

Video Article

Viability of Bioprinted Cellular Constructs Using a Three Dispenser Cartesian Printer

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Abstract

Tissue engineering has centralized its focus on the construction of replacements for non-functional or damaged tissue. The utilization of three-dimensional bioprinting in tissue engineering has generated new methods for the printing of cells and matrix to fabricate biomimetic tissue constructs. The solid freeform fabrication (SFF) method developed for three-dimensional bioprinting uses an additive manufacturing approach by depositing droplets of cells and hydrogels in a layer-by-layer fashion. Bioprinting fabrication is dependent on the specific placement of biological materials into three-dimensional architectures, and the printed constructs should closely mimic the complex organization of cells and extracellular matrices in native tissue. This paper highlights the use of the Palmetto Printer, a Cartesian bioprinter, as well as the process of producing spatially organized, viable constructs while simultaneously allowing control of environmental factors. This methodology utilizes computer-aided design and computer-aided manufacturing to produce these specific and complex geometries. Finally, this approach allows for the reproducible production of fabricated constructs optimized by controllable printing parameters.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53156/>

Introduction

Tissue engineering uses the principles of biology and engineering in the development of functional substitutes to maintain, restore, or enhance native tissue and . The capability of generating three-dimensional biomimetic constructs on demand would facilitate scientific and technological advances in tissue engineering as well as in cell-based sensors, drug/toxicity screening, tissue or tumor models, and other . The three-dimensional organization of tissue-engineered constructs is a fundamental component of the fabrication method because it must closely mimic the highly organized interaction of cells and extracellular matrix in native tissue.

Biodegradable and shape-forming three-dimensional scaffolds are critical factors in generating novel tissue constructs because cells migrate to form a two-dimensional layer of cells, but lack the ability to grow in favored three-dimensional . The scaffold serves as a temporary foundation for cell attachment and proliferation, so it must be constructed from materials with controllable porosity and biodegradability, and sufficient mechanical integrity. The scaffold materials should not be cytotoxic or create an adverse response from the host. Hydrogels have been commonly used in tissue engineering techniques, and due to their hydrophilicity, hydrogels permit fluid and gas exchange throughout the structure. By combining different hydrogels, the synthesized hydrogel's properties are modifiable to meet distinct application requirements.

The conventional tissue engineering approach involves the creation of acellular porous sacrificial scaffolds that are seeded with cells post-fabrication. Many techniques have been employed, such as fiber bonding, solvent casting, and melt molding, but proved to be minimally successful for tissue engineering applications. Fiber bonding methods allow fibers to be aligned in specific shapes, but they are only capable of producing very thin scaffold. Solvent casting methods produced highly porous constructs, however the largest produced membrane was only 3-mm thick. Therefore, creating three-dimensional constructs is not feasible using these techniques. Melt molding techniques proved successful in producing three-dimensional scaffolds, but such high temperatures are required that biological materials cannot be incorporated during the production process. Scaffolds seeded post-fabrication are limited in their ability to meet the requirements of tissue engineering to produce three-dimensional scaffolds with pre-defined or controllable microstructures and . Another major issue with solid scaffold seeding technologies is the deficiency of vascularization and poor mechanical .

Bioprinting has since been extended to three dimensions through the use of nontoxic, biodegradable, thermo-reversible gels to overcome the disadvantages of conventional . A few of the solid freeform fabrication techniques currently being employed are laser-assisted bioprinting and inkjet printing. Laser-assisted bioprinting techniques use a pulsed laser source, a target plate, and a receiving substrate to generate three-dimensional . However, this technique is limited due to low throughput, low cell viability, and can only produce limited arrangements of fabricated

structures because only photocrosslinkable prepolymers can be used to form a crosslinked hydrogel. Inkjet printing was developed as a non-contact methodology that reproduces digital image data on a substrate by depositing picoliter ink. However, inkjet printing does not produce a high-resolution construct, constructs experience rapid protein denaturation, and many of the cells are lysed during the deposition.

Currently, new additive manufacturing bioprinting methods have been developed. In these systems cells, proteins, growth factors, and biomimetic hydrogels are typically integrated into matrix materials during the fabrication process and concurrently deposited using computer-controlled actuators to generate three-dimensional scaffold-based cell-laden constructs that closely mimic the microarchitecture of native. The cell-laden hydrogels constitute the bioink, which can be heterogeneous, consisting of multiple cell types, or homogeneous. Additive manufacturing systems deposit bioink drop-by-drop or layer-by-layer via disposable syringes and tips onto a computer-controlled stage capable of moving in the x, y, and z directions. Through computer software, the architecture of printed scaffolds can be easily manipulated depending on requirements of the application. Unlike conventional techniques, three-dimensional medical technologies (magnetic resonance imaging, computer tomography) can be incorporated into the designs, generating patient-specific construct. These methods also allow the possibility of producing vascularized replacements because constructs are produced with a higher local cell density, allowing cell-cell interactions and improving the likelihood of post-implantation survival.

The Palmetto Printer is a custom built three-dimensional multi-dispenser system that uses programmable robotic manufacturing methods to generate three-dimensional heterogeneous tissue constructs (**Figure 1**). It allows the use of a plurality of materials in unique combinations to produce heterogeneous structures. The initialization of the bioprinter is one of the most important steps in bioprinting because it allows you to set a variety of parameters to optimize the printability of the bioprinted constructs.

The bioprinter comprises a batch type process with startup, operation and shutdown sequences controlled by a programmable logic controller (PLC), which the user operates through an interactive touch screen control panel (**Figure 1, A**). To prevent contamination of biological materials the bioprinter is enclosed in a positively-pressured poly(methyl methacrylate) (PMMA) chamber with a high-efficiency particulate arrestance (HEPA)-filtered air circulation system (**Figure 1, B,C**). The interior of the printer can be sterilized using the built-in ultraviolet light sources (**Figure 1, D**). The central component of the bioprinter is a fully programmable positioning robot that can reproducibly place a dispenser tip with an accuracy of 10 micrometers (**Figure 1, E**). There are three dispensers, which are able to deposit volumes as small as 230 nL using a rotary screw (**Figure 1, F**). They are independently programmable using separate computers that govern printing parameters for each dispenser (**Figure 1, G**). Rotary-screw dispensing utilizes the rotation of a motor-driven screw to move bioink down a syringe and out of the syringe tip. These dispensers are mounted onto a pneumatically controlled Tool Nest (**Figure 2A, B**), allowing the robot to switch dispenser mounted onto the Z-axis robotic arm under programmed control (**Figure 1, H**).

