Published in final edited form as:

Bioprinting. 2019 September ; 15: . doi:10.1016/j.bprint.2019.e00048.

Scaffold-free Bioprinting of Mesenchymal Stem Cells with the Regenova Printer: Optimization of Printing Parameters

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Abstract

The Kenzan bioprinting method provides a high-resolution biofabrication process by facilitating the fusion of submillimeter cell aggregates (spheroids) into larger tissue constructs on a needle array that is removed upon spheroid fusion. Although the method is relatively straightforward in principle, Kenzan method bioprinting relies on a complex 3D bioprinter (Regenova Bio 3D Printer, Cyfuse, K.K., Japan) implementing an advanced vision system to verify the microscopic spheroids' geometry and high-precision mechatronics to aseptically manipulate the spheroids into position. Due to the complexity of the operation, the need for aseptic conditions, and the size of the spheroids, proficiency with the Regenova Bio 3D Printer and the Kenzan method requires development of best practices and troubleshooting techniques to ensure a robust print and minimize the use of resources. In addition, managing the construct post-bioprinting both in culture and for surgical implantation requires careful consideration and workflow design. Here, we describe methods for generating a competent tissue construct and optimizing the bioprinting process. Optimization resulted in a 4-fold reduction in print times, a 20-fold reduction in the use of

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bioprinting nozzles, and more robust constructs. The results and procedures described herein will have potential applications for tissue engineering, research, and clinical uses in the future.

Keywords

Osteogenesis; Mesenchymal Stem Cells; Spheroid Formation; Bioprinting; Scaffold-Free; Tissue Engineering; Regenova

Introduction

The Kenzan bioprinting method provides the highest resolution biofabrication method available in the known literature. With this method, 500 μm cellular spheroids can be placed in close proximity to one another such that they fuse into a larger tissue construct[123]. Spheroids can be made with homogenous or heterogenous cell populations and any spheroid combination can be printed onto the Kenzan array (figure 1((a), (b) and (c)). The Kenzan array is an array of stainless steel needles, 10 mm in length and 170 μm in diameter, spaced 400 μm apart in either a 9×9 or a 26×26 pattern. Kenzan Bioprinting occurs on the Regenova Bio 3D Printer (Cyfuse, K.K., Japan; figure 2) which relies on a camera-based machine vision system, plate handling platform (not shown), disposal chamber (not shown), a container holding a Kenzan needle array submerged in PBS, and a nozzle connected to a pressure system on a 6 axis gantry. During the printing process, a spheroid is first located and inspected by the vision system. Next, the spheroid is picked up from the culture plate with suction from the nozzle, “skewered” onto a needle on the Kenzan array, and placed on the needle according to a preprogrammed 3D pattern. Therefore, tissue constructs can be printed with diverse cell populations in anatomically correct structural scales found in natural tissues. For example, channels can be generated either at the scale of the needle diameters (~200 μm) or by omitting spheroids from a particular location in the array. Despite the relative simplicity of Kenzan method biofabrication, proficiency with the Regenova requires development of best practices and troubleshooting to minimize operator error, optimize print time, reduce cost of use, and ensure a robust print. In addition, maturation of the bioprinted construct and its transfer from the culture environment to the surgical site must be carefully considered so as to avoid damaging the construct or introducing contaminants. Here we describe practical methods for streamlining the Kenzan biofabrication workflow with the Regenova, troubleshooting tips, and post-bioprinting topics necessary for generating a competent tissue construct which could eventually be implanted. Bioprinting has several applications in tissue replacement and regeneration, including bone repair. Therefore, this study was performed in the context of regenerating bone in a calvarial defect model immortalized bone marrow stromal cells (BMSCs) that were differentiated to the osteogenic lineage.

Methods

Bone marrow derived stromal cell (BMSC) culture

Immortalized BMSCs were originally isolated from C57BL/6 mice which were alkaline phosphatase positive and could mineralize[4]. BMSCs were expanded and cultured in control

medium. Control medium was composed of: Alpha-Minimum Essential Medium (α-MEM, Gibco ®, NY, USA), 10% fetal bovine serum (Atlas Biologicals, CO, USA), 1% penicillin-streptomycin-glutamate (Gibco ®, NY, USA), and 0.001% amphotericin B (Sigma-Aldrich, MO,USA). Cells were cultured until they were approximately 90% confluent and were seeded into new dishes for the generation of spheroids as detailed below.

