccm. The mineralized volume in the single-layered triangle, hexagon and diamond shaped configurations was measured to be 0.051, 0.056 and 0.081 mm³, respectively. Uniform mineralization was also observed in the 3-layered diamond topography, with no significant alternation in its shape. The mineralized volume in first, second- and third-layer of the 3-layered geometry was measured at 0.163, 0.036 and 0.004 mm³, respectively.

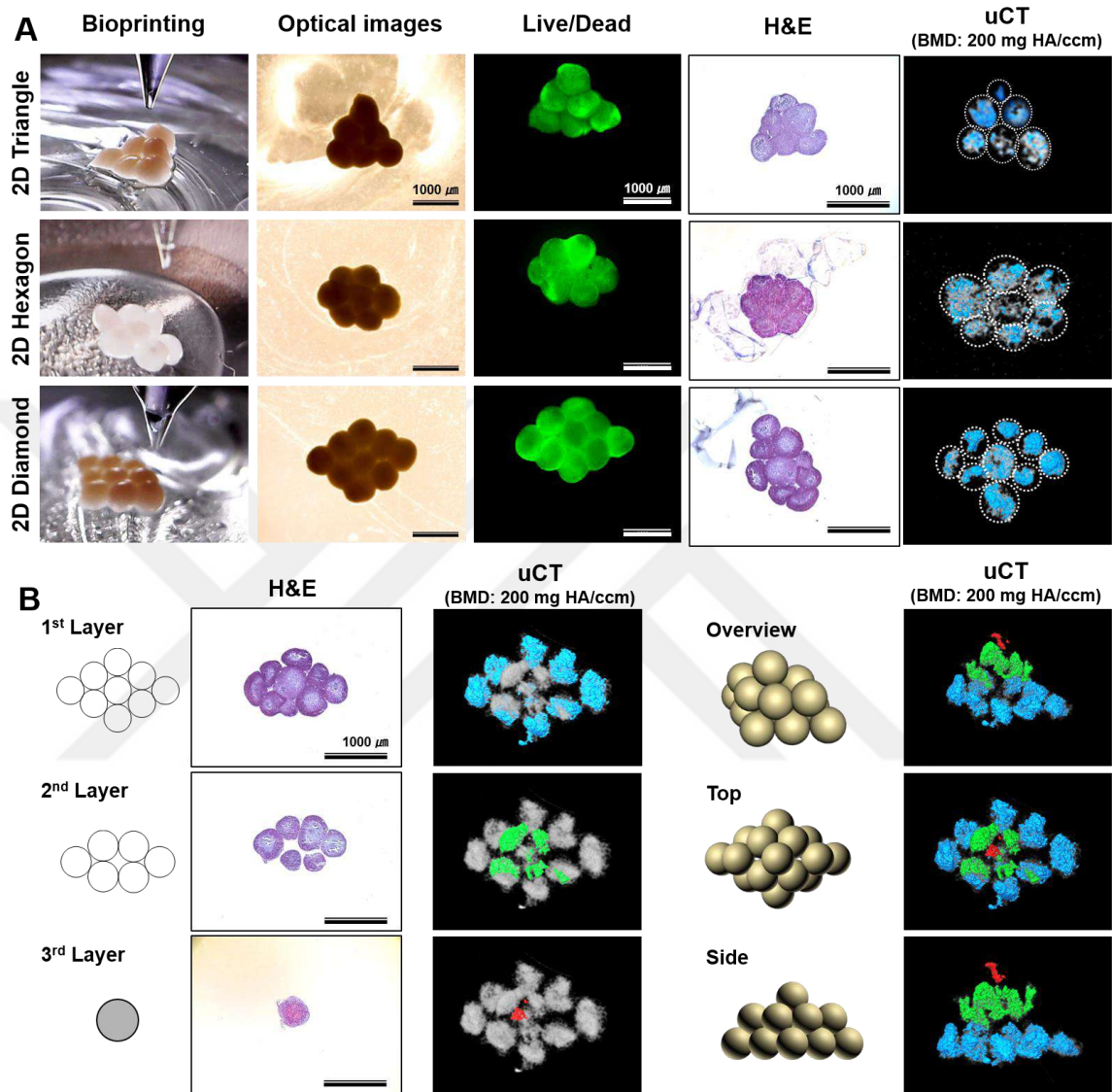


Figure 3.5: 3D biofabrication of (A) triangle, hexagon, and diamond-shaped topographies in a single layer (A) and 3 layers (B) using mid-term osteogenic differentiated hMSC/HUVEC spheroids. The bioprinted tissue constructs were characterized by optical microscopy, LIVE/DEAD assay, H&E and µCT showing bone formation and mineralization two days post bioprinting. The red, green and blue colors in the overview µCT scans depict the 3rd, 2nd and 1st layer, respectively, of the 3-layered diamond-shaped bioprinted tissue. We show negligible shape changes in the 3D tissue constructs post-bioprinting utilizing pre-differentiated hMSC/HUVEC spheroids.

Thus, in this study, we presented a new approach to fabricating scaffold-free bone tissue constructs using the AAB system with mid-term osteogenic hMSC/HUVEC spheroids as building blocks. The constructs retained the bioprinted shape and demonstrated uniform mineralization throughout the entire 3D configuration.

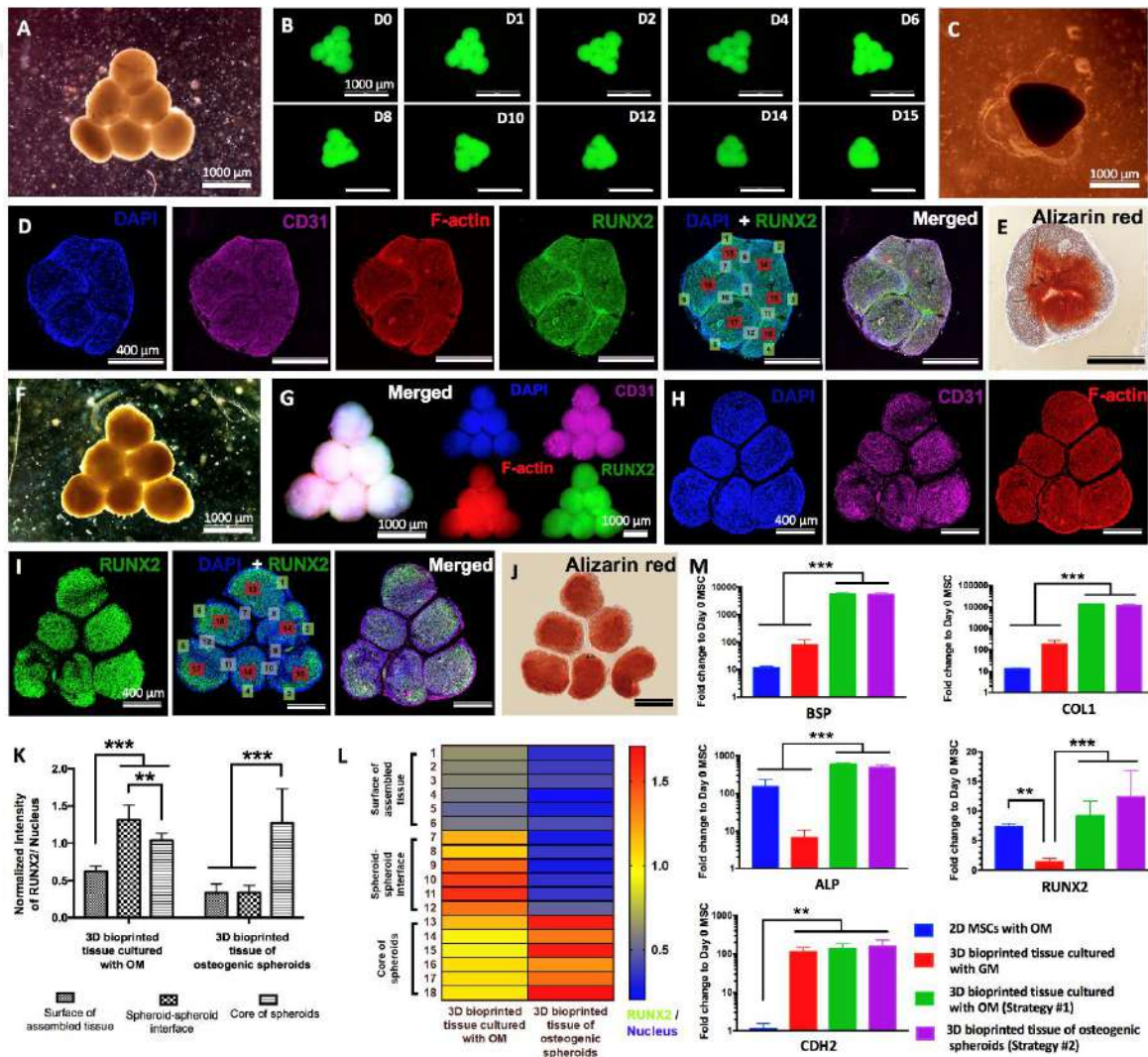


Figure 3.6: Biofabrication of osteogenic tissues. Strategy #1: (A) Triangle-shape tissue complexes were bioprinted using MSC/HUVEC spheroids and cultured for three days in growth media (GM) and 12 days in osteogenic media (OM). (B) Time-lapsed images showing fusion of GFP+ spheroids

up to Day 15 (D15) after bioprinting. (C) An optical image showing the assembled tissue at Day 15 post bioprinting. (D) Immunofluorescence staining (DAPI, CD31, F-actin, RUNX2, DAPI+RUNX2), and (E) Alizarin red staining of the sectioned tissue. Strategy #2: (F) The final shape of the bioprinted tissue of osteogenic spheroids (cultured 10 days in OM before bioprinting) and two days in OM after bioprinting. Immunofluorescent images of (G) the bioprinted tissue and (H) confocal images of its histological sections stained for DAPI, CD31, and F-actin, and (I) RUNX2 and DAPI+RUNX2. (J) Alizarin red staining of the tissue section. (K) Quantification of normalized RUNX2 intensity at different regions including the surface of assembled tissue, spheroid-spheroid interface and core of spheroids ($n=50$; $**p < 0.01$, and $***p < 0.001$). (L) A representative heatmap figure showing RUNX2/DAPI distribution in the surface of assembled tissue, spheroid-spheroid interface, and core of spheroids for Strategy #1 and Strategy #2. (M) BSP, COL1, ALP, RUNX2 and CDH2 gene expressions of 2D MSCs cultured in OM (control), 3D bioprinted tissues cultured in GM (control), and 3D bioprinted tissues cultured using Strategy # 1 and #2 ($n=5$; $**p < 0.01$, and $***p < 0.001$).