The XYZ robot receives printing instructions from a computer running design software (**Figure 1, I**). Each program contains dispensing locations, calibration routines, and dispenser-changing protocols. The design of generated constructs primarily consists of the XYZ coordinates where each dispenser will deposit material. The bioprinter comprises two optical light sensors (**Figure 2C**) that determine the XYZ coordinates of the syringe tip end. These sensors send coordinate information to the robot, which uses these to calculate positions of the dispenser tip ends. There is an additional displacement laser (**Figure 2D**) that projects a 633 nm diode red laser beam of spot size 30 x 100 micrometers to measure distance with an accuracy of 0.1 micrometers. When the beam is highly focused the robot determines the Z distance of the printing surface. This measurement, and the optical light sensors measurement of the tip end in Z, allows calculation of accurate Z coordinates used to place the dispenser tip in relation to the printing surface. The dispenser tips move laterally and vertically through the X-axis oriented optical light sensor to find the Y and Z centers, and laterally through a Y-axis sensor to find the center of the X-axis. The printing surface is mapped using the formula for a flat plane in xyz space: $ax + by + cz = d$ to determine where the surface is relative to the position of the dispensing tip end. The printer stage (**Figure 1, J**) holds a sample Petri dish up to 80 mm in diameter and uses a recirculating water bath to maintain the set temperature (**Figure 1, K**). Stage temperature can be set within a range of -20 and remains stable within. There is a USB camera mounted onto the robot Z-arm to provide a magnified view of the dispensing tip during the printing process (**Figure 1, L**). There is a second camera mounted towards the top of the chamber interior that provides a complete view of the bioprinter during the printing process (**Figure 1, L**).

A computer-aided design drawing software determines the deposition pattern and permits the user to generate incrementally spaced droplets and complex structures (**Figure 3**). Three-dimensional pathways can be manually coded into the printer-compatible design software or imported from a separate computer-aided design drawing software (**Figure 4, Table 1**). The printer-compatible software allows variations of printing parameters such as the deposition method (single droplet deposition or continuous pathway deposition), three-dimensional geometry of the pathways, deposition rate, distance between the syringe tip end and substrate printing surface, the amount of time to deposit an individual drop, and the height and speed the syringe is lifted between deposition of the drops. Each program contains XYZ dispensing locations, tip calibration routines, and dispenser-changing protocols to provide a sterile environment, without operator intervention, during printing. The programmable logic controller (PLC) of the robot receives instructions from the computer running the design software and controls the timing of events from the external controllers (e.g., the dispensers). To do this, the PLC uses a looping mechanism to control the dispensers, robotic positioning device, and environmental factors.

Three-dimensional direct-write bioprinting utilizing a rotary-screw, liquid-dispensing system allows the process of depositing cells to be more efficient, accurate, and easier than previous methods. This study shows the custom built bioprinter is capable of generating cell-laden hydrogel constructs with high cell viability.

Protocol

1. Preparation of Gelatin Containing Substrate for Three-Dimensional Bioprinting of Alginate Hydrogels

1. Prepare the calcium/gelatin substrate following the calcium/gelatin substrate method described by Pataky *et al*¹¹ to avoid reduced viability associated with high content. The calcium/gelatin substrate method is listed below.

1. Combine calcium chloride dehydrate (1.5 wt%), sodium chloride (0.9 wt%), and porcine gelatin (2 wt%) in distilled water and boil for 2 min to create a 100 mM gelatin solution.
2. Pour 5 ml of the gelatin/calcium solution into 100 mm standard petri dishes, swirl the solution around to make an even coating on the surface, and place on a flat surface in the fridge to gel O/N (allow to gel at least 8 hr before use).
3. To increase the opacity of the substrate surface, add titanium dioxide (0.3 wt%) to the gelatin/CaCl₂ solution. Stir for 10 min. Autoclave the gelatin/TiO₂ solution on the liquids cycle for 30 min to sterilize it.
 1. Add 3 ml of the gelatin/TiO₂ solution to the surface of the previously prepared gelatin plates. Swirl the mixture to ensure it is spread evenly across the surface. Allow to gel in the 4 °C fridge O/N (allow to gel at least 8 hr before use). The substrates must be used within 3 days.

2. Alginate Oxidation

1. Oxidize the sodium alginate bioink following the method for partially oxidized alginate by Bouhadir *et al*³⁰ described below.
 1. To make a 5% oxidized alginate solution, dissolve 1 g of sodium alginate in 100 ml of distilled water. Add an aqueous solution of sodium periodate (0.25 M, 0.25 mmol), the oxidizing agent, to produce a 5% oxidation solution. Stir for 19 hr at RT. Add 40 ml ethylene glycol to the solution after 24 hr to end the reaction.
 2. Dissolve 2.5 g of sodium chloride in the solution. Add an excess amount of ethyl alcohol (2:1 ratio) to precipitate the oxidized alginates. Centrifuge the solution at 1,000 x g to collect the precipitates and re-dissolve them in distilled water. Repeat the ethanol wash.
 3. Freeze-dry the oxidized alginate pellets and store at -20 °C until ready for use.
2. Determine the degree of oxidation by measuring the percentage of sodium periodate consumed before being terminated by the ethylene glycol.
 1. Prepare a potassium iodide solution (20% w/v, pH 7.0 sodium phosphate buffer) and a thyodene solution (10% w/v, pH 7.0 sodium phosphate buffer). Mix the two solutions with the oxidized alginate at RT.
 2. Gradually drop the reacted alginate and sodium periodate solution into the mixture of potassium iodide and theodyne solutions. Measure the absorbance of the mixture spectrophotometrically at 426 nm. When it has reached a maximum, record the used volume of alginate and sodium periodate solution as V₁.
 3. The reaction is $IO_4^- + 2I^- = I_2 + IO_3^-$. The amount of unreacted sodium periodate is $20\% \left(\frac{w}{v}\right) \times 10 \text{ mL} \times 100 \frac{\text{mL}}{V_1}$
 4. Subtract the amount of unreacted sodium periodate from the original concentration to determine the amount of sodium periodate consumed. Using the previous formula, determine the final oxidation degree of the alginate.