Spheroid formation

BMSC passage number 24-37 were seeded at 20,000 or 40,000 cells/well in ultra-low attachment (ULA) U-bottom 96 well plates (SBIO™, Japan). The BMSCs collected at the bottom of the well either under the force of gravity or under centrifugation for 5 min at 300G, forming spheroids. BMSC spheroids were cultured in control medium for three days prior to printing.

3D Bioprinting

We attempted 4 separate “Trials” of printing constructs using the Kenzan biofabrication method and the Regenova Bio 3D Printer. Throughout the remainder of the manuscript, specific experimental conditions will be described in reference to sequential trial number (e.g. Trial 1, Trial 2, Trial 3, or Trial 4).

Prior to printing, a 5 mm diameter construct with a total of 324 spheroids in 2 layers (~800 μm thick) for Trial 1 (not shown) or with a total of 648 spheroids in 4 layers (~1600 μm thick) for Trials 2-4 (figure 3(a)) was designed using the Bio 3D Designer software provided with the Regenova Bio 3D Printer. To set up each print, the container containing a 26×26 needle Kenzan in PBS, the reservoir system (which is used to transfer negative pressure during spheroid pickup), and the nozzles were autoclaved separately, allowed to cool, and installed aseptically into the printer (figure 2 and 3(b)). The pressure system can be autoclaved with a nozzle installed onto it, although there is a risk of damage to the needle during handling. To begin a bioprint, the vision system first scans the needle array to ensure the needles are not bent or missing and can be visualized by the vision system. The vision system then scans the nozzle for straightness, nozzle diameter, and to calibrate nozzle location. If these elements are within specification, the plate handler will remove a 96- well plate from the plate magazine and place it into the printing position where the vision system identifies a spheroid within a well and quantifies spheroid parameters (distance from the well center, diameter, roundness, and smoothness (figure 3(c)). The roundness is determined by measuring the radius of the smallest circumscribed circle of the spheroid (R) and the radius (r) of an inscribed circle, concentric with the first circle, and contacting the spheroid perimeter. Roundness is then calculated using equation (1):

$$Roundness[\,\%\,] = \left[100 - \left(\frac{(R-r)}{R}\right)\right] * 100 \quad (1)$$

Smoothness percentage is calculated by measuring the area of the regions deviating from the average of the minimum and maximum contour areas of the spheroid (DA) and then dividing DA by the spheroid area (SA):

$$Smoothness[\,\%\,] = \frac{DA}{SA} * 100 \quad (2)$$

Spheroid specifications can be set by the user, and for all trials were 450-550 μm diameter, 76-100 % roundness, and 0-10.0% smoothness.

If the spheroid is within specification, the gantry will lower the nozzle into the well and exert low negative pressure, suctioning the ~500 μm spheroid onto the tip of the 26 gauge nozzle (figure 3(d)). The nozzle then moves away from the well and the vision system scans the well for the spheroid. If the spheroid is no longer observed by the vision system, the spheroid is presumed to be on the nozzle and the nozzle will then move into position over the needle array, lower itself onto a preselected needle (using position control), impaling the spheroid onto the needle (figures 3(d) and (e)). The pressure on the nozzle is then released, releasing the spheroid which stays in position. If a spheroid is observed in the well by the vision system after an attempted pickup, this is considered a failed pickup and the system will try to pick up the spheroid as many times as determined by the user. Once the preselected number of pickup attempts has passed, the system will stop and inform the operator. Upon spheroid placement the system will move to the next spheroid, in the next well, and will continue the printing process until the construct is completed. Construct bioprinting progression is observed by the machine vision system (figures 3(f), (g), and (h)) and can be observed directly from the side of the Kenzan container (figure 3(i)).

Nozzle installation—Nozzles used for manipulating spheroids during printing must be sterile. The manufacture's nozzle installation procedure calls for securing the first nozzle to the reservoir system prior to autoclaving. For subsequent needle changes, the manufacturer calls for securing the nozzle onto the reservoir system using forceps. This awkward grip and hand motion required for nozzle installation resulted in dropped nozzles which would result in nozzle damage and would potentially damage the printer, and specifically, the vision system which is housed, in part, under the nozzle. The manufacturer's method also added considerable time to the printer setup time. To mitigate nozzle installation issues, each nozzle was sheathed with a 1000 μL pipette tip. These sheathed needles could be autoclaved and gripped by the pipette tip such that each nozzle could be aseptically installed using a gloved hand and finger tightened (Supplemental video 1).