This practical AAB approach could be refined in the near-future to enhance the throughput and scalability and has the likelihood to be used as a reproducible tissue bioprinting platform, for various applications, including organ-on-a-chip devices, microfluidics, organoid engineering and tissue engineering. In addition, since lack of vascularization is one of the major roadblocks for functional bone tissue constructs [125] leading to tissue necrosis, long-term bone tissue viability has to be addressed in the future by the fabrication of scaffold-free constructs with an extensive and efficient network of vascular micro-vessels is desirable, prior to preclinical testing.

After fabrication, two different osteogenic differentiation culture strategies were used, both with equal total exposure time of osteogenic induction. This was performed to decode the role of

mid-term osteogenic induction of stem cell-based spheroids (prior to bioprinting) on the mineralization and assembly behavior of generated tissues. In Strategy #1, 2-day cultured spheroids were bioprinted into a triangle pattern (Figures 3.6A and 3.7). Post bioprinting, triangle-shape tissue complexes maintained their integrity and transformed into a more compact and dense structure after three days in proliferation culture conditions (growth media (GM)). Spheroids in this triangle-shape pattern self-assembled over time, where cells exhibited viability of 83% after 5 days of incubation, and the assembled structure was further cultured for a period of 12 days in osteogenic media (OM) (Figure 3.6B-C). In general, hMSCs cultured in OM express high levels of osteogenic markers [126].

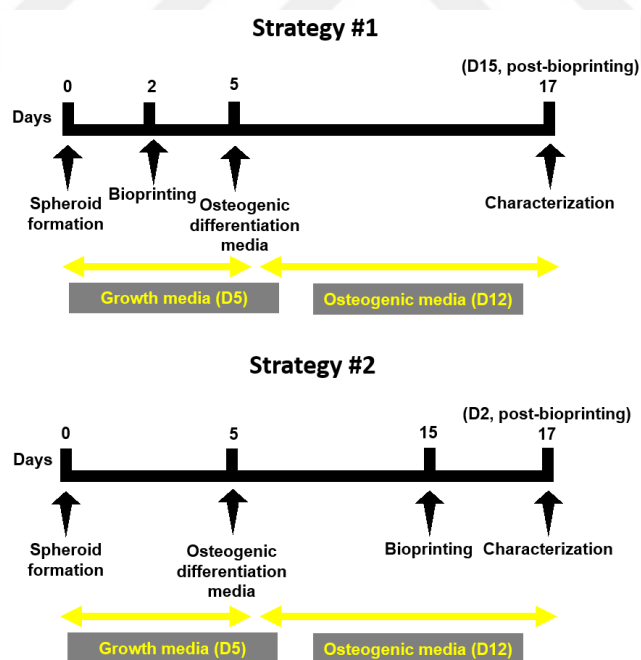


Figure 3.7: Culture strategies: A schematic of osteogenic culture strategies including strategy nos. 1 and 2.

In order to confirm the osteogenic differentiation of bioprinted tissues, the early osteogenic differentiation marker, RUNX2, was used for immunohistochemical staining. At the same time, the presence of HUVECs was confirmed by CD31 staining. As shown in Figure 3.6D, bioprinted tissues expressed osteogenic- and endotheliogenic-specific markers, as indicated by positive staining of RUNX2 and CD31. Calcium deposition of osteogenically-differentiated tissues was also confirmed by Alizarin red staining. As shown in Figure 3.6E, substantial calcium deposition was observed after 12 days of osteogenic induction, particularly at the core of the assembled tissue. As significant contraction was observed after the fusion of hMSC/HUVEC spheroids and the originally-bioprinted triangular shape was not maintained (Figure 3.6C) as well as the mineralization was not uniform throughout the tissue boundary (Figure 3.6E), we also followed an alternative strategy (Strategy #2) in order to preserve the bioprinted shape of osteogenic tissues (Figure 3.7). In this regard, hMSC/HUVEC spheroids were first maintained in GM for five days followed by inducing them with OM for 10 days. Next, spheroids were bioprinted and then kept another two days in OM. In both strategies, the total exposure time to GM and OM was identical. At the end of culture (Figure 3.6F), bioprinted tissues using Strategy#2 exhibited strong expression of RUNX2 with more uniform distribution of mineralization demonstrated by Alizarin red staining although RUNX2 staining was stronger in cores of spheroids (Figures 3.6G-J). Intensity analysis of RUNX2 revealed that RUNX2 staining was the strongest in the core of tissues or at the interface between spheroids, and the weakest on the surface of the assembled tissues (Figures. 3.6K-L, Figure 3.8A) while CD31 staining was uniformly distributed throughout the histological sections of bioprinted tissues under both strategies (Figure 3.8B).

A previous study demonstrated that HUVEC-mediated paracrine of VEGF and the inflammatory mediator prostaglandin E(2) (PGE₂) promoted osteogenesis of periodontal ligament stem cells under hypoxic conditions regulated by MEK/ERK and p38 MAPK pathways [104]. As

the core of tissues and interface between spheroids were more hypoxic compared to the surface of the assembled tissues, the osteogenic differentiation of hMSCs could be further promoted in those regions by PGE₂ and VEGF paracrine factors secreted by HUVECs. In addition, limited shape change was observed. This could be due to the fact that hMSCs in hMSC/HUVEC spheroids, cultured in OM for 10 days before bioprinting, were in the osteogenic differentiation pathway and their proliferation capability was diminished as reported in a previous study [124], and hence limited contraction was observed in spheroids. Another advantage of Strategy #2 is that the culture duration after bioprinting was reduced, where 2-day cultured tissues after bioprinting were still structurally stable

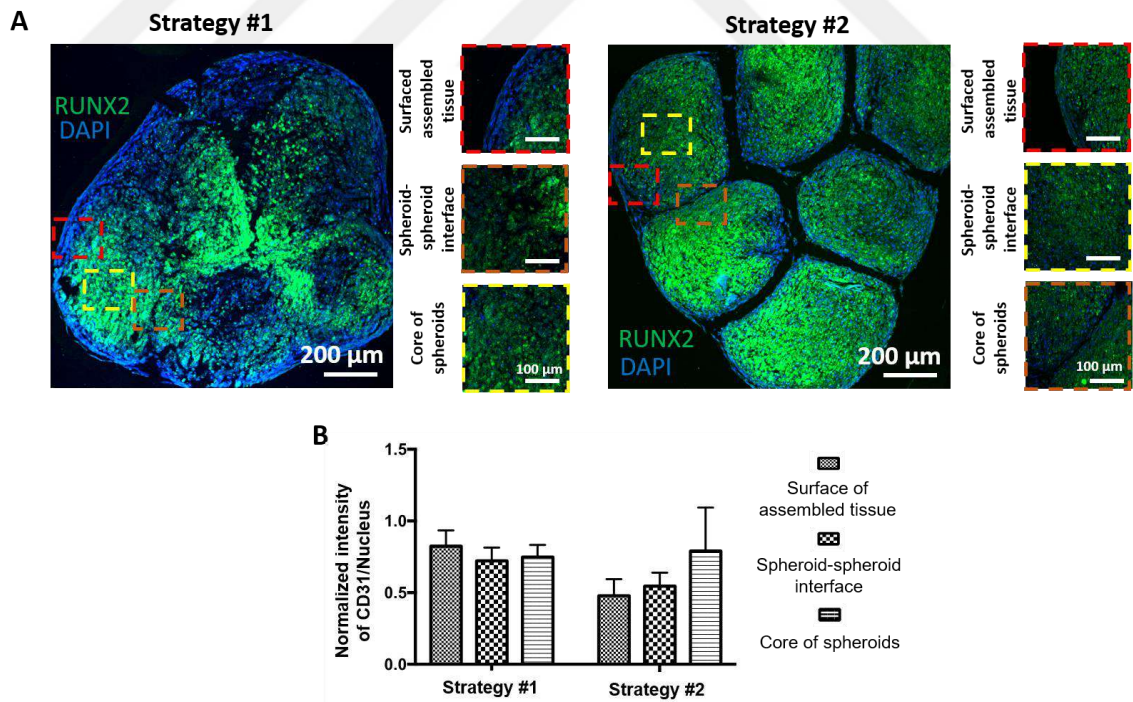


Figure 3.8: Osteogenic tissue biofabrication using strategy nos. 1 and 2.(A) Confocal images of bioprinted tissues stained for RUNX2 and DAPI (10X) along with representative zoom-in sections (20X) at the surface of assembled tissue, interface between spheroids, and core of spheroids. (B)

Normalized intensity of CD31 expression at different regions, including the surface of assembled tissue, interface between spheroids, and core of spheroids, on the histological sections of 3D bioprinted tissues cultured using both strategies ($n=50$).

We also analyzed the expression levels of BSP, COL1, ALP, RUNX2, and CDH2 (N-cadherin) genes for both strategies and bioprinted tissues cultured in GM (labelled as '3D Bioprinted tissue cultured with GM') and hMSCs differentiated in 2D for 12 days (labelled as '2D MSCs cultured with OM') were used as control groups. As shown in Figure 3.6M, expression levels of BSP, COL1, ALP, and RUNX2 genes for both strategies were similar to each other and significantly higher than those of control groups. In addition, the expression level of CDH2 gene (encoding N-cadherin protein) for all 3D bioprinted tissue groups were similar to each other and higher than that of the 2D control group. Overall, by changing the osteogenic induction window under the same total osteogenic induction duration, the shape of bioprinted tissues and uniformity of mineralization could be controlled although no differences were detected in the expression levels of osteogenic genes.

3.5 Conclusion

In conclusion, we present an effective spheroid bioprinting strategy using the AAB system, which facilitates bioprinting of osteogenic hMSC/HUVEC spheroids to fabricate scaffold-free 3D bone tissue constructs. This study addresses the limitations of the previously explored spheroid bioprinting techniques by enabling precise positioning of spheroids onto a hydrogel (11% with respect to the spheroid diameter), preservation of the geometrical shape of the fabricated construct by reducing compaction, and induction of uniform bone formation across the entire spheroid.