3. Alginate Peptide Conjugation

1. Conjugate ligands with an exposed arginine-glycine-aspartate sequence (peptide) into the previously prepared oxidized alginate by following the RGD-Alginate conjugation method by Rowley *et al*³¹ described below to promote cell attachment and spreading.
2. Use aqueous carbodiimide chemistry with G₄RGDSPto conjugate³¹.
3. Dissolve 1 g of 5% oxidized alginate in a 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH = 4. Add 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC, 0.54 mmol) and N-Hydroxysuccinimide (NHS, 0.27 mmol) at 2:1 ratio to form amide intermediate.
4. Add 0.28 mmol peptide, coupling to the backbone of the alginate polymer via the terminal amine. Stir at RT O/N.
5. Stop the coupling reaction by adding 2.5 g sodium chloride to the solution. Add an excess amount of ethyl alcohol (2:1 ratio) to precipitate the oxidized alginates. Centrifuge the mixture at 4,000 x g for 5 min to collect the precipitates. Aspirate the media in the cell culture hood and re-dissolve the precipitates in distilled water. Repeat the ethanol wash.
6. Freeze-dry the precipitates until it becomes completely dried (will appear as a white powdery substance) and store in the -20 °C fridge for later use.

4. Human Adipose Tissue Stromal Cells (hADSC's) Cell Culture

1. Culture human adipose tissue stromal cells (hADSC's) in 75 cm treated cell culture flasks (T75 flasks), covered with 15 ml low glucose DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin, 1% glutamine, and 1% antimycin. Change the media, in the cell culture hood, every two days until they have reached confluency (80-90%).
2. Once confluent, transfer the T75 flasks to the cell culture hood and suspend the hADSC's using the trypsin enzyme digestion method.
 1. In the hood, aspirate all of the cell culture media off of the cells. Rinse with 5 ml of Dulbecco's Phosphate-Buffered Saline with calcium and magnesium (DPBS ++). Aspirate the DPBS++ off of the cells.
 2. While in the hood, make a solution of trypsin and DPBS++ by mixing 1 ml trypsin and 4 ml DPBS++. Each flask requires 5 ml of the solution, so make the appropriate volume for the number of confluent flasks. Add 5 ml of the trypsin/DPBS++ to each flask and put them in the incubator for 2 min.
 3. After 2 min, remove the flasks and lightly tap the sides of them to loosen the cells from the bottoms. Look at each flask under a microscope to ensure the cells are suspended. Place the flasks back in the cell culture hood and add 3 ml of appropriate cell culture media to each flask. This ends the trypsin reaction.
 4. Transfer the cell-laden media from each flask and put in a 50 ml conical. Centrifuge them at 1,000 x g for 5 min. The cells should appear as a little white pellet in the bottom of the conical. Transfer back to the cell culture hood and aspirate the media. Resuspend the cells in 2 ml of cell culture media.
 5. Count the cells using a hemocytometer under the microscope. Once the cells have been counted, in the culture hood, aliquot the amount of media containing ~1.3 million cells and transfer to a 15 ml conical. Centrifuge the 15 ml conical containing the cells again for 5 min at 1,000 x g.

6. In the culture hood, reseed the remaining cells in multiple T-75 flasks, adding a concentration of ~350,000 cells to each flask. Add 15 ml of DMEM media and return to the incubator until confluent again.
7. Once the centrifuge cycle is complete, return the 15 ml conical to the cell culture. Aspirate the media from the cell pellet, and resuspend the cells in aqueous alginate solution at a concentration of 1.3 million cells per milliliter of bioink, terteriating the solution often so there is a homogeneous distribution of cells throughout the bioink. Load the cell-laden solution into a sterile printer-compatible 3 ml syringe and screw on the sterile 22 G plastic tip.

5. Bioprinter Setup

1. Turn on the bioprinter, each of the dispenser computers, and the recirculating water bath.
 1. Manually set the recirculating water bath temperature to for the gelation mechanism.
 2. Manually set printing parameters for each dispenser on the correlating dispenser computer. Set the dispense volume to 230 nl, number of backsteps to 0, and the dispense rate to 10 μ l –sec.
2. Open the design software and the program for viewing the USB camera's display on the computer.
 1. Using the software, manually enter the coordinates for a 5 x 5 dot array with 2.4 mm spacing between drops.
 2. Set the printing parameters to be: distance between tip end and substrate surface = 0.1 mm; height syringe is lifted between depositions = 20 mm; the amount of time per deposition = 1 sec.
 3. Save the program and send it to the robot.
3. Place the gelatin/TiO₂-containing Petri dish on the 4 °C printer stage. Close and lock the chamber door.
4. Use the PLC to initialize the ultraviolet light sources, and sterilize the chamber for 90 sec.
5. Once sterilization is complete, open the chamber and load the syringe containing hADSC's suspended in alginate into Gun 1. Close and lock the chamber door.
6. Use the PLC to turn on the fan system, wait 30 sec for equilibrium internal pressure.
7. On the computer, run the program containing the geometrical pathway and printing parameters.
8. Throughout the printing process, watch the USB camera's display on the computer to confirm accurate and uniform printing.
9. Once printing has finished, allow the constructs to gel for 40 min.

6. Cell Viability Assessment

1. Cover the constructs that are not going to be imaged immediately post-printing in DMEM and store in the incubator until time of imaging.
2. To quantify the viability of the constructs, stain them using a fluorescent-based viability/cytotoxicity assay, and image using confocal microscopy.
 1. Following the kit instructions, prepare a staining solution containing calcein AM and ethidium homodimer-1. To make 10 ml of staining solution, add 20 μ l of the ethidium homodimer-1 and 5 μ l of the calcein am to 10 ml of sterile, tissue culture-grade Dulbecco's Phosphate-Buffered Saline (+magnesium, +calcium; DPBS++).
 2. Immerse the bioprinted constructs in the stain solution for 15 min in the dark.
 3. Image the stained constructs using a confocal microscope system at days 0 and 8. Take multiple pictures of each bioprinted construct, using Z-stack parameters of 30 optical slices over a 300 μ m depth, and manually count the cells. If cells appear yellow or green count them as alive, and if red, count them as dead.
3. Calculate the cell viability percentage as the number of live cells divided by the total number of cells in the construct; Cell Viability = number of live cells (green+yellow)/ number of total cells (green+yellow+red) x 100%.
4. Calculate the amount of cell proliferation for each sample as the cell number of day 8 divided by the cell number on day 0; Cell Proliferation = live cell count on day 8/ live cell count on day 0 x 100%.