Failed nozzle scans after component exchanges—When plates or Kenzan needle arrays were being exchanged in the middle of a print, some fluid remained on the nozzle tip. When the vision system scanned the nozzle at printing restart, fluid was registered as part of the nozzle, resulting in the nozzle being out of specification. To mitigate this issue in Trials 3 and 4 a sterile Pasteur pipette was manually touched to the nozzle, which draws the fluid away from the fluid away from the nozzle by electrostatic force. Once the fluid was removed from the tip, the nozzle scanned with no errors. The fluid could also be allowed to dry so that nozzle diameter was back in specification but this would lengthen the print time considerably.

Thresholds and blind pickups—The Regenova vision system allows the user to adjust the thresholds for spheroid distance from the well center, diameter, percent smoothness, and percent roundness. While these are used to indicate that the spheroids are within a preselected specification, cellular debris and matrix secreted from the spheroid during maturation can be registered by the Regenova. As an example, cellular and secreted extracellular matrix debris from spheroids can take on a round form with a diameter smaller than that of the spheroid. When the nozzle picks up a spheroid and the vision system checks the well again for spheroid presence and registers the debris as a spheroid, the system will attempt to pick up the debris despite a spheroid already being held by the nozzle. These "blind pickups" can result in the loss of the picked up spheroid and a delay in printing. To mitigate these errors, the diameter threshold was adjusted to within 50 μm of the minimum spheroid diameter. For example, the spheroids utilized in this study had a minimum viable spheroid diameter of 450 μm. Therefore, the spheroid presence threshold was set to 400 μm.

Failed spheroid pickups—Despite the use of low-binding plates, possible poor coverage of the low-binding surface in the well bottom and possible scraping of this surface with pipette tips during culture may result in the spheroids adhering to the wells strongly enough that the nozzle cannot pick them up. In other cases, the nozzle pressure ruptures the spheroid such that part of the spheroid is drawn into the nozzle or the spheroid is ruptured in a way that the entire spheroid is drawn into the nozzle. Both scenarios can block the nozzle. Originally, the plates were lightly tapped to loosen them (Trial 1, Table 1). However, vigorous motion while tapping the plates spilled medium onto the lids, which increased risk of contamination without fully loosening all spheroids. Medium leakage onto the lid also causes a vacuum between the plate and lid which prevented the Regenova from removing the lid from the plate during the printing process, potentially damaging the machine. Therefore, to avoid failed pickups, spheroid rupture, and medium leakage, each spheroid was loosened by hand-pipetting 50 μl of medium in each well containing a spheroid several times with a micropipette just prior to printing. This flushing procedure loosened the spheroids so they could be easily picked up.

When weak or ruptured spheroids or other cellular debris are caught in the nozzle, generating a blockage and preventing spheroid pickup, the Regenova's disposal feature is utilized to remove blockages. The disposal routine involves the nozzle moving into position over a small waste container with the nozzle tip being placed through the container opening. Air is forced through the nozzle at up to ~10 kPa. During the procedure, the digital nozzle pressure gauge should be observed. If the gauge reads ~4 to 7 kPa after a disposal step, the blockage has been cleared and printing can resume. If the gauge reads "FFF", the pressure has exceeded the range of the reservoir system failsafe and the blockage is not clear. If, after several disposal routines, the gauge continues to read "FFF" and the blockage remains, the nozzle should be replaced.

If the spheroids are loosened and the disposal routine does not show an increase in pressure, there may be a loose connection in the nozzle reservoir system. The connections along the pressure line should be checked and reconnected and then a print step attempted.

Maturation time—After printing, Trial 1 constructs with Kenzans were placed in a Magenta GA-7 Vessel (Carolina® Biological Supply Company, NC, USA) with control medium for one week. Medium was replaced 2 times/week. During medium changes, half of the medium was removed from the vessel, and an equivalent amount of fresh medium was added to the vessel. After one week of maturation, the constructs were carefully removed from the Kenzans by using forceps to grip the platen supporting the bottom of the construct and sliding the platen along the needles and off of the Kenzan, taking the construct with it (figure 1(a)). The constructs were separated from the platen and placed in a petri dish for two weeks in control medium. Half medium changes were continued on alternating days for an additional two weeks.

For Trial 2, constructs loaded on Kenzans were placed in a Magenta GA-7 Vessel for two weeks. One construct was placed in control medium. The second construct was placed in the same medium but supplemented with 200 mM β-glycerophosphate disodium salt hydrate (BGP) (Sigma-Aldrich, MO, USA) and 50 mg/ml of L-ascorbic acid 2-phosphate (AA) (Sigma-Aldrich, MO, USA), referred to as osteogenic medium. Half medium changes were completed twice a week. After 2 weeks in the containers, the constructs were carefully removed from the Kenzan with forceps using the methods described in Trial 1. They were placed in different Petri dishes that were previously coated with 2% agarose to prevent cell adhesion to the dish. Half medium changes were continued on alternating days for an additional week.