HUVECs were introduced into hMSCs to investigate their influence on spheroid formation and compaction, osteogenic differentiation and reduction of shape deformation after differentiation. Our findings demonstrate that osteogenically-differentiated hMSC/HUVEC spheroids, with as little as 8% of HUVECs, could be used as building blocks for bone tissue fabrication. These spheroids demonstrated reduced necrosis, increased cell viability in the core of the spheroid, enhanced differentiation into osteogenic lineage, and improved mechanical properties. Additionally, our bioprinting strategy exploited the cohesive nature of spheroids and reduced the proliferative potential of hMSCs after differentiation; this enabled building a scaffold-free 3D construct without any major shape change after bioprinting. Such a bioprinting approach, to facilitate fabrication of 3D geometries with negligible shape changes, using spheroids composed of differentiated stem cells and introduced with endothelial cells to enhance cell viability and differentiation, provides a new direction in bottom-up, scaffold-free bone tissue fabrication.

Chapter 4

Aspiration-assisted Bioprinting of Osteochondral Interface^{*†}

4.1 Chapter Summary

Osteochondral defects contain damage to both the articular cartilage and underlying subchondral bone, which remains a significant challenge in orthopedic surgery. Layered structure of bone, cartilage and the bone-cartilage interface must be taken into account in the case of biofabrication of the osteochondral (OC) interface. In this study, a dual layered OC interface was bioprinted using a newly developed aspiration-assisted bioprinting (AAB) technique, which has been the first time that scaffold-free bioprinting was applied to OC interface engineering. Tissue spheroids, made of human adipose-derived stem cells (ADSCs), were differentiated in three dimensions (3D) into chondrogenic and osteogenic spheroids, which were confirmed by immunostaining and histology qualitatively, and biochemistry assays and gene expression, quantitatively. Remarkably, the OC interface was bioprinted by accurate positioning of a layer of osteogenic spheroids onto a sacrificial alginate support followed by another layer of chondrogenic spheroids overlaid by the same support. Spheroids in individual zones fused and the maintenance of phenotypes in both zones confirmed the successful biofabrication of the histomorphologically-relevant OC interface. The biofabrication of OC tissue model without the use of polymeric scaffolds

^{*} This content is under revision in *Scientific Reports* entitled as “Aspiration-assisted bioprinting of the Osteochondral Interface” by Ayan, B, Wu, Y., Karuppagounder V., Kamal F., Ozbolat I.T. with minor changes.

[†] Yang Wu assisted with tissue characterization, Vengadeshprabhu Karuppagounder and Fadia Kamal assisted with characterization of bioprinted tissue.

unveils great potential not only in regenerative medicine but also in drug testing and disease modeling for osteoarthritis.

4.2 Introduction

Osteoarthritis (OA) is a degenerative disease that occurs in millions of individuals over the age of 60, which is characterized by significant joint pain, swelling and stiffness because of the loss of hyaline cartilage in synovial joints [127,128]. Osteochondral (OC) defects occur most frequently in the knee and affect both the articular cartilage and subchondral bone. Most current treatments for OA are limited to painkillers and anti-inflammatory drugs providing symptomatic relief [129]. Numerous efforts have been put to investigate for new therapeutic strategies that could prevent or cure OA. The development of such interventions is complex and challenging. Previous studies have identified enzymes, which are associated with the breakdown of the extracellular matrix (ECM) of the articular cartilage during OA, such as matrix metalloproteinases (MMPs) [130]. Growth factors, such as bone morphogenetic protein 7 (BMP-7), have been considered to enhance ECM synthesis and preserve cartilage in OA [131]. In addition, several drugs and proteins for the treatment of subchondral bone and cartilage have drawn interest including strontium ranelate, calcitonin, bisphosphonates, cathepsin K, and transforming growth factor-beta (TGF β) [132–136]. Several *in vitro* and *in vivo* models have been reported for OA drug testing in normal and pathological conditions [129]. Although there have been a plethora of advancements in drug screening, a high attrition rate remains as the main reason for the tremendous time and cost incurred in pharmaceutical research [53].

Given the high failure rate in drug discovery cycle, technological advancements should provide more precise and reproducible preclinical models, which closely recapitulate the native microenvironmental factors. Compared to conventional two-dimensional (2D) culture, three-

dimensional culture (3D) is more favorable in imitating cell bioactivities, tissue microstructure, and cell-matrix interactions [137]. 3D bioprinting is an emerging technology that builds targeted tissues which resemble histomorphologic, micro-architectural and biomechanical properties of native ones [138,139]. Recently, several shear-thinning bioinks have been developed including native cartilage matrix components such as hyaluronic acid (HA) and chondroitin sulphate [140]. Moreover, gelatin-methacrylamide (GelMA) [46,141], alginate [142], nanocellulose-alginate [143], and Pluronic F127 diacrylate crosslinked by methacrylated hyaluronic acid (PF127DA-HAMA) [144] are some examples of biomaterials used in scaffold-based bioprinting of the OC interface. Even though significant progresses have been made, the heterogeneity of OC interface in terms of structural and biomechanical properties is difficult to recapitulate using current approaches [145]. In scaffold-based approaches, cell-cell interactions and signaling are limited as cells are confined in the scaffold matrix, which is essential for cell differentiation and mechanotransductive signaling between cells in order to regenerate the OC interface with heterogenic structural, biological and mechanical characteristics [146,147]. Tissue strands, honeycombs, cell aggregates or spheroids are some examples of bioink used for scaffold-free biofabrication of tissues [19].

The OC tissue, is constituted of bone, cartilage and the interface, which is not trivial to manufacture easily because of its heterogeneous and anisotropic architecture [145,148]. Aspiration-assisted bioprinting (AAB), which is recently reported by our group, is a novel method for precise positioning of viscoelastic tissue spheroids in both scaffold-free and scaffold-based manners [106]. Taking the advantage of accurate positioning of tissue spheroids, this study is the first time to resemble the human OC interface using 3D scaffold-free bioprinting. This bottom-up approach with high precision renders a compact and stratified tissue arrangement, which is more biomimetic in terms of cell density and ECM organization, as compared to previously reported methods [149]. AAB enabled picking of spheroids from the cell media with aspiration forces and bioprint them

onto/into hydrogel substrates without any damage. In this study, patient-derived adipose-derived stem cells (ADSCs) were used to fabricate spheroids, which were then differentiated towards osteogenic and chondrogenic lineages in three weeks. Osteogenic and chondrogenic spheroids were then 3D bioprinted in multiple zones and then self-assembled into a single patch of tissue, which recapitulated the OC interface with histologically-relevant morphologies. The bioprinted OC interface has the potential to be used not only in tissue engineering and regenerative medicine but also in OA disease modeling for drug testing and the development of new therapeutic strategies.

4.3 Materials and Methods

4.3.1 Spheroid preparation and differentiation

To obtain human ADSCs, surgically discarded adipose tissues were obtained from patients who underwent an elective adipose tissue removal process (e.g., panniculectomy) at the Pennsylvania State University (Hershey, PA). The human study was approved by the Pennsylvania State University Institutional Review Board (protocol #4972). Patients' informed consent was obtained for study participation. All methods were performed in accordance with the relevant guidelines and regulations. ADSCs were isolated according to the protocol described in our previous work with verification of flow cytometry against CD73 and CD90 [80]. The sorted ADSCs were cultured in DMEM/F12 supplement with 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C with 5% CO₂. Cell medium was changed every three days. Expanded ADSCs were then used for the preparation of spheroids. ADSCs were trypsinized and centrifuged to cell pellets. Cells were then suspended in the media to obtain a density of 1×10^6 cells/mL. Afterwards, 200 µL of cell suspension per well was transferred to a 96-well plate (Greiner Bio One, NC) in order to obtain an average density of $\sim 2 \times 10^4$ cells per well. After 5-day culture with ADSC growth media, the growth media was replaced with Human Chondrocyte Differentiation Media and Human Osteoblast

Differentiation Medium (Cell Applications, CA) for chondrogenic and osteogenic differentiation of spheroids for another 21 days, respectively. As a control group, ADSC spheroids were also cultured for another 21 days using the ADSC growth media.

4.3.2 Determination of the physical properties of spheroids

Spheroids were cultured in differentiation media from Day 5 and the diameters of spheroids were measured by an EVOS[®] microscope (Invitrogen, MA) until Day 26. Surface tension of spheroids was also measured according to the protocol described in our recently published work [52]. Briefly, after collecting the spheroids from well plates, they were washed with phosphate buffer saline (PBS) for three times. Customized straight micropipettes, fabricated from glass pipettes (VWR, PA) using a P1000 Flaming/Brown micropipette puller (Sutter Instrument, CA), were used for surface tension measurements. Aspirated spheroids were monitored via a STC-MC33USB monochromatic camera (Sentech, Japan). The surface tension for ADSC, chondrogenic and osteogenic spheroids were measured at Day 26.

4.3.3 Scanning electron microscope (SEM) imaging

Field Emission SEM (FEI Navo NanoSEM 630, Nanolab Technologies, CA) was used to probe the surface topography of fabricated ADSC, chondrogenic, and osteogenic spheroids. Spheroids were fixed in 4% paraformaldehyde (Santa Cruz Biotechnology, TX) overnight, carefully washed with PBS, and dehydrated using graded ethanol solutions (25%, 50%, 75%, 95% and 100%, sequentially). To ensure the complete removal of water, samples were further dried in a critical point dryer (CPD300, Leica EM, Germany). Upon complete dehydration, they were sputter coated with iridium (Leica, Germany) and imaged at an accelerating voltage of 3-5 keV.

4.3.4 Cell viability analysis

ADSC, chondrogenic, and osteogenic spheroids were collected from 96-well plates, and cell viability was assessed using LIVE/DEAD viability assay kit (Life Technologies, NY). First, spheroids were washed for three times using PBS and then incubated in a fluorescent reagent consisting of 1 μ M calcein AM and 1.6 μ M ethidium homodimer-1 in PBS for 30 min. Live cells were able to take up and retain the calcein dye resulting in bright green fluorescence of their cytoplasm. The ethidium homodimer could only enter dead cells, where it binds to nucleic acids producing a bright red fluorescence. Z-stack images were taken on the EVOS[®] microscope. ImageJ software (National Institute of Health, USA) was used for quantitative analysis for red- and green-fluorescent cells.