7. RGD Peptide Conjugation Analysis

1. To analyze the success of RGD peptide conjugation on the alginate, compare RGD-conjugated alginate and non-conjugated alginate. To do this, image the printed constructs using (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) (DAPI) and phalloidin stains.
 1. Make the phalloidins working solution by diluting 5 μ l of the methanolic stock solution with 200 μ l of DPBS++. Store at -20 °C until use.
 2. Make a 300 μ M stock solution of the DAPI stain following the equation: $(0.10509 \text{ g/L}) / (350.3 \text{ g/mol}) = 3 \times 10^{-4} \text{ M} = 0.0003 \text{ M} = 0.300 \text{ mM} = 300 \mu\text{M}$. Make the DAPI working solution by diluting the stock solution 1:100 in DPBS++ to obtain 3 μ M solution. Store at -20 °C until use.
2. Completely submerge the sample in 4% paraformaldehyde. Incubate for 1 hr at RT. Wash three times with DPBS++, allowing the solution to sit for 5 min each wash. Transfer the gel sample from the well to a glass slide, flipping the gel over in the process. Immerse the gel in 0.1% Triton X-100 (0.1 g/ 100 ml) in DPBS++ for 10 min. Wash three times with DPBS++, allowing 5 min for each wash.
3. Stain the printed constructs with phalloidin by immersing them in the working solution. Cover with foil and incubate for 4 hr. Remove the phalloidin stain and wash three times with DPBS++. The first wash should be fast, the latter washes should sit for 5 min each.
4. Stain the printed constructs with DAPI by immersing them in the DAPI working solution. Cover with foil and incubate at RT for 30 min. Wash three times with DPBS++, allowing each wash to sit for 5 min. Observe and image the samples on a confocal microscope system.

Representative Results

The results demonstrate the bioprinter is capable of depositing cell-laden hydrogels in specific three-dimensional locations accurately and consistently using computer-aided software. These softwares determine the placement of each droplet and control many of the parameters for dispensing (**Figure 3,4**). The repeatability of the bioprinter to appropriately deposit biomaterials is fundamental to its success in tissue engineering applications.

Cell viability, one of the requirements of a successful bioprinting technique, was analyzed 1 hr and 8 days post-printing. High cell viability is essential for fabricating biomimetic constructs and is a direct representation of an adequate bioink. RGD peptide conjugation improves cell viability over extended periods of time by promoting cell spreading. Fluorescent microscopy was used to quantify cell viability in constructs after the printing process. Alginate bioink with a concentration of 15% and oxidation of 5% had a day 0 viability of 98%, day 4 of 96%, and day 8 of 95% (**Figure 5**). These results indicate the deposition technique of the direct-write bioprinter extrudes cells gently enough to produce constructs that remain viable during and after the printing process (**Figure 1, 2**). The high cell viability shows the 5% oxidation and 15% concentration alginate bioink was a suitable vehicle for cell deposition and provided an adequate environment for cell-survival. Similar cell counts in each of the areas showed a homogeneous cell distribution in the alginate bioink, a fundamental aspect of printing resolution.

Most tissues have complex combinations and gradients of extracellular matrix constituents, each with specific biological and mechanical influences. A biomaterial should be biomimetic of the native environment and facilitate cellular functions. The high porosity of the alginate scaffold allows the cells to communicate and network with each other, and may also facilitate the flux of nutrients and metabolites between the scaffold and its surrounding environment. Cell adhesion to the extracellular matrix is a preliminary phase of tissue formation that happens before cell proliferation and the organization of extracellular matrix molecules into functional tissue. The proliferation of cells plays a vital role in wound healing and tissue growth, and is therefore a very important factor when analyzing bioprinted constructs for tissue engineering applications. The RGD-conjugated alginate enhanced cell attachment in printed constructs, leading to improved cell spreading and proliferation. The proliferation of cells in the printed scaffolds was quantified by counting three separate areas on days 0 and 8 (**Figure 6**). The overall cell proliferation was found to be 219.674% after 8 days of culture. These results signify the scaffold has adequate biocompatibility to be used as a synthetic extracellular matrix for delivering cells to repair damaged or nonfunctional tissue.

To analyze the success of RGD peptide conjugation on the alginate bioink, a comparison experiment was performed using cell-laden, RGD-conjugated 15% concentration, 5% oxidation alginate bioink and cell-laden, non-conjugated 15% concentration, 5% oxidation alginate bioink. DAPI staining for nuclei and phalloidin staining for actin were used to analyze the cell spreading in printed constructs on day 8. Images of each sample (at least three random pictures per sample) were taken using a confocal microscope system using Z-stack parameters of 30 optical slices over a 300 depth (**Figure 7**). The cell spreading shown in the sample with RGD-conjugated alginate proves the successful incorporation of the peptide on the alginate. Cell migration is an important step in tissue development; therefore the conjugation of RGD peptides on alginate improves the likelihood of *in vivo* application using this bioink.