Trial 3 constructs with Kenzans were placed in a Magenta GA-7 Vessel for three weeks. Osteogenic medium was added to one construct on day 0 (Construct 1); osteogenic medium was added to the second construct on day 3 (Construct 2). Maturation time on the Kenzan within the vessel for both constructs was three weeks. Medium was changed twice a week (half medium changes). After 3 weeks, constructs were removed from the Kenzan and placed in a 2% agarose coated petri dish.

Trial 4 constructs with Kenzans were placed in a Magenta GA-7 Vessel for 3 weeks. Osteogenic medium was added to three constructs on day 3; osteogenic medium was added to the other three constructs on day 7. Osteogenic medium was changed twice a week (half medium changes). After 3 weeks, constructs were removed from the Kenzan. This was accomplished by using sterile gloves and forceps to aseptically slide the top platen (figure 1(a)) from the needle array such that the construct remained on the platen. The back-side of a surgical spatula was then used to slide the construct from the platen (figure 3(j) and (k)).

Results

Sheathing nozzles with pipette tips reduced the number of dropped and damaged nozzles considerably, though the number of nozzles lost during bioprinter installation was not recorded. Loosening the spheroids prior to printing led to a reduction in failed pickups and in blockages leading to nozzle changes. In Trial 1, an average of 65 spheroids were printed per hour. Loosening the spheroids with the pipettes increased the print rate to 87-93 spheroids per hour in Trials 2 and 3. This also reduced the number of nozzle changes.

In Trial 3, the additional step of cleaning fluid from the nozzle contributed to further reducing the printing time. However, light reflecting from the spheroids interfered with the machine vision and image processing such that the needle position could not be resolved for spheroid placement. Therefore, manual spheroid placement was employed for Trial 3, contributing to a print time which was only slightly decreased compared to Trial 2. Lighting adjustments made prior to Trial 4 reduced the need for manual spheroid placement. As detailed in Table 1, reducing failed pickups, the number of nozzle changes, and cleaning the nozzle contributed to a 20-fold decrease in the number of nozzles used (nozzles are single use), and print rate of spheroids increased by a factor of four from 65 spheroids per hour (Trial 1) to 260 spheroids per hour (Trial 4).

For Trials 1 and 2, the constructs were removed from the Kenzans 1 to 2 weeks after printing. The constructs did not have much structural rigidity and folded over on themselves and became misshapen in the week following removal from the Kenzan. Since the Kenzan needles provide structural support, Trial 3 constructs were removed from the Kenzans three weeks after printing (figure 4(a) and (b)) and were subjected to osteogenic medium beginning at 0 or 3 days post-bioprinting. The 0-day constructs almost completely disintegrated at the spheroid level during handling following Kenzan removal (figure 4(a) and 4(c)) while 3-day constructs broke into large pieces and also showed some spheroid-level disintegration (figure 4(b), 4(d) and 5(a-c)). Histology of tissue fragments confirmed high cellularity in both constructs (figure 4 (e) and (f)).

To determine the influence of the media on spheroid fusion, Trial 4 constructs were transitioned from control medium to osteogenic medium after 3 or 7 days. The 7-day constructs, compared to the 3-day constructs, could be handled without disintegrating (figures 3(j), 3(k) and 5(d-f)) , suggesting that constructs should be kept on the Kenzan needle array and in control medium (as opposed to osteogenic medium) long enough for spheroids to fuse to one another prior to differentiation and ECM deposition. Moreover, the 7-day constructs were robust enough to withstand handling while the 3-day constructs had to be scooped from the Kenzan platen to avoid construct damage (figure 5(f)). Also of note were the microchannels left in Trial 4 constructs by the Kenzan needles.

Discussion

Over the course of our investigations and biofabrication projects, we have learned several best practices that can significantly improve bioprinting efficiency and bioprinted construct quality while also reducing bioprinting waste and costs. Here we summarize our findings. While the culture plates used to culture spheroids have been treated to reduce cell adhesion, ULA coverage on the well surface is not ideal and may be prone to removal by scratches from pipette tips during cell seeding and medium exchange. It is also possible that the ultra-low adhesion coating was not as effective as intended. Therefore, a spheroid loosening procedure was employed to release the spheroids from the well floor. Additionally, high spheroid adhesion had an adverse effect on spheroid pick up, resulting in spheroids being ruptured and portions of the ruptured spheroids being drawn into the nozzle, causing a blockage. Blockages cause costly delays in printing time and increase costs due to the need

for nozzle replacement. In some cases, ruptured or partial spheroids are placed onto the Kenzan, undermining the final print.