4.3.5 Immunohistochemistry study

All primary monoclonal antibodies were purchased from Abcam (MA) and fluorescence-conjugated secondary antibodies were purchased from Life Technologies (CA). Sections of ADSC and chondrogenic spheroids were treated using Triton-X 100 (0.1 % in PBS) for 10 min and blocked with normal goat serum (NGS, 10 % in PBS) for 1 h. Samples were then incubated with monoclonal rabbit anti-human collagen type II (COL-II, 1:200), mouse anti-human aggrecan (1:50) and NGS (negative control) for 1 h, respectively. Samples were washed twice with PBS and incubated using secondary antibodies (goat anti-rabbit IgG (H+L)-Alexa Fluor 647 for COL-II, and goat anti-mouse IgG (H+L)-Alexa Fluor 488 for aggrecan, both at 1:200 dilution) for 1 h. After rinsing thrice with PBS, samples were incubated with Hoechst 33258 (1:200, 5 min) for nucleus visualization and mounted with Fluoromount-G[™] (Invitrogen, MA). Using the same protocol, sections of ADSC and osteogenic spheroids were stained for bone-specific primary antibodies, including rabbit anti-human bone sialoprotein (BSP, 1:200) and runt-related transcription factor 2 (RUNX2, 1:200), followed by incubation with the secondary antibodies (goat anti-rabbit IgG (H+L)-Alexa Fluor 647

for BSP, and goat anti-mouse IgG (H+L)-Alexa Fluor 488 for RUNX2, both at 1:200 dilution). Images for each marker were taken using a Zeiss Axiozoom microscope (Carl Zeiss Microscopy, LLC, Germany).

4.3.6 Histological analysis

ADSC, Chondrogenic, and osteogenic spheroids were fixed with 4% paraformaldehyde and embedded in paraffin using an automatic tissue processor (TP 1020, Leica, Germany). Next, spheroids were gradually dehydrated in alcohol, cut into 8 μ m sections, and placed onto charged slides. Sections were then stained with hematoxylin and eosin (H&E) using Leica Autostainer XL (Leica, Germany). Coverslips were mounted on the slides with Xylene Substitute Mountant (Thermo Fisher Scientific, PA).

Sections were also separately stained for Alizarin Red S, Toluidine Blue O, and Picrosirius Red according to standard protocols for detection of calcium, sulfated proteoglycan, and collagen, respectively. For Alizarin Red S staining, sections were incubated with Alizarin Red solution (EMD Millipore Corp., USA) at room temperature for 20 min. The dye was removed and dehydrated with acetone and acetone-xylene (1:1) solutions, sequentially. Samples were then cleaned with xylene and mounted with Xylene Substitute Mountant. For Toluidine Blue O staining, sections were incubated in a Toluidine Blue solution (0.1% in deionized (DI) water) (Sigma Aldrich, MO) at room temperature for 2 min. The dye was removed and then the samples were washed twice with DI water. After dehydration with 95% and 100% alcohol (sequentially) and clearing with xylene, coverslips were mounted to slides. For Picrosirius Red staining, the solution was prepared by dissolving 0.1% Direct Red 80 and 0.1% Fast Green FCF dissolved in saturated aqueous picric acid (1.2% picric acid in water, Sigma Aldrich, MO). The solution was then applied to sections and then the sections were incubated for 60 min. Samples were rinsed with DI water, dehydrated with

alcohol, cleaned with xylene, and then mounted. Finally, samples were imaged using the EVOS® microscope.

4.3.7 Biochemical assays

For samples undergoing chondrogenic differentiation, sulfated glycosaminoglycan (sGAG) content was determined by 1,9-dimethylmethylene blue (DMMB) dye-binding assay. For each sample, 10 chondrogenic spheroids were collected and rinsed with PBS. Next, spheroids were digested in 500 μ L solution of 0.1 mg/mL papain extraction reagent at 65 °C for 18 h. 20 μ L of digested samples were mixed with 200 μ L DMMB solution and the absorbance was measured at 525 nm using a microplate reader (PowerWaveX, BioTek, VT). A series of solution of chondroitin 4 sulfate was prepared as a standard and sGAG contents of samples were calculated according to the standard curve. For samples undergoing osteogenic differentiation, alkaline phosphatase (ALP) activity assay was conducted using an assay kit (K412-500; BioVision, Inc., CA) according to the manufacturer's instructions. 10 spheroids per sample were re-suspended in an assay buffer and subsequently centrifuged at 13,000 g for 3 min at 4°C to remove the insoluble material. The supernatant was mixed with p-nitrophenyl phosphate (pNPP) substrate and then incubated at 25°C for 60 min. The optical density of the resultant pNPP at 405 nm was determined at 405 nm.

In order to determine the total collagen content of each spheroid type, a hydroxyproline colorimetric assay kit (BioVision, BioVision Inc, CA) was utilized according to manufacturer's instructions. Fifty spheroids per sample were collected at Day 26 and washed with DPBS. Afterwards, samples were homogenized in 100 μ L of distilled water and transfer to tight vials. 100 μ L of 12 N hydrochloric acid was added and incubated at 120°C for 3 h. All measurements were taken using a Powerwave X-340 spectrophotometer (Biotek, Winooski, VT) at 560 nm and the results were normalized to the collagen amount expressed by the ADSC group.

A Quant-iT™ PicoGreen dsDNA Assay Kit (Molecular Probes Inc., OR) was used to determine the DNA amount, according to the manufacturer's instructions. Fluorescence intensity was determined by a SpectraMax multi-detection microplate reader (Molecular Devices, Inc., CA), using a wavelength of 480 nm (excitation) and 520 nm (emission). sGAG content and ALP activity were normalized to dsDNA content. ADSC spheroids were used as a control group. For sGAG content, the fold change of ADSC group was set as 1-fold and values of chondrogenic and osteogenic groups were further normalized with respect to ADSC group.

Table 4.1 Primer information for RT-qPCR

Gene		Primer
Aggrecan	Forward	5'-TCCCCTGCTATTTTCATCGAC-3'
	Reverse	5'-CCAGCAGCACTACCTCCTTC-3'
SOX9	Forward	5'-AGCGAACGCACATCAAGAC-3'
	Reverse	5'-CTGTAGGCGATCTGTTGGGG-3'
COL2A1	Forward	5'-CCAGATGACCTTCCTACGCC-3'
	Reverse	5'-TTCAGGGCAGTGTACGTGAAC-3'
COL1	Forward	5'-CAGAACGGCCTCAGGTACCA-3'
	Reverse	5'-CAGATCACGTCATCGCACAAAC-3'
BSP	Forward	5'-AACGAAGAAAGCGAAGCAGAA-3'
	Reverse	5'-TCTGCCTCTGTGCTGTTGGT-3'
RUNX2	Forward	5'-GGTTAATCTCCGCAGGTCAC-3'
	Reverse	5'-CACTGTGCTGAAGAGGCTGTT-3'
ALP	Forward	5'-AGCTGAACAGGAACAACGTGA-3'
	Reverse	5'-CTTCATGGTGCCCGTGGTC-3'
GAPDH	Forward	5'-CACATGGCCTCCAAGGAGTA-3'
	Reverse	5'-GTACATGACAAGGTGCGGCT-3'

4.3.8 Gene expression study using quantitative real-time polymerase chain reaction (RT-qPCR)

In order to evaluate cartilage- and bone-specific gene expression levels, 30 differentiated spheroids per sample at Day 26 were homogenized in TRIzol reagent (Life Technologies, CA),

followed by adding 0.2 mL chloroform per 1 mL TRIzol reagent and centrifuging the mixture at 12,000 g for 15 min at 4°C. The upper aqueous phase with RNA was transferred and the RNA was then precipitated by adding 0.5 mL isopropyl alcohol per 1 mL TRIzol reagent, followed by centrifuging at 12,000 g for 10 min, at 4°C. Subsequently, the precipitated RNA was rinsed twice by 75% ethanol, air-dried for 10 min and dissolved in 50 µL diethyl pyrocarbonate (DEPC)-treated water. RNA concentration was measured using a Nanodrop (Thermo Fisher Scientific, PA). Reverse transcription was performed using AccuPower® CycleScript RT PreMix (BIONEER, Korea) following the manufacturer's instructions. Gene expression was analyzed quantitatively with SYBR Green (Thermo Fisher Scientific, PA) using a 7500 RT-qPCR system (Applied Biosystems®, Life Technologies, USA). Cartilage-specific genes tested for chondrogenic spheroids included collagen type II (COL2A1), Aggrecan, collagen type I (COL1), and the chondrogenic transcription factor SOX9. Bone-specific genes tested for osteogenic spheroids included BSP, RUNX2, COL1, and ALP. The reader is referred to Table 4.1 for the gene sequences. Expression levels for each gene were then normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The fold change for the ADSC group was set as 1-fold and values in chondrogenic and osteogenic groups were normalized with respect to that of the ADSC group.

4.3.9 Bioprinting of the OC interface

Prior to bioprinting, sodium alginate, sodium citrate and calcium chloride (CaCl₂, Sigma Aldrich, MO) were sterilized with ultraviolet (UV) light for 30 min. Solutions, including 1% (w/v) sodium alginate, 4% (w/v) CaCl₂ and 4% (w/v) sodium citrate, were prepared by dissolving the powder in sterile deionized (DI) water and subjected to magnetic stirring until the powder was completely dissolved. In order to 3D bioprint the OC interface, the AAB technique was used as described in our recent study. Briefly, alginate droplets were deposited onto 35 × 10 mm tissue culture dishes (Corning, NY) using a micro-valve (INKX0517500, Lee Company, TN) equipped

with 250 μm nozzles (INZA3100914K, Lee Company, CT). A unipolar wave pulse with a dwell voltage (amplitude) of 5 V and a dwell time (valve opening duration) of 1,000 μs was used for actuating the micro-valve dispenser, where a positive back pressure of ~ 103 kPa was used for driving the flow of sodium alginate inside the tubing. CaCl_2 vapor was fumed onto alginate for partial crosslinking purposes. In order to fabricate custom-made glass pipettes, borosilicate Pasteur pipettes (VWR, PA) were pulled using P-2000 Flaming/Brown micropipette puller (Sutter Instrument, Novato, CA). Using ~ 80 μm glass pipettes, spheroids were then lifted from the reservoir via aspiration at their critical back-pressure (74 and 95 mmHg for chondrogenic and osteogenic spheroids, respectively), which was controlled by 1/4" NPT 12VDC normally-closed solenoid valves (USS2-00051, U.S. Solid, OH). They were then transferred and positioned onto the alginate support. The procedure was repeated as many times as needed in order to build the construct. At the last step, bioprinted constructs were overlaid with alginate using micro-valve bioprinting and CaCl_2 vapor was then applied to crosslink alginate. After 1-week culture in hybrid media (chondrogenic and osteogenic media mixed in 1:1 ratio) spheroids were completely fused and alginate was de-crosslinked using 4% sodium citrate solution, leaving the fused OC interface behind.