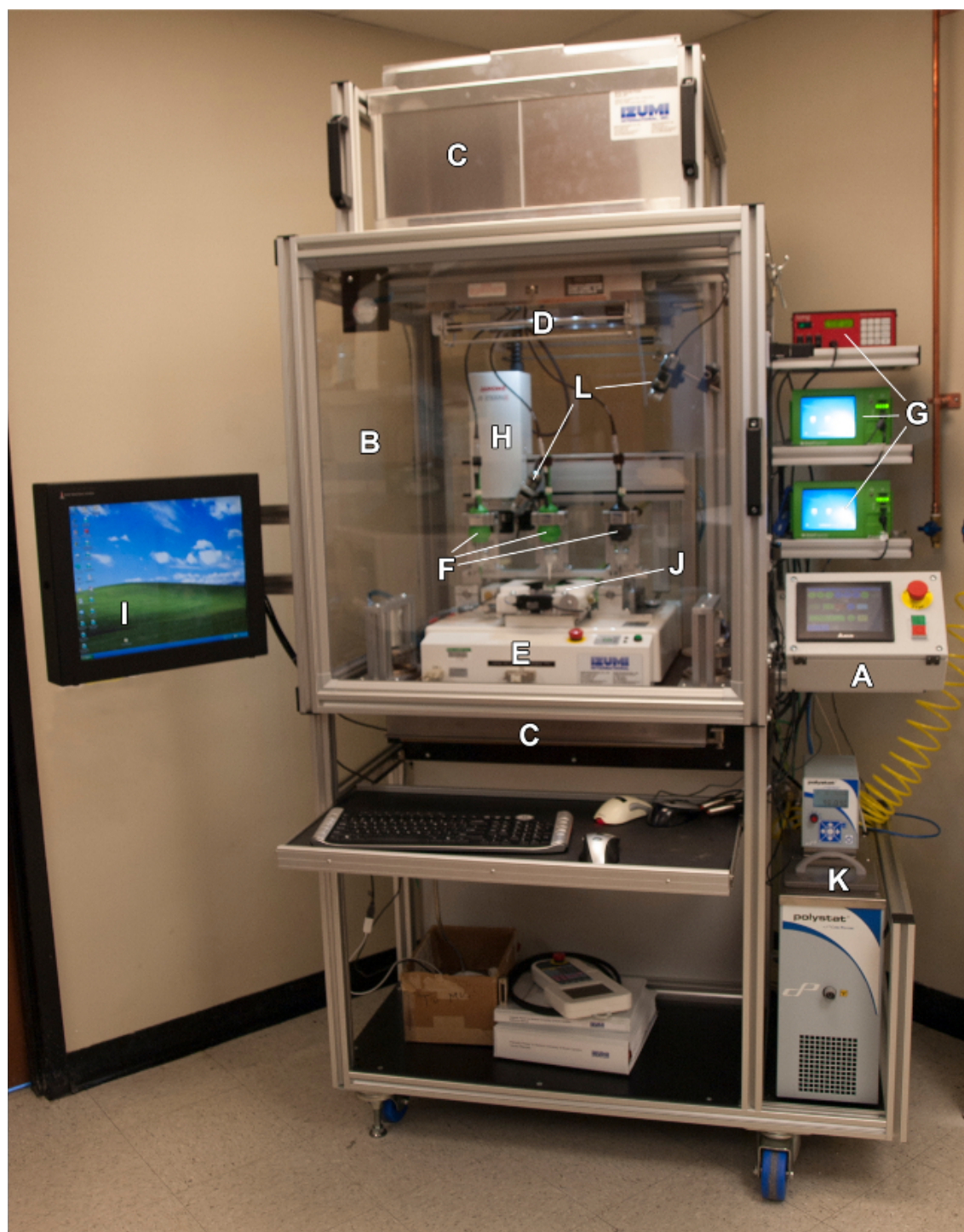


Figure 1. Palmetto Printer. Programmable Logic Controller coordinates the actions of all printer functions (A). An airtight containment chamber (B) with filtered intake (C) and exhaust (C) maintains a regulated internal positive pressure to reduce the chance of contamination. Dual UV lights (D) mounted in the chamber ceiling can be programmed to operate at safe intervals. A Janome 2300N XYZ Robot (E) is programmed and controlled by an integrated computer (I). Three dispenser controllers (G) are programmed to regulate the output of dispenser guns (F) available to be loaded onto the robot Z-axis arm (H) under computer control. The temperature of the robot sample holder (J) is set between 4 and 40 °C by a water bath controller (K). Dual digital cameras (L) are available to monitor printer activity and sample formation. One camera is mounted onto the robot Z-axis arm and provides a magnified image of the dispenser tip of the loaded gun. [Please click here to view a larger version of this figure.](#)

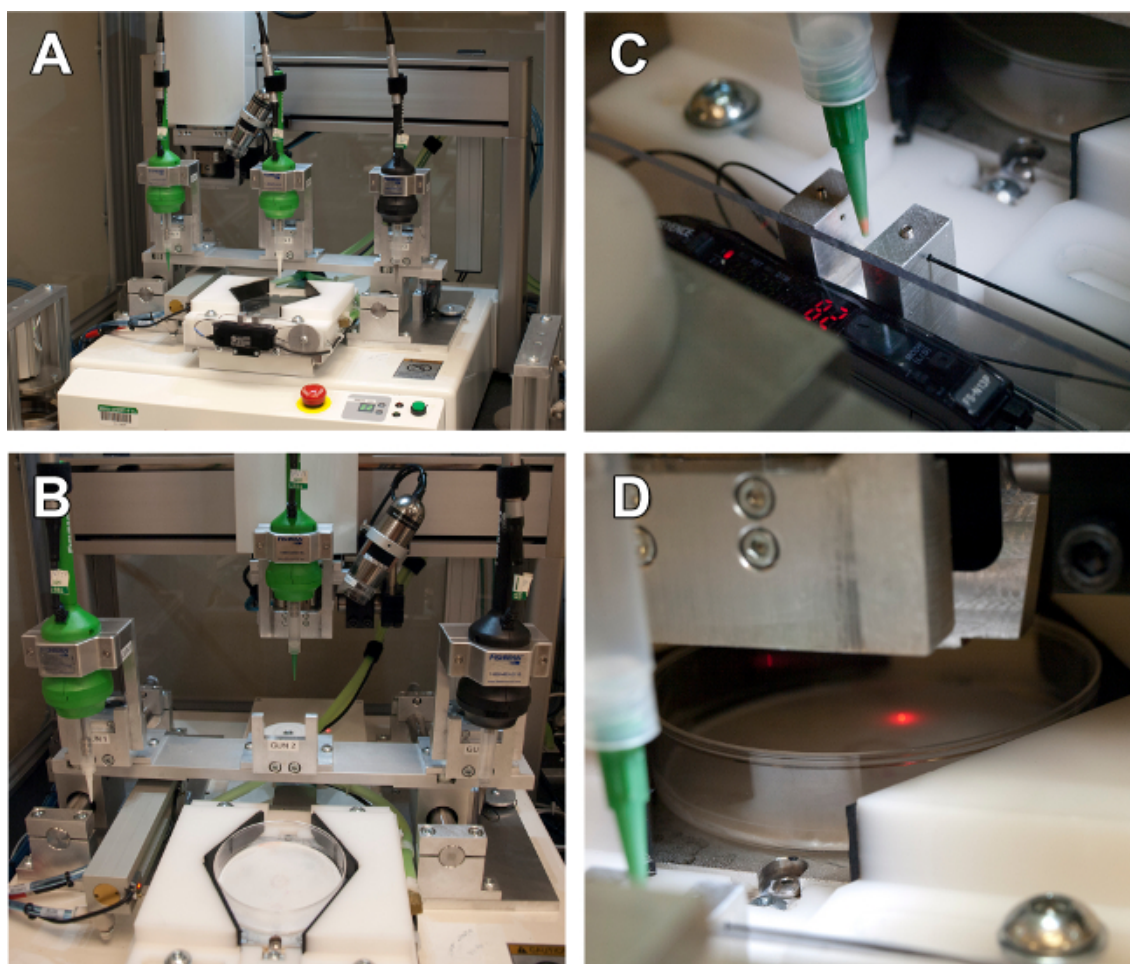


Figure 2. Tool Nest, Optic Sensors, and Displacement Laser of Bioprinter. **A.** Unloaded tool nest view from the front. **B.** Loaded tool nest view from the front. **C.** Optic Sensors measuring the dispensing tip end in three-dimensional space. **D.** Distance laser measuring the Z coordinate of the printing surface. [Please click here to view a larger version of this figure.](#)