The constructs are bioprinted on a Kenzan submerged in PBS since culture media supplemented with serum may interfere with the Regenova's machine vision system. Since the PBS has no nutrients, keeping the constructs in PBS long term, as printing issues (i.e., repeated failed pickups, repeated disposal routines to clear nozzle blockages, repeated nozzle replacements, and thresholding) are resolved, can compromise cell viability and alter cell differentiation or metabolism, confounding print results. Increased printing time, particularly in events that require manually manipulating Regenova components, exposes the constructs to risk of contamination. The Regenova is fitted with a sash, a HEPA filter, and negative air pressure to reduce the chance for contaminants to enter the build envelope. However, risk of contamination increases with the number of times elements near the Kenzan are manipulated and the number of times the Regenova's sash is opened.

Constructs freshly printed in the Regenova are extremely fragile, requiring best practices in work flow to prevent damage prior to implantation. The first step to reducing construct damage is to ensure that spheroids within the construct are adequately fused prior to handling for culture or implantation. In initial Trials, constructs were subjected to osteogenic medium 0 or 3 days post-bioprinting. The 0-day constructs almost completely disintegrated at the spheroid level during handling following Kenzan removal (figure 4(a) and 4(c)) while 3-day constructs broke into large pieces and also showed some spheroid-level disintegration (figure 4(b) and 4(d)). Poor fusion suggests that secretion of extracellular matrix (ECM) induced by osteogenic medium may have prevented cell-cell adhesion between spheroids, or that differentiation altered cell-cell adhesions. Previous studies have demonstrated this phenomenon, concluding that more mature spheroids, defined as having increased ECM, result in slower spheroid fusion[5]. To generate competent tissues, it is critically important to appreciate that fusion behavior is different for different cell populations. Thus, spheroid fusion behavior must be properly characterized and optimized for each cell type so that procedural, timing, and biochemical regimens can be developed to achieve robust bioprinted tissues. Since ECM concentration is shown to regulate spheroid fusion[5], controlling ECM deposition rate, which can also be described as speeding-up or slowing-down maturation, may result in better control of spheroid fusion, and thus, tissue integrity. For example, Mironov et al. demonstrated that fibrillogenic agents such as transforming growth factor beta 1 (TGFβ1) increased spheroid cohesion, which reduced the spheroid's ability integrate with other spheroids.[1] As a result, spheroids produced without fibrillogenic agents, which accordingly had lower ECM concentrations, placed adjacent to TGFβ1-matured spheroids were able to envelop the more mature spheroids. Therefore, biochemical methods for slowing down spheroid maturation should be explored to enhance spheroid maturation. Once the spheroids are adequately fused, fibrillogenic biochemical agents can be applied to enhance integrity of the bioprinted tissue. On the other hand, ECM deposition for some cell types may be low or slow enough that spheroids fuse quickly but do not possess the mechanical integrity to remain intact post-bioprint. In these cases, it may be necessary to provide fibrillogenic agents to rapidly increase post-bioprint construct strength. With regard to generating complex tissues, further characterization and nuanced post-bioprinting

maturation regimens must be accomplished for heterospheroidal bioprints to account for differences in spheroid maturation and thus interspheroid fusion[1,5].

Therefore, a separate set of constructs (Trial 4) were exposed to osteogenic medium 3 or 7 days after post-printing. The 7-day constructs, compared to the 3-day constructs, could be handled without disintegrating (figures 3(j) and 3(k)), suggesting that differentiation and ECM deposition can significantly affect spheroid fusion. Construct culture immediately after printing may need to be optimized for cell-cell interaction, adhesion, and spheroid fusion. Handling the fragile constructs may also lead to damage. Initial Trials involved construct transfer from the Kenzan array to an intermediate culture chamber during post-printing maturation. These constructs not only began to fall apart, but extra handling put them at risk for contamination. Keeping the constructs on the Kenzan until just before implantation would reduce the number of times and the degree to which the construct is handled, limiting potential damage to the constructs.

Keeping the construct on the Kenzan until just before implantation could be beneficial with the microchannels left by the Kenzan needles. While the Kenzan method was originally intended for these microchannels to close during long-term culture[67], maintaining them would provide Haversian canal-like channels through which nutrients can interact with the interior of the construct. These microchannels are most visible in figure 4(d). Ongoing studies are investigating methods for maintaining the patency and direct perfusion of these Haversiancanal-like channels to enhance nutrient delivery during long-term culture.