4.3.10 Histomorphological analysis of the bioprinted OC interface

The bioprinted OC interface samples were then fixed, embedded in paraffin, and then sectioned for histological analysis. Samples were sectioned transversely in chondrogenic and osteogenic layers separately. H&E Staining was performed according to the protocols explained in Section 4.3.6. Sagittal sectioning was also performed across the osteochondral interface and sections were stained for Toluidine Blue O and Alizarin Red S using the protocols described in Section 4.3.7. For quantification of cartilage and bone zones, total cartilage or bone area was first measured by selecting the entire region of interest using the area tool in OsteoMeasure software

(OsteoMetrics Inc., GA, USA), while the stained areas were selected with the thresholding tool. The thresholding selection was set by sampling from the most darkly stained region of either bone or cartilage, then smoothing/filtering the selection. Any selected areas that were not in the region of interest were deselected before gathering the total stained area. The percentage of stained areas in the bioprinted OC interface were calculated as below:

$$\% \text{ of stained area} = \frac{\text{Stained area}}{\text{Total area of bone or cartilage zone}} \times 100 \quad (1)$$

4.3.11 Statistical analysis

All data were presented as the mean $\pm$ standard deviation unless stated otherwise, and were analyzed using Student's t-test and one-way analysis of variance (ANOVA) to test for significance when comparing the data. Post-hoc Tukey's multiple comparison test was used to determine the individual differences among the groups. Differences were considered significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All statistical analysis was performed by Statistical Product and Service Solutions software (SPSS, IBM, USA).

4.4 Results and Discussion

4.4.1 Formation of spheroids

In this study, ADSC spheroids were obtained with a density of 2×10^4 cells/spheroid using 96 U-shape well-plates. After five days of culture, formation of compact ADSC spheroids were observed. Afterwards, spheroids were cultured with osteogenic or chondrogenic induction media for 21 days before bioprinting the OC interface. The average diameter of ADSC spheroids were $\sim 650 \mu\text{m}$ at Day 5 (Figure 4.1A). One week after differentiation, the average diameter of ADSC, chondrogenic and osteogenic spheroids were measured to be ~ 510 , 663 , and $541 \mu\text{m}$, respectively. In another words, the average diameter of ADSC and osteogenic spheroids decreased by ~ 20 and

19% at Day 12, respectively. However, no significant changes were observed for the average diameter of chondrogenic spheroids. After the second week of differentiation, the average diameter of ADSC, chondrogenic, and osteogenic spheroids were measured to be ~555, 764, and 580 μm at Day 19, respectively, in which the average diameter of chondrogenic spheroids displayed an increase of ~15%. Overall, the average diameter of ADSC and osteogenic spheroids decreased by ~9 and 13% at Day 26, respectively, while, the average diameter of chondrogenic spheroids increased by ~19% during the prolonged culture time with significant differences (*** $p < 0.001$ shows significance between ADSC and chondrogenic spheroids, and ### $p < 0.001$ shows significance between osteogenic and chondrogenic).

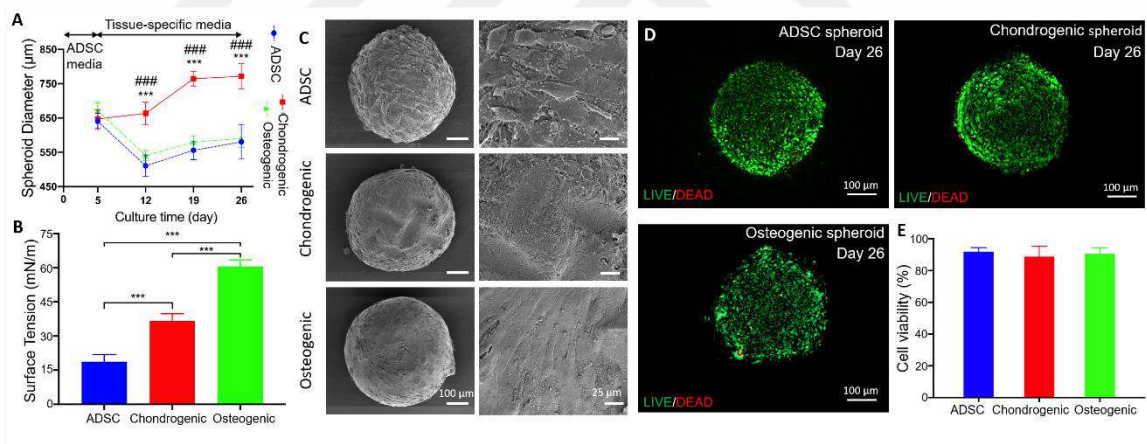


Figure 4.1: Structural, mechanical, and cell viability analysis of spheroids: (A) Diameter change over 26 days ($n=8$, *** $p < 0.001$ shows significance between ADSC and chondrogenic spheroids, and ### $p < 0.001$ shows significance between osteogenic and chondrogenic spheroids), (B) surface tension at Day 26 ($n=5$, *** $p < 0.001$), and (C) SEM images of ADSC, chondrogenic and osteogenic spheroids. (D) LIVE/DEAD images (E) and cell viability in ADSC, chondrogenic, and osteogenic spheroids at Day 26.

The surface tension of ADSC, chondrogenic and osteogenic spheroids, representing the mechanical properties of viscoelastic spheroids, were measured to be ~19, 37, and 60 mN/m, respectively (Figure 4.1B). After 26 days of culture, all groups exhibited compact morphologies (Figure 4.1C). After the differentiation of each tissue type, cell viability was investigated at Day 26, which could be considered long term. LIVE/DEAD staining demonstrated a high level of cell viability (Figure 4.1D), which was further confirmed by the quantitative measurements, showing ~92, 94 and 96% of live cells in ADSC, chondrogenic and osteogenic spheroids, respectively, without significant difference ($p > 0.05$, Figure 4.1E).

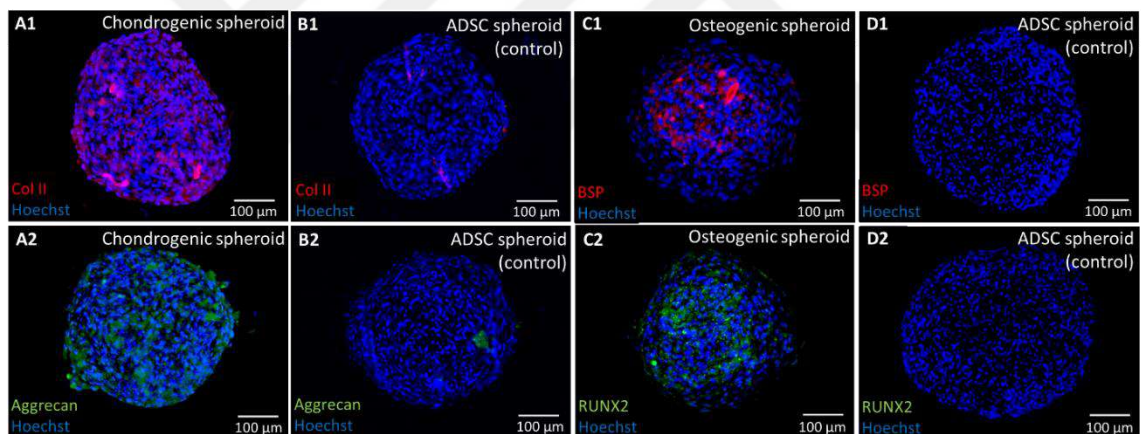


Figure 4.2: Expression of cartilage-specific markers including (A) COL-II and (B) AggreCAN for chondrogenic and ADSC spheroids, and bone-specific markers including (C) BSP and (D) RUNX2 for osteogenic and ADSC spheroids.

4.4.2 Qualitative and quantitative analysis of chondrogenic and osteogenic spheroids

In order to fabricate the OC interface, ADSC spheroids were differentiated into chondrogenic and osteogenic lineages. Immunohistochemistry staining was performed to evaluate the expression of cartilage-specific markers, including COL-II and AggreCAN, in order to verify the

chondrogenesis of ADSC spheroids (Figure 4.2A1-B2). Images show substantial expression of COL-II and Aggrecan distributed throughout the chondrogenic spheroids as compared to the ADSC control. The results demonstrated that ADSCs were able to chondrogenically differentiate in the form of spheroids and exhibit chondrogenic phenotype. Similarly, osteogenesis was evidenced by the superior expression of bone-specific markers, including the early-stage marker RUNX2 and late-stage marker BSP, in osteogenic spheroids as compared to ADSC spheroids (Figure 4.2C1-D2).

H&E images show that cells in all groups were densely packed without any visible necrotic core (Figure 4.3A). Particularly, for osteogenic spheroids, higher cell density and ECM deposition were observed around the surface of spheroids. In contrast, chondrogenic spheroids demonstrated more homogeneous cell distribution of cells compared to their osteogenic counterparts. Osteogenic spheroids exhibited a strongly positive signal for Alizarin Red indicating the abundance of calcification, as compared to ADSC and chondrogenic spheroids (Figure 4.3B).

Toluidine Blue staining of chondrogenic spheroids showed violet color demonstrating higher level of sulfated glycosaminoglycan (sGAG) deposition while ADSC and osteogenic were negative (Figure 4.3C). In addition, a limited amount of collagen was observed in ADSC spheroids (Figure 4.3D). In contrast, a moderate level of collagen fibers was identified in osteogenic spheroids. Chondrogenic spheroids exhibited dense and thick collagen fibers, especially around the edge of the spheroids.