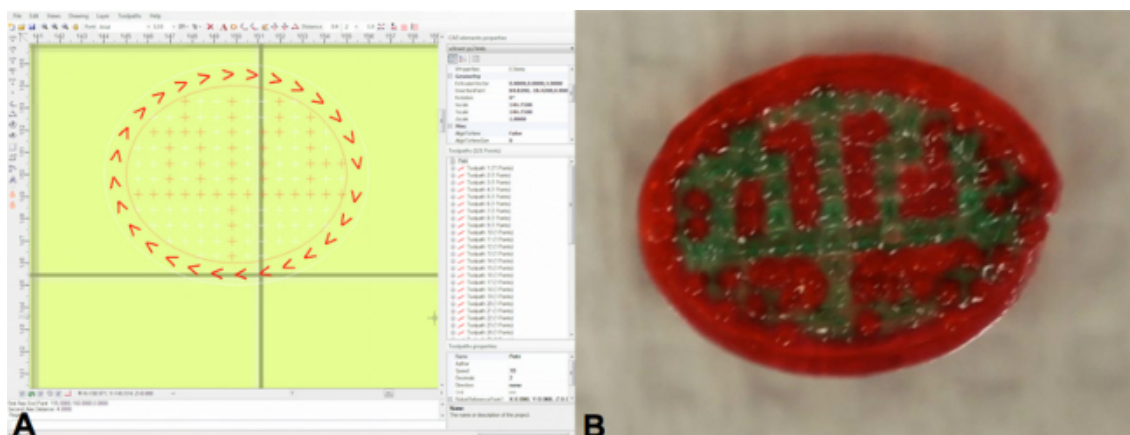


Figure 3. Visual PathBuilder Software. Image of computer-aided design drawing software used for designing the external architecture of bioprinted constructs. This program provides the ability to generate incrementally spaced droplets and complex geometries. [Please click here to view a larger version of this figure.](#)

360_1_10 - JR-C-Points(Dispensing)

File Edit View Program Data Robot Set T.M.C. Customize Account Help

23 Contact Angles1

Point	Type	X[mm]	Y[mm]	Z[mm]	Speed[mm]	Circle An	Condition N	BJob No.	MJob No.	Job No.	Pallet No.	Work Adj. No.	Execute No.	Tag Code	
1	PTP Point	150.000	150.000	0.000	-	-	-	-	-	-	-	-	-	-	-
2	Find_Base_Z	0.000	0.000	0.000	-	-	-	5	Me	-	-	4	SICK_L	-	Tip_Gap[mm] 0.2
3	Get_1	0.000	4.460	0.000	-	-	-	-	-	-	-	-	-	-	Z1_Down1[mm] 53.5
4	Find_Tip1_Y	0.000	310.000	0.000	-	-	-	-	-	-	-	-	-	-	-
5	Find_Tip1_X	268.000	158.715	0.000	-	-	-	-	-	-	-	-	-	-	-
6	PTP Point	150.000	140.000	0.000	-	-	-	-	-	-	-	-	-	-	-
7	Point Dispen	150.000	140.000	0.000	-	-	-	-	2	Adju	15	CA_	1	Gun1	Disperse Time[s] 0.5
8	PTP Point	165.000	140.000	0.000	-	-	-	-	-	-	-	-	-	-	-
9	Point Dispen	165.000	140.000	0.000	-	-	-	-	-	-	15	CA_	1	Gun1	Disperse Time[s] 0.5
10	PTP Point	150.000	160.000	0.000	-	-	-	-	-	-	-	-	-	-	-
11	Point Dispen	150.000	160.000	0.000	-	-	-	-	-	-	15	CA_	1	Gun1	Disperse Time[s] 0.5
12	PTP Point	165.000	160.000	0.000	-	-	-	-	-	-	-	-	-	-	-
13	Point Dispen	165.000	160.000	0.000	-	-	-	-	-	-	15	CA_	1	Gun1	Disperse Time[s] 0.5
14	PTP Point	150.000	150.000	0.000	-	-	-	-	-	-	-	-	-	-	-
15	Store 1	0.000	4.460	0.000	-	-	-	-	-	-	-	-	-	-	Z1_Down2[mm] 53.5

Figure 4. JR-C Points Software. Screenshot of the printer-compatible design software. This program allows the user to control the deposition method (*i.e.*, single drop deposition or continuous pathway deposition), deposition speed, distance between syringe tip end and printing substrate surface, the allotted time for deposition of each drop, and the three-dimensional placement of droplets (refer to **Table 1**). [Please click here to view a larger version of this figure.](#)

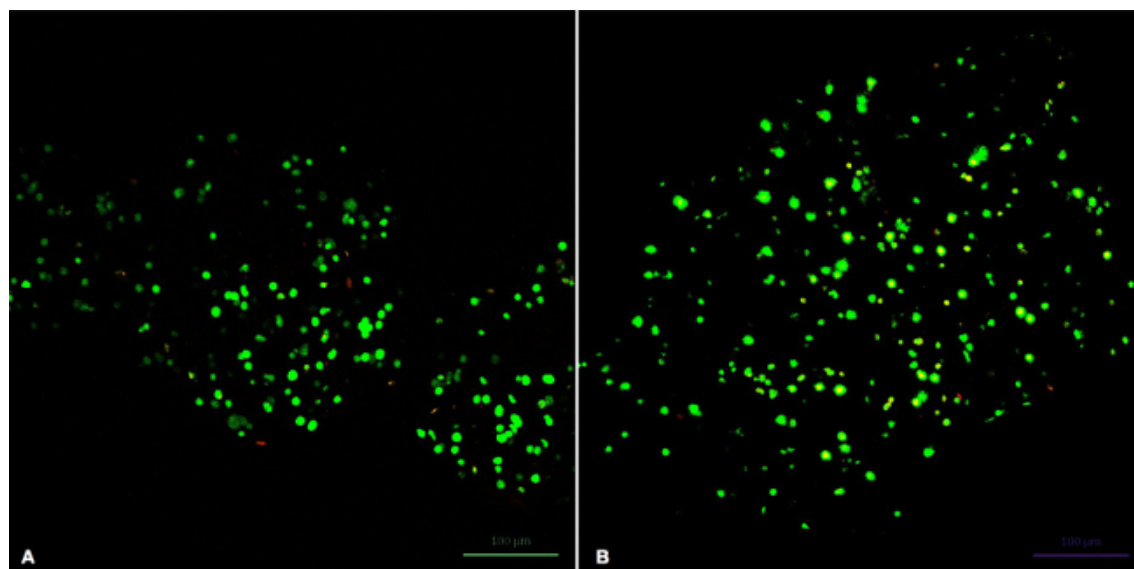


Figure 5. Fluorescent Image of Stained hADSCs Post-Printing. Cell viability/cytotoxicity fluorescent images of hADSC's in bioprinted construct taken using a confocal microscope system (Z-stack parameters of 30 optical slices over a depth) after 0 (**A**) and 8 (**B**) days. The hADSC's were labeled post-printing using a mammalian cell viability/cytotoxicity assay. Live cells are stained green, and dead cells are stained red. [Please click here to view a larger version of this figure.](#)