Properly clearing fluid droplets from the nozzle and loosening the spheroids to ensure complete pickup reduced the number of nozzle failures/changes which considerably reduced the printing time (Table 1). The bioprint for Trial 1 resulted in 2 constructs (2 layers each) on the same Kenzan. Trial 1 therefore printed at an average rate of 65 spheroids per hour which increased to 260 spheroids per hour in Trial 4. With increasing demand for use of the Regenova, the cost of consumable components, and bioprinting facility charges for using the bioprinter, the reduction in printing time and reduced use of resources as a result of streamlining the bioprinting technique will allow greater user access, ensure reproducibility, reduce the risk of contamination and cell death, reduce operating costs, and increase Regenova output.

Conclusion

The Kenzan method of biofabricating constructs generated on the Regenova represents an advance in biofabrication due to the system’s ability to generate dimensionally-accurate scaffold-free tissue constructs with submillimeter resolution and the ability to place individual cell populations (spheroids) adjacent to one another, approximating the apposition of diverse populations in complex tissues. Generating tissue construct models exhibiting cell populations heterogeneity modeled after natural tissue compositions can provide valuable information about cell-cell interactions and lead to effective therapies[8]. Therefore, spheroid-based bioprinting has applications in tissue engineering, studying tissue development and tissue disease models, and, potential clinical uses[1]. However, operating the Regenova proficiently requires development of best practices to ensure quality prints since operating

the machine without streamlining the bioprinting workflow can quickly become prohibitively expensive. The constructs generated may also be fragile and prone to contamination, meaning careful consideration of the post-bioprinting and implantation workflow are required to ensure that Kenzan method bioprints are competent for *in vivo* use. Continuing to advance bioprinting and post-bioprinting techniques with the Kenzan method and the Regenova will lead to research discoveries in tissue engineering, improved tissue models for development and disease research, and clinical applications where robust, high resolution, scaffold-free biofabrication is desired. An additional level of sophistication is to perfuse the constructs using a perfusion bioreactor such as the FABRICA bioreactor platform to ensure that the constructs are receiving adequate nutrition[9].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Department of Orthopaedic Surgery, Indiana University School of Medicine (MAK), NIH T32AR065971 (INA), NIH S10OD023595 (LJS), and NIH T32 DK007519 (DJO). This research was also made possible by Indiana University Health and the Indiana Clinical and Translational Sciences Institute, funded in part by grant #ULI TR001108 from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award and The Advanced in Medicine grant from Cook Medical (LJS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or Cook Medical. We would like to thank Dr. David Burr, Dr. Nicanor Moldovan, Dr. Marta Alvarez, and the 3D Bioprinting Core for their support. We would also like to thank Riley Gorden, Ghazal Hosseini, Huseyin E. Arman, Kevin A. Maupin, and Ruben Dario Aguilar for their technical assistance, and Shatoria Lunsford for her administrative support.