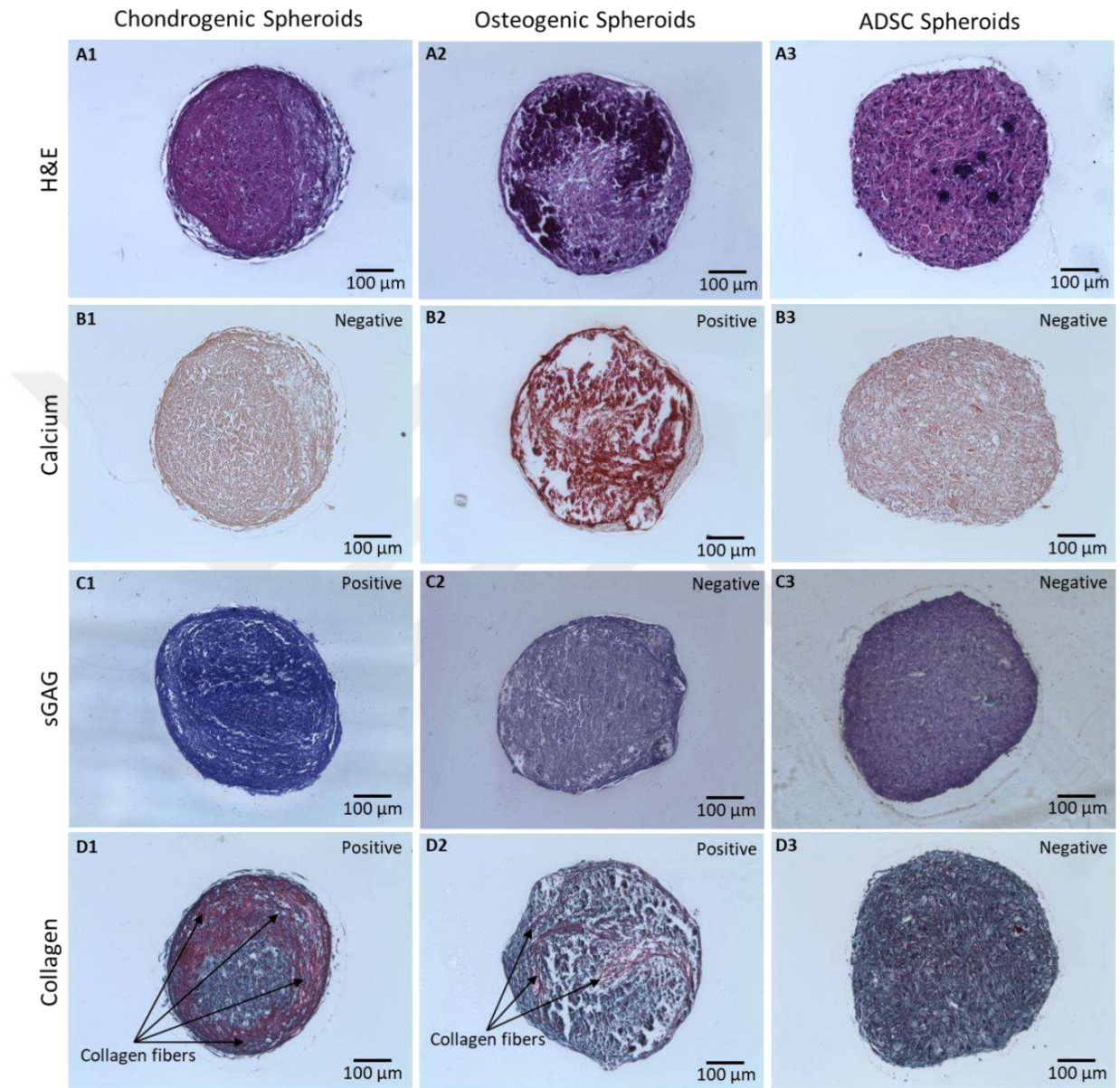


Figure 4.3: Histological staining of ADSC, osteogenic, and chondrogenic spheroids for (A) H&E, (B) Alizarin Red, (C) Toluidine Blue, and (D) Picrosirius Red/Fast Green.

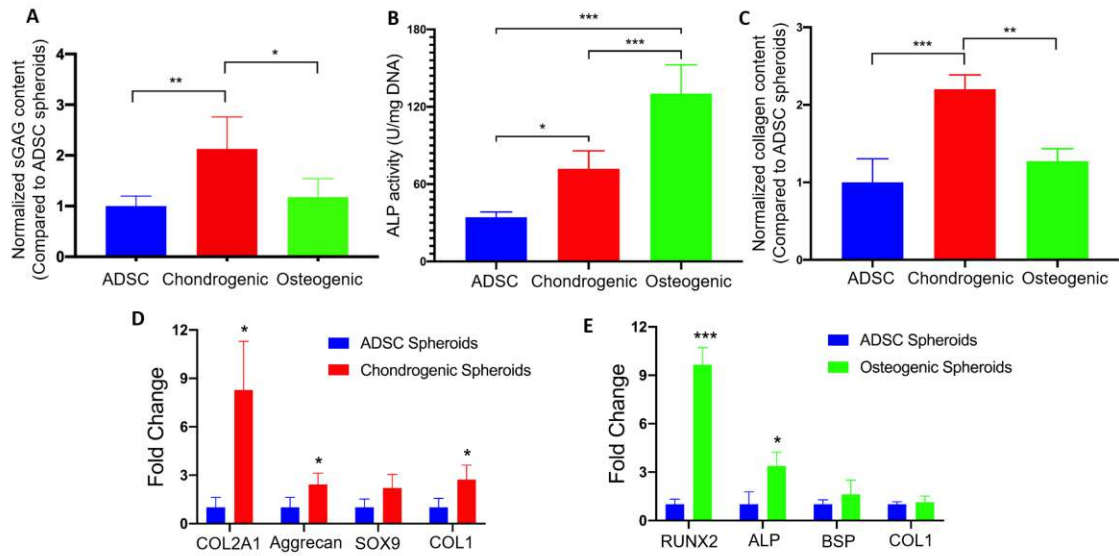


Figure 4.4: Quantification of protein and gene expression in different types of spheroids. (A) sGAG content measurement normalized to DNA amount and ADSC group ($n=3$). (B) ALP activity normalized to DNA amount ($n=3$). (C) Collagen deposition. RT-PCR analysis demonstrating the expression of (D) cartilage- and (E) bone-specific genes ($n=3$ for all analysis, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

The chondrogenic and osteogenic induction of ADSCs in the 3D spheroid culture was further confirmed quantitatively. Chondrogenic spheroids yielded significantly greater dsDNA normalized sGAG content as compared to ADSC and osteogenic spheroids (with ~2.1 and 1.8-fold increase, respectively, $p < 0.05$, Figure 4.4A). As regards to osteogenesis, the biochemical assessment showed that ALP activity of osteogenic spheroids was significantly superior with respect to both ADSC and chondrogenic spheroids with a ~3.8 and 1.8-fold increase, respectively ($p < 0.05$, Figure 4.4B). The collagen expression analysis revealed that chondrogenic and osteogenic spheroids possessed higher collagen matrix deposition than ADSC spheroids. Chondrogenic spheroids demonstrated a ~2.2-fold increase in collagen expression compared to

ADSC spheroids, while osteogenic spheroids showed only a ~ 1.3 -fold increase, which was consistent with the Picrosirius Red staining results.

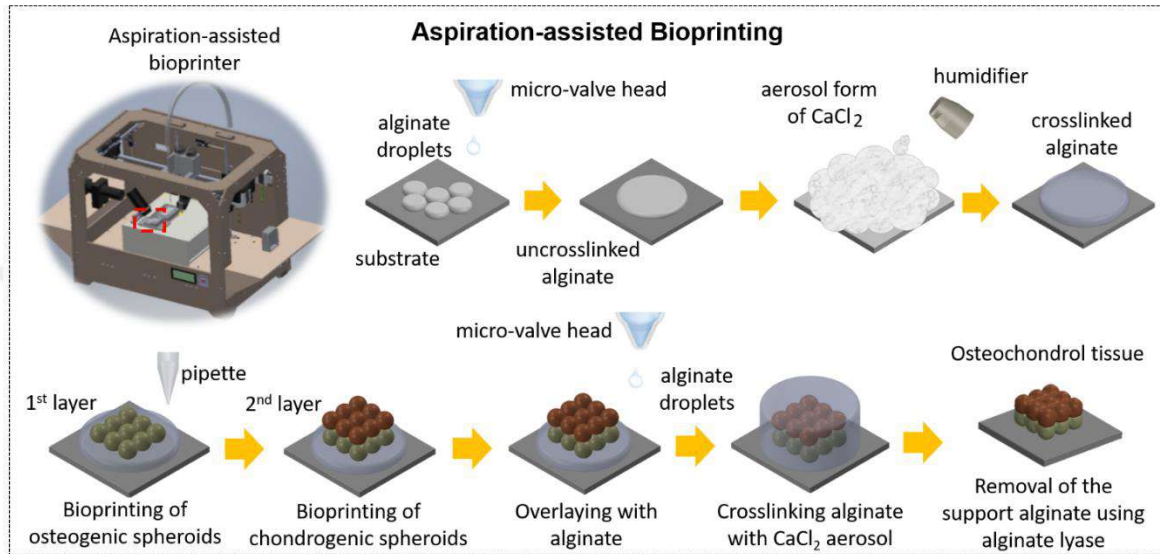


Figure 4.5: A schematic illustration showing the aspiration-assisted bioprinting (AAB) of the OC interface with chondrogenic and osteogenic zones.

In terms of cartilage-specific gene expression, chondrogenic spheroids had a significantly up-regulated expression of COL2A1, Aggrecan and COL1 (with a ~ 8.3 , 2.4 and 2.7-fold increase, respectively $p < 0.05$) compared to the ADSC group (Figure 4.4D). In addition, the gene expression in chondrogenic spheroids revealed a ~ 2.2 -fold increase with regard to SOX9, a transcription factor required for maintaining the chondrocyte phenotype during chondrogenesis, against the ADSC group. In terms of bone-specific gene expression, osteogenic spheroids exhibited significantly higher gene expression levels of RUNX2 and ALP (~ 9.6 and 3.4-fold increase, respectively, $p < 0.05$, Figure 4.4E), and at the same time, demonstrated greater expression of BSP and COL1 as compared to the ADSC group. These results demonstrate that ADSC spheroids were successfully

differentiated into chondrogenic and osteogenic lineages after a three-week induction of differentiation media in the 3D spheroid culture.