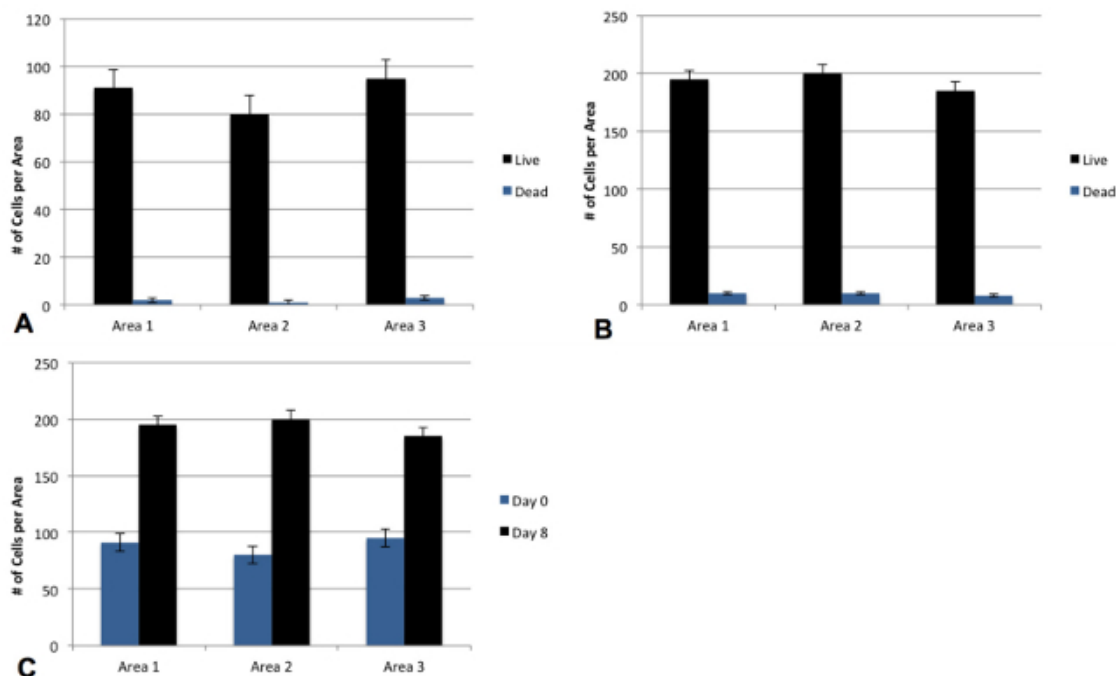


Figure 6. Quantified Viabilities of Biprinted Constructs. The number of live and dead cells was quantified using a viability/cytotoxicity assay. The live/dead cell counts for Day 0 are shown in (A), and the counts for Day 8 in (B). The number of live cells counted for each area on days 0 and 8 are shown in (C) and were used to quantify cell proliferation. [Please click here to view a larger version of this figure.](#)

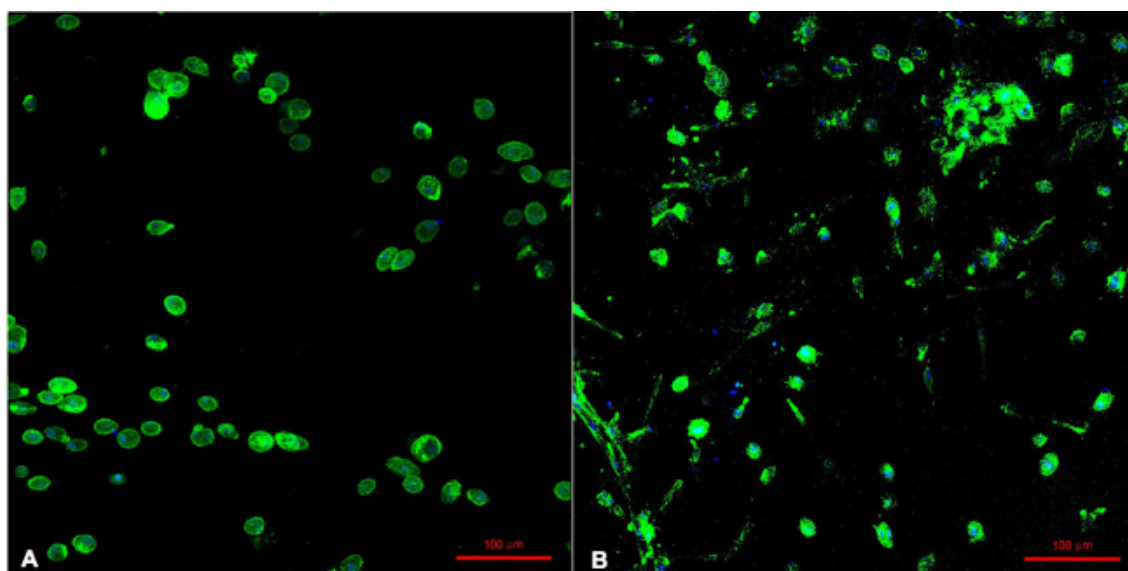


Figure 7. Comparison of Cell-Laden, Non-Conjugated and RGD-Conjugated Alginates. Fluorescent images of bioprinted hADSC's in non-conjugated (A), and in RGD-conjugated (B) 15% concentration 5% oxidation alginate bioink taken using a confocal microscope system (Z-stack parameters of 30 optical slices over a depth). The hADSC's were stained with phalloidin and DAPI stains to analyze the cell spreading in each of the constructs. [Please click here to view a larger version of this figure.](#)

Table of Commands	
Command	Robot Response
PTP Point	Robotic arm moves to indicated position in X, Y, Z space
Find_Base_Z	Uses the SICK laser to measure the printing surface of the substrate; The distance between the syringe tip end and substrate surface is manually set.
Work Adj. No. (Work Adjustment Number)	Commands robot to use SICK laser (for determining substrate surface), Gun 1, Gun 2, or Gun 3.