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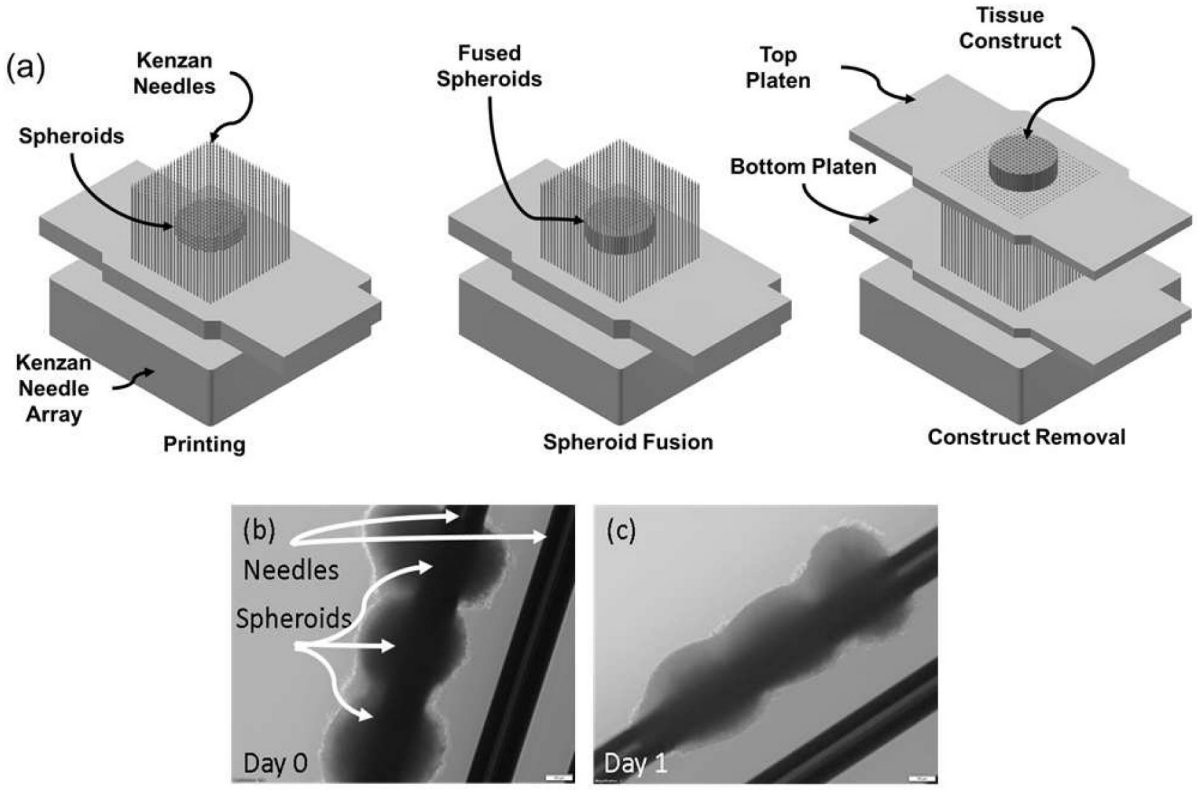

Figure 1: Kenzan Method Biofabrication.

(a) The Kenzan needle array is comprised of several needles arrayed in a 9×9 or a 26×26 pattern. A 26×26 Kenzan is shown in this image. Spheroids are impaled onto the Kenzan needles such that spheroids contact one another and fuse. Fused spheroids form a tissue construct. Tissue constructs can be removed by sliding the top platen from the bottom platen, keeping the construct intact. (b) Image of three spheroids on a Kenzan needle just after printing. (c) Image of fused spheroids on a Kenzan needle a day after printing. Needle diameters are 200 μm.

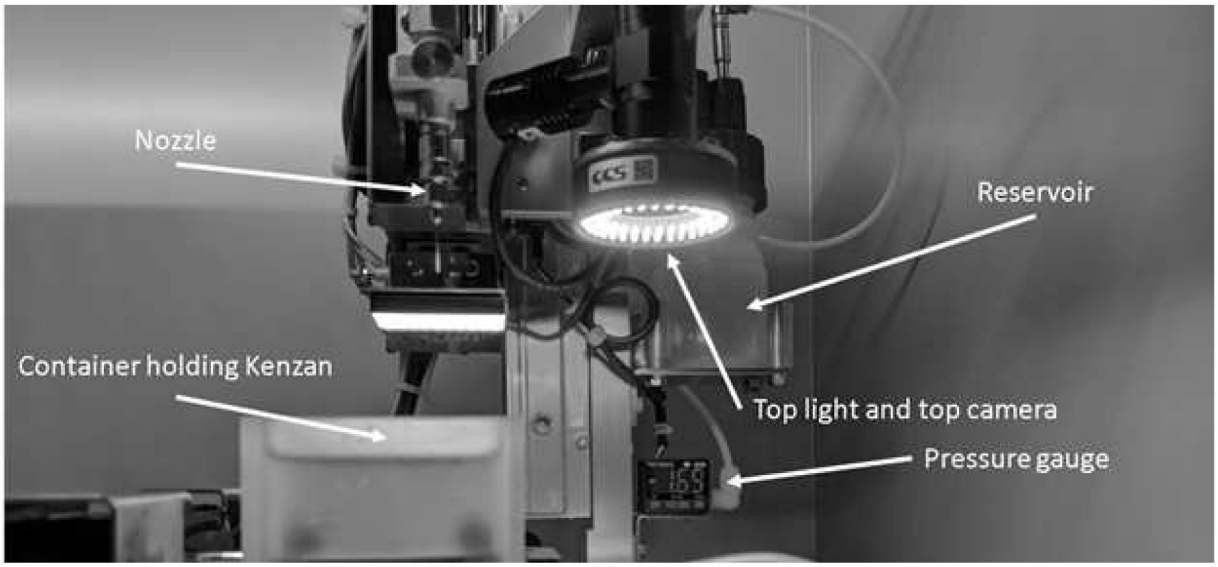

Figure 2: Regenova Biofabrication Components

Primary components of the Regenova Bio 3D Printer include camera and lighting components as part of the machine vision system, a pressure gauge to determine pressure applied to the spheroids as they are picked up, a nozzle connected to the reservoir system and the pressure gauge, and container holding the Kenzan needle array.