4.4.3 3D Bioprinting of the OC interface

In this study, a newly developed AAB process [106] was utilized to fabricate the OC interface. AAB enabled precise positioning of viscoelastic tissue spheroids in 3D, and when coupled with micro-valve bioprinting, it facilitated the self-assembly of these spheroids in a sacrificial alginate support. In the AAB process (Figure 4.5A), the first step was to pick a spheroid and then lift it and drag it rapidly outside the culture media by aspiration forces. After lifting spheroids from the cell media and transferring them to the bioprinting stage, the back pressure was released and the spheroids were partially submerged into the partially-crosslinked alginate support as going deeper could result in their breakage due to penetration of the pipette tip into the spheroid. When the nozzle moved up, the spheroid was deposited due to adherence between the spheroid and alginate. The procedure was repeated as many times as needed in order to build the OC interface. At the last step, bioprinted constructs were overlaid with alginate using micro-valve bioprinting and CaCl_2 vapor was then applied to crosslink alginate. During overlaying, alginate droplets were gently bioprinted to prevent the dislocation of deposited spheroids, where a positive back pressure of ~ 103 kPa was used for driving the flow of sodium alginate inside the tubing and a unipolar wave pulse with a dwell voltage (amplitude) of 5 V and a dwell time (valve opening duration) of 1,000 μs was utilized for actuating the micro-valve dispenser. After bioprinting of spheroids, the construct was maintained in the alginate support for a week to facilitate complete fusion of spheroids and alginate was then de-crosslinked leaving the assembled OC interface behind. In order to successfully bioprint of the OC interface, we utilized aspiration pressure of 95 and 74 mmHg for osteogenic and chondrogenic spheroids, respectively, as these pressure levels were sufficient to

transfer spheroids and did not induce major stress on them that could result in their complete aspiration, breakage, or substantial cell death.

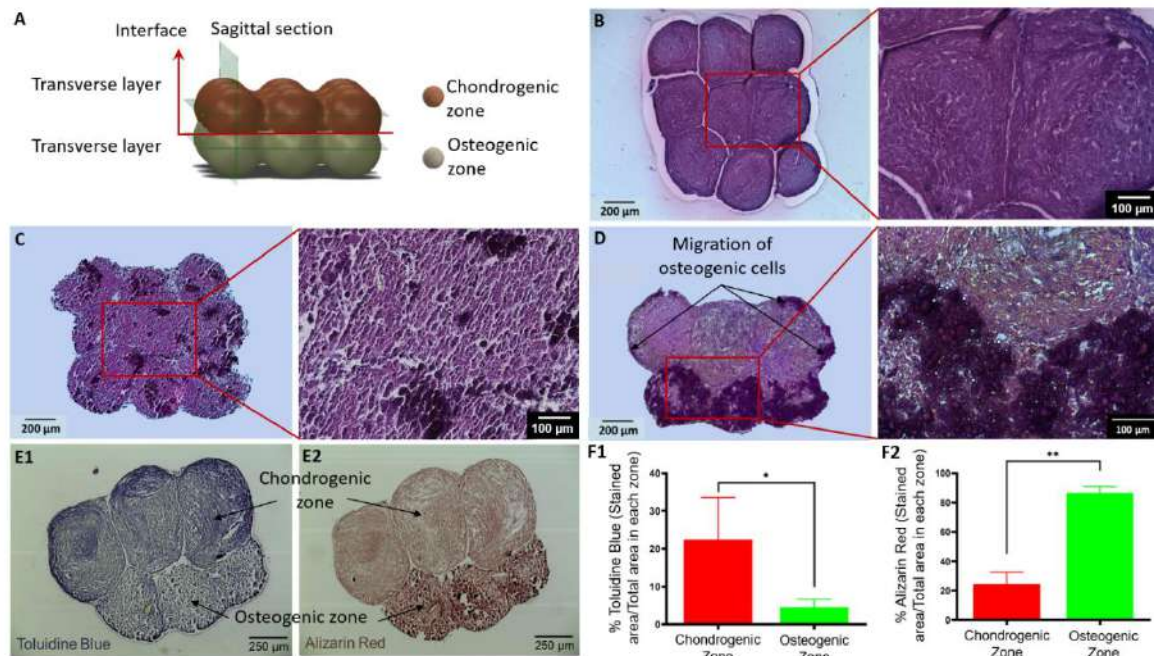


Figure 4.6: Bioprinting of osteochondral interface (A) A schematic diagram showing positions of histological sectioning in the construct. (B-D) H&E staining of the chondrogenic, and osteogenic zones, and the interface. Histomorphological characteristics of the bioprinted OC interface indicated by (E1) Toluidine Blue and (E2) Alizarin Red staining. Quantification of stained areas for Toluidine Blue (F1) and Alizarin Red (F2) in chondrogenic or osteogenic zones ($n=4$, $*p < 0.05$ and $**p < 0.01$).

The OC interface was bioprinted and then sectioned according to the model given in Figure 4.6A in order to visualize the chondrogenic and osteogenic zones and the interface. The cross-sections of chondrogenic and osteogenic zones in images of H&E staining exhibited compact

tissue with fused spheroids in a 3×3 arrangement (Figure 4.6B-D). Similar to the uniform morphology of individual spheroids, the chondrogenic zone also showed more uniform cellular and ECM distribution as compared to the osteogenic layer. Most importantly, chondrogenic and osteogenic zones fused completely with the absence of gaps in between (Figure 4.6D). In addition, both zones were apparently distinguishable with osteogenic zone showing darker purple color and chondrogenic zone displaying more even matrix, demonstrating the maintenance of their respective phenotypes after fusion.

In order to confirm the histomorphological characteristic of the OC interface, Toluidine Blue and Alizarin Red staining were performed. As expected, the chondrogenic zone was dyed with violet color showing expression of sGAG, when it compared to the pale staining on the osteogenic zone (Figure 4.6E1). The osteogenic zone exhibited ample deposition of calcium component, which was indicated by the dark orange color in Alizarin Red staining (Figure 4.6E2). Further quantification based on area measurement revealed that Toluidine Blue stained area in the chondrogenic zone (~22%) was significantly greater than that in the osteogenic zone (~5%, $p < 0.05$, Figure 4.6F1). On the contrary, Alizarin Red stained area in the osteogenic zone (~86%) outnumbered that in the chondrogenic zone (~24%, $p < 0.05$, Figure 4.6F2).

In this study, a double-layer OC interface was 3D biprinted using the AAB technique, which mimics the native counterpart that is composed of two main compartments, subchondral bone and articular cartilage [150]. The OC interface plays a critical role in maintaining the integration between bone and cartilage, and transfer the forces from the joint to subchondral bone [151]. The OC tissue exhibits a thickness of ~3 mm in adults, of which about 90% consists of articular cartilage, 5% calcified cartilage, and 5% the subchondral bone plate [152]. The chondrogenic and osteogenic zones in 3D biprinted tissues had a thickness of ~1 mm, which was in the same order of magnitude as the native OC interface.

Chondrocytes and osteoblasts are often used for *in vitro* studies of osteochondral repair [145]. However due to the small number of chondrocytes and osteoblasts found in the body, making up 2% of the cartilage volume and 4-6% of total resident bone cells, respectively, primary chondrocytes and osteoblasts may not be translational [153–155]. Particularly, chondrocytes are very difficult to isolate since their collagen matrix requires thorough digestion by collagenase, which might be harmful to cells [156]. Most importantly, although adult chondrocytes are capable of cell division in tissue culture, they exhibit telomere shortening and dysfunction after multiple passages, which is related to replicative senescence and senescent secretory phenotype [157]. Hence, an alternative and readily available cells is desired for the OC repair. In this study, ADSCs were used as a cell source, which are convenient to harvest as compared to mature cells and other adult stem cells.

In terms of surface tension that represents the mechanical properties of viscoelastic spheroids [106], the highest value was observed for osteogenic spheroids, which could be attributed to the mineralized surface compactness of spheroids. Chondrogenic and osteogenic spheroids exhibited more compact organization as compared to ADSC spheroids, which could be because of the abundant ECM production on the differentiated spheroids [158]. It has been well acknowledged that 3D culture is more sufficiently mimic the *in vivo* tissue formation with regard to the level of differentiation as compared to 2D culture [159]. Especially for chondrocytes, 3D culture prevented them from dedifferentiation and preserved their chondrogenic phenotype [160]. In addition, the formation of osteogenic spheroids using differentiated ADSCs or ADSCs undergoing osteogenic differentiation was not quite possible in our initial experiments as cells did not tend to assemble into spherical aggregates (data not shown here). Therefore, we preferred to differentiate the cells in the form of spheroids. As hypoxia usually occurs in cell aggregates with diameter over 500 μm , we formed ADSCs spheroids with a diameter of $\sim 500 \mu\text{m}$ [80]. The successful differentiation of

ADSCs in the form of 3D spheroids were proven by immunostaining, in which COL II and Aggrecan for chondrogenesis and BSP and RUNX2 for osteogenesis were present throughout the spheroids. Similar results in histological staining were also observed, with calcium component presented in osteogenic spheroids, sGAG expressed in chondrogenic spheroids, and collagen fibers observed in both. More uniform cell distribution was observed in chondrogenic spheroids compared to other two groups due to the anaerobic nature of chondrocytes as consistent with our previous findings [80]. The presence of live cells and abundant amount of ECM in the core of spheroids indicated the absence of hypoxic core and the diffusion of induction media in the spheroids. In addition, the quantitative results, including biochemistry and RT-qPCR, further confirmed that differentiated phenotypes were maintained.

In the context of OC tissue engineering, biphasic or triphasic scaffolds have been developed, displaying either discrete or continuous gradients [145,153,161]. Besides naturally-derived materials including collagen type I and II, hydroxyapatite, GAG, HA, gelatin and chondroitin sulfate, other synthetic materials, such as poly(lactic-co-glycolic acid), polycaprolactone (PCL) and poly(ester-urethane), have been widely used to resemble the bone and cartilage compartments [21,162–170]. With the advances in bioprinting, investigations of bioprinted OC constructs have been reported recently. Levato *et al.* [46] utilized microcarriers of polylactic acid (PLA) to load mesenchymal stem cells (MSCs), followed by encapsulating cell-laden microcarriers in GelMA-gellan gum (GG). The cartilage zone was bioprinted with GelMA-GG and the bone zone was represented by GelMA-GG with encapsulated microcarriers. In 2017, Levato *et al.* [171] also reported another strategy for fabrication of zonally-stratified arrangement using a recently identified articular cartilage-resident chondroprogenitor cells (ACPCs). Targeting at the reconstruction of the OC tissue, Shim *et al.* [172] used PCL as a supporting frame for extrusion of hydrogel-laden human MSCs. The construct (5 mm in height) was divided into two

distinct zones including the subchondral bone 3D printed using atelocollagen with MSCs and recombinant human bone morphogenic protein (rhBMP)-2, and the superficial cartilage comprised of Cucurbit[6]uril-conjugated hyaluronic acid (CB[6]-HA), 1,6-diaminohexane-conjugated HA (DAH-HA), MSCs and transforming growth factor beta (TGF- β). Different from the above-mentioned scaffold-based strategies, the OC interface presented in this work was successfully reconstituted using a scaffold-free strategy, which was comprised of single layer of chondrogenic spheroids and another layer of osteogenic spheroids in a 3×3 matrix arrangement. Cell density in scaffold-based methods is usually in the order of 10^6 cells/mL [173–175], while it can reach 10^7 - 10^8 cells/mL equivalently in scaffold-free tissues due to the absence of scaffolding hydrogels [80]. The spheroids were bioprinted in a pick-and-place fashion using the AAB bioprinter, to accurately position them ensuring their successful fusion to form a well-defined interface arrangement. Since the spheroids were merely comprised of cellular components, considerations in scaffold-based methods, such as the choice of biomaterials, material degradation and cell-matrix interactions, were no longer concerns. Interestingly, osteogenic cells were observed at the edge of the chondrogenic zone in some of the bioprinted OC interface samples (Figure 5E). This observation might be attributed to the migration of cells from the bone zone to the cartilage zone due to the use of chondrogenic and osteogenic media with 1:1 ratio, resulting to the calcification of chondrogenic zone. Such interaction could probably be a model for investigation for calcification of articular cartilage, which is commonly observed during aging or osteoarthritis [176]. In addition, the determination of bioactivity for each zone is quite challenging in the bioprinted OC interface. Therefore, we performed bioactivity assessment of single spheroids before bioprinting as they could be representative for the individual zones in the bioprinted OC interface.