Get_1	Commands robot to retrieve and load Gun 1
Find_Tip1_YZ	The robot finds the tip end of Gun 1 in the Y and Z directions
Find_Tip1_X	The robot finds the tip end of Gun 1 in the X direction
Point Dispense	The robot will dispense one droplet of bioink in the determined X, Y, Z position
Pallet No. (Pallet Number)	Incorporates a manually coded design for printing, e.g., an array.
Dispense Time	Is the time allotted for the deposition of each individual drop.
Store_1	Commands the robot to return Gun 1 to the toolnest, and return to home position: (0,0,0).

Table 1. Programmable Computer Software Commands. This chart outlines the programmable computer software commands, which are used to control the robotic arm and optimize printability parameters.

Discussion

The primary focus of tissue engineering is to bridge the gap between organ shortages and transplantation needs by developing biological substitutes capable of restoring, maintaining, or improving native tissue function. This has led to the direct fabrication of scaffolds with a complex, anatomically correct external geometry, and precise control over the internal geometry. Three-dimensional bioprinting is a methodology used for generating three-dimensional constructs of various sizes and shapes from a digital model using a layer-by-layer approach. The fabrication of three-dimensional biomimetic constructs plays an essential role in the advancement of tissue engineering.

There are critical aspects of the design process that impact the generated construct's biomimetic function. The ability to control the temperature of the biomaterial and substrate is essential for the gelling mechanism of the hydrogels and the maintenance of their mechanical properties, therefore influencing cell distribution, proliferation, and differentiation within the hydrogel. Organs consist of many cell types, so the multiple dispensers are critical for producing heterogeneous, tissue-like structures. The computer-aided design of the external architecture allows the production of custom tissue substitutes for distinct wounds or tissues. This is essential for the development of patient-specific replacements. The internal architecture is equally important because it affects the cell-cell relationships within the structure by placing the proper cells in intimate contact with each other and allowing them to form *in vivo*-like cell-cell junctions. Precise placement of cells determines how the cells communicate and network with each other to form vascular networks and mimic their bioactivity in native tissues. Three-dimensional bioprinting provides homogeneously dispersed cells within the bioink, as well as excellent precision on spatial placement of the cells. Generated scaffolds also have high local cell densities, which is essential for cell differentiation and the formulation of extracellular matrix.

Presented here is a 3D robotic bioprinter that reliably and consistently dispenses homogeneous drops of individual cells or cells mixed with biomimetic hydrogel. Similar bioprinters have used a pre-fusion technology. In accordance with the differential adhesion hypothesis, individual cells when placed in a mold or similar device, will fuse and organize based on the concentration and distribution of adhesion molecules on the cell surface. These devices create pre-fused rods or other geometric shapes that are then loaded into the dispenser and placed in close proximity to other pre-fused or pre-made rods, which then fuse into a larger geometric shape. End geometries are limited by what can be constructed from these pre-made entities. The bioprinter implemented here comprises a unique temperature-controlled environment in which the cells and cell-hydrogel mixtures are not limited by the necessity of pre-fusion. Under these conditions, the bioprinter is not solely reliant on the differential adhesion hypothesis. The inclusion of hydrogel materials can help guide the cell distribution and allow the cells to fuse, or not, depending on the properties desired for specific experimentation. The selection of biomimetic hydrogels for cell-encapsulation also has a profound effect on cell phenotype. Materials are known to have an effect on cell attachment, as well as cell size and morphology. The rheological characteristics, such as viscosity, of hydrogels dictate their influence on the cellular microenvironment. Native alginate is inert and does not readily communicate with cells participating in the control of phenotype. However, using alginates that are chemically modified via peptide conjugation and oxidation, the resulting constructs display controlled degradability and increased cell attachment, migration, and proliferation. Altering the physicochemical properties of a biomaterial can influence tissue development.

Three-dimensional bioprinting using a fluid-dispensing, direct-write machine is limited by the degree of resolution of printed constructs, the availability of hydrogel materials, initial cell death post-printing, and the ability to vascularize the biomimetic. An important feature of the bioprinting is its resolution. Every printing method is defined by the lower technical limit size of the smallest achievable details. There is a dynamic relationship between the lower limit size and attainable scale of the printed construct: the higher the resolution of the minute details, the smaller the maximum construct. The bioprinter is capable of depositing volumes as small as 230 nL in highly specific and organized patterns, giving it a higher resolution than similar machines. Hydrogels have been commonly used in bioprinting due to their hydrophilicity, biocompatibility, structural similarity to the extracellular matrix, and ease of modification. The high water content of hydrogels improves their biocompatibility, but greatly reduces their mechanical strength and. There is a lack of optimal hydrogels with the appropriate mechanical properties for fluid delivery during bioadditive-manufacturing extrusion. Therefore, there is a large demand for developing hydrogels that are immunologically inert, have cytocompatible gelation mechanisms that can be successfully extruded using fluid delivery, and also produce a cell-laden matrix with an optimal range of mechanical. Before the printing process, the cell-laden hydrogel bioink must be stored in the syringes for an amount of time, compromising the cell's. During the printing process, the shear stress induced on cells during extrusion can also be harmful to their. The bioprinter is able to produce highly viable (>90%) constructs, therefore overcoming the issue of initial cell death. Vascularization plays a vital role in transmitting, supporting, or preserving the biomimetic function of bioprinted. The diffusion of oxygen is limited; therefore in larger bioprinted constructs hypoxia is a. Conventional techniques are incapable of producing constructs with embedded vasculature, greatly limiting the size of producible scaffolds. The cell viability assessment of the bioprinter showed significant cell proliferation in the printed constructs over 8 days. Therefore, the technique proves its ability to generate scaffolds that allow cell growth, communication, and the formation of networks, requirements of vascularization.

The bioprinter provides the ability of using a variety of materials to quickly deposit cell-laden hydrogels in specific patterns. While this technique produces heterogeneous constructs with tunable properties, it is incapable of concurrent deposition and reactive mixing. For some biomaterials, this deposition method would enhance the gelation mechanism and shorten the time for scaffold production. The addition of a multi-syringe dispenser could allow a broader range of biomaterials for the biofabrication technique. Investigation of cell activity in bioprinted constructs over a longer period of time would provide more information about hydrogel characteristics, cell network formation, and vascularization of the constructs.

The bioprinter's deposition method described can further involve robotically positioning and driving the three dispensers to deposit a plurality of biological materials on top of previously deposited materials in a predetermined pattern. This step can be repeated using ascending patterns until a three-dimensional organ or tissue is produced. Therefore, the Palmetto Printer is suitable for reliably dispensing cell-laden hydrogels to create a three-dimensional construct that is capable of retaining vasculature and high cell viability, and could be used in tissue engineering applications.

Disclosures

The authors have nothing to disclose.

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