Since healthy human articular chondrocytes are difficult to obtain because of ethical issues, *in vitro* and *in vivo* models have been investigated to explain the molecular mechanisms in joint

pathophysiology [129]. It has been suggested that OA results from a failure of the entire joint and early changes can be detected both in the cartilage and subchondral bone. In addition, interaction between bone and cartilage cells plays a key role in driving OA pathological processes, which was evidenced by the co-culture of osteoblasts and chondrocytes isolated from the OA subchondral bone and cartilage [177]. The bioprinted OC interface in this study contained osteogenic and chondrogenic zones with high cell density enabling close cell-cell interactions and histomorphologically-relevant of 3D interface arrangement, which offers great potential of the presented OC interface for use in disease modeling and drug testing.

4.5 Conclusion

Herein, it has been the first time that an OC interface was bioprinted using a scaffold-free methodology. After chondrogenesis and osteogenesis in a 3D spheroid culture, differentiated ADSC spheroids exhibited cartilage- and bone-specific gene and protein expressions, which were then precisely bioprinted to generate a double-layer arrangement with the top and bottom zone imitating the cartilage and bone, respectively. The bioprinted OC interface exhibited a tightly-integrated organization between chondrogenic and osteogenic zones, with the preservation of their histomorphological characteristic in terms of tissue-specific protein deposition. This study has opened a new avenue for the biofabrication of the OC interface model without the use of polymeric scaffolds, which has great potential to be utilized not only in regenerative medicine and tissue engineering but also in drug testing and disease modeling.

Chapter 5

Conclusion and Future Work

5.1 Summary and Future work

Taken together, we demonstrated the ability of AAB approach to bioprint a wide range of biologics, which can allow one to explore a myriad of avenues such as tissue-tissue interactions, tissue-material interactions and angiogenesis in the rapidly growing field of tissue engineering and regenerative medicine. Also, AAB provides an outstanding platform for the bottom-up assembly of various spheroids having diverse applications in tissue engineering. Precise positioning of spheroids can also be critical for applications such as building organ-on-a-chip devices or micro-physiological systems where the proximity of spheroids to each other or a perfusion channel can be crucial for the viability and function of spheroids as well as the robustness of the developed system. Organoids or spheroids are currently loaded using manual approaches, which may reduce the repeatability of system outcome measurements (such as measured insulin in circulating perfusion media in a pancreas-on-a-chip model). To the best of our knowledge, the presented approach is the first bioprinting method that allows high-precision bioprinting of spheroids in both scaffold-free and scaffold-based manner. Additionally, the underlying mechanism explaining the physical behavior of viscoelastic spheroids and their interactions with physical governing forces during aspiration, lifting and bioprinting has not been documented in literature till date, and with our custom made bioprinter, we were able to exploit it for new applications enumerated throughout this paper. In particular, mediating angiogenic sprouting and its directionality through precise positioning of spheroids in a gel medium and the impact of paracrine signaling in response to such positioning has not been presented elsewhere. Besides, the role of mid-term osteogenic induction to stem cell-based spheroids (prior to bioprinting) on the mineralization and assembly behavior has

not been reported before. Therefore, the presented bioprinting approach can be employed in detailed understanding of fundamental biology as well as studying angiogenesis, histogenesis and organogenesis.

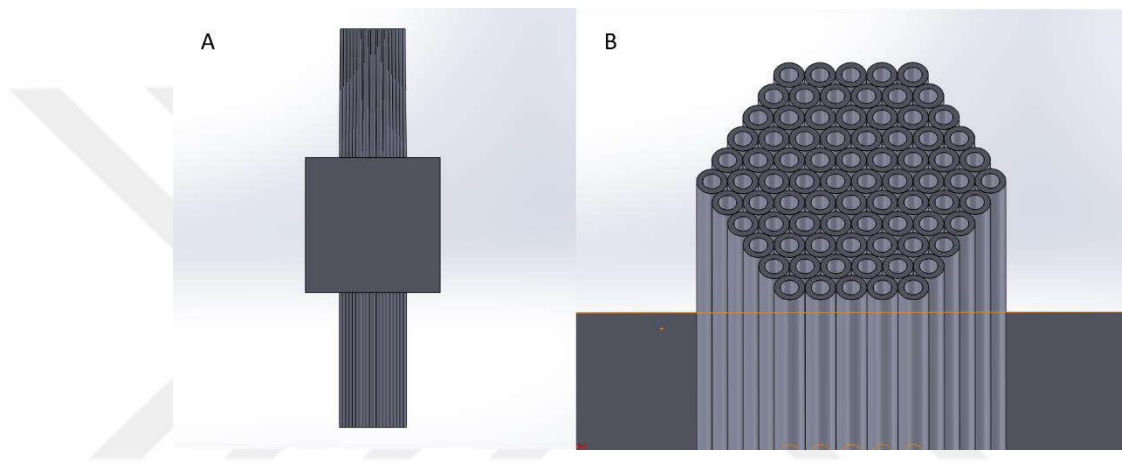


Figure 5.1 Schematic illustrations of needle array system for high-throughput spheroid bioprinting

The presented approach paves the way for bioprinting several tissue types and a wide range of spheroids in 2D and 3D in gel substrates with multiple applications presented throughout this dissertation, including (i) the development of physiologically relevant culture environments to demonstrate the collective angiogenic sprouting behavior of spheroids in a scaffold medium (ii) fabrication of osteogenic tissues to decode the role of midterm osteogenic induction of stem cell-based spheroids (before bioprinting) on the mineralization and assembly behavior in a scaffold-free environment, and (iii). In addition to spheroids, other living cells and tissue building blocks, including electrocytes from electric eel and tissue strands, can be bioprinted for a wide variety of applications, such as but not limited to tissue engineering, regenerative medicine, drug testing and pharmaceuticals, disease modeling, microphysiological systems, biophysics, and biocomputing.

In near future, our AAB platform could be advanced to be able to bioprint an array of spheroids simultaneously for scale up tissue engineering (Figure 5.1). In this regards, digitally-controlled nozzle array platform will be fabricated using micro-tubes and each pipeline can be controlled with individual solenoid valves, where spheroids can be picked and bioprinted in a high-throughput manner. With a nozzle array of 10x10, 100 of spheroids can be bioprinted in 30 seconds with a throughput of 200 spheroids/min. It will enable us to bioprint scalable tissues and we will establish the interactions between bioprinting parameters and tissue self-assembly.

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Selected Journal Publications

1. **B. Ayan**, N.D. Heo, Z. Zhang, M. Dey, A. Povilianskas, C. Drapaca, I.T. Ozbolat, Aspiration-assisted bioprinting for precise positioning of biologics, *Science Advances* 6.10 (2020) eaaw5111.
2. **B. Ayan**, Y. Wu, K. Vengadeshprabhu, F. Kamal, I.T. Ozbolat, Scaffold-free bioprinting of osteochondral interface, *Scientific Reports* (Under Revision).
3. N.D. Heo, **B. Ayan**, M. Dey, D. Banerjee B. Wee, G. Lewis, I.T. Ozbolat., 3D bioprinting of co-cultured osteogenic spheroids for bone tissue fabrication (Submitted).
4. N.D. Heo, M. A. Alioglu, Y. Wu, V. Ozbolat, **B. Ayan**, M. Dey, I.T. Ozbolat, 3D Bioprinting of carbonyldiimidazole-modified gelatin into microparticle-suspended oxidized alginate for fabrication of complex-shaped tissue constructs, *ACS Applied Materials & Interfaces* 12,18 (2020).
5. P. Datta, **B. Ayan**, I.T. Ozbolat, Bioprinting for vascular and vascularized tissue biofabrication, *Acta Biomaterialia* (2017).
6. W. Peng, P. Datta, **B. Ayan**, V. Ozbolat, D. Sosnoski, I.T. Ozbolat, 3D bioprinting for drug discovery and development in pharmaceuticals., *Acta Biomaterialia* 57 (2017) 26–46.
7. P. Datta, V. Ozbolat, **B. Ayan**, A. Dhawan, I.T. Ozbolat, Bone tissue bioprinting for craniofacial reconstruction, *Biotechnol. Bioeng.* 114 (2017) 2424–2431.
8. V. Ozbolat, M. Dey, **B. Ayan**, A. Povilianskas, M.C. Demirel, I.T. Ozbolat, 3D Printing of PDMS Improves Its Mechanical and Cell Adhesion Properties, *ACS Biomater. Sci. Eng.* (2018) acsbomaterials.7b00646.
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11. V. Ozbolat, M. Dey, **B. Ayan**, I.T. Ozbolat, Extrusion-based printing of sacrificial Carbopol ink for fabrication of microfluidic devices, *Biofabrication*. 11 (2019) 034101.
12. A.N. Leberfinger, M. Hospodiuk, A. Pena-Francesch, **B. Ayan**, V. Ozbolat, S. V. Koduru, I.T. Ozbolat, M.C. Demirel, D.J. Ravnicek, Squid ring teeth-coated mesh improves abdominal wall repair, *Plast. Reconstr. Surg. – Glob. Open.* (2018).