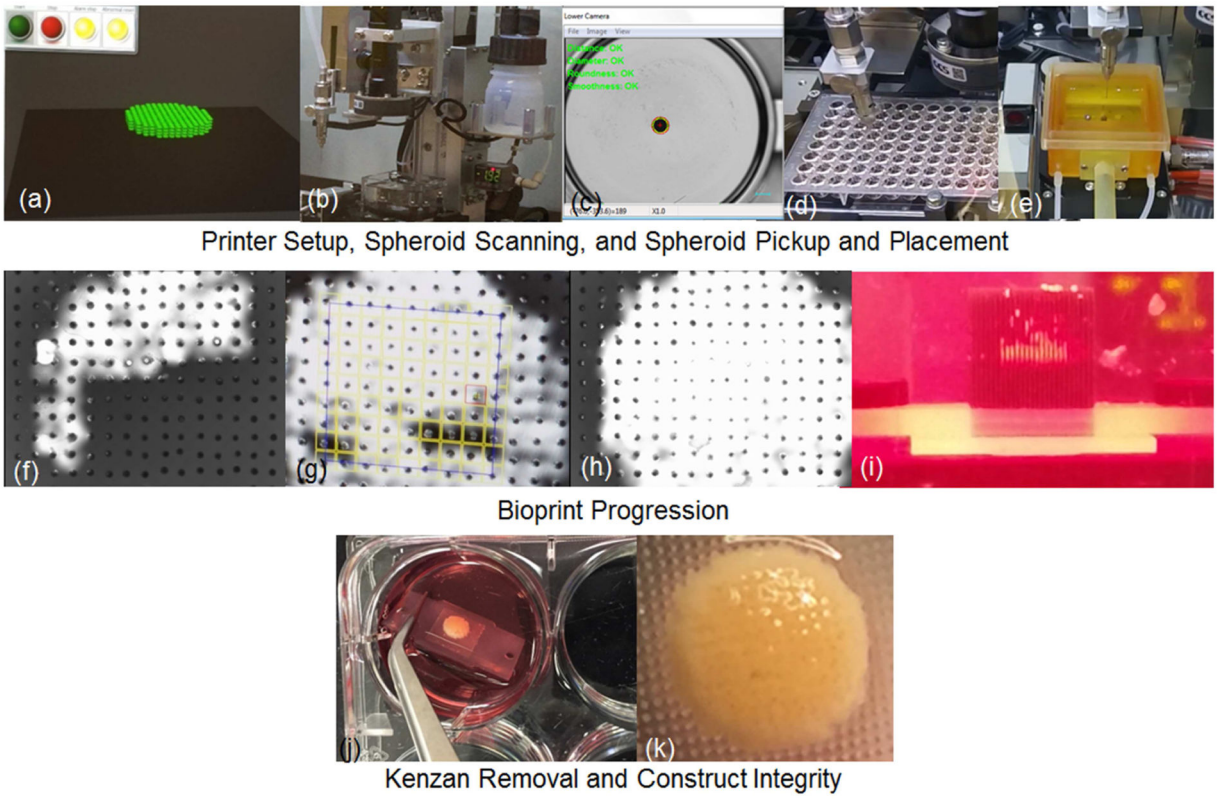

Figure 3: Bioprinting Preparation, Progression, and Construct Integrity.

(a)The construct is designed using “Bio 3D Designer”, software provided by the Regenova manufacturer. (b) The bioprinting components are installed into the bioprinter. The nozzle on the far left and the reservoir system bottle on the far right are used to aspirate each spheroid on to the nozzle. (c) The machine vision system inspects each spheroid for location, diameter in micrometers, roundness, and smoothness based on thresholds specified by the user. (d) Once a spheroid passes inspection, it is aspirated lightly onto the nozzle tip using the pressure system. (e) The nozzle, which is mounted onto a 6-axis gantry, is then placed over the Kenzan needle array and placed onto a preselected needle at the height corresponding with the construct design set forth using Bio 3D Designer. (f,g, and h) Bioprint progress is observed after each spheroid placement using the Regenova machine vision system. (i) Construct progression can also be observed manually from the side of the Kenzan container. (j and k) For Trial 4, the constructs were removed from Kenzans after 3 weeks to minimize handling and avoid construct disintegration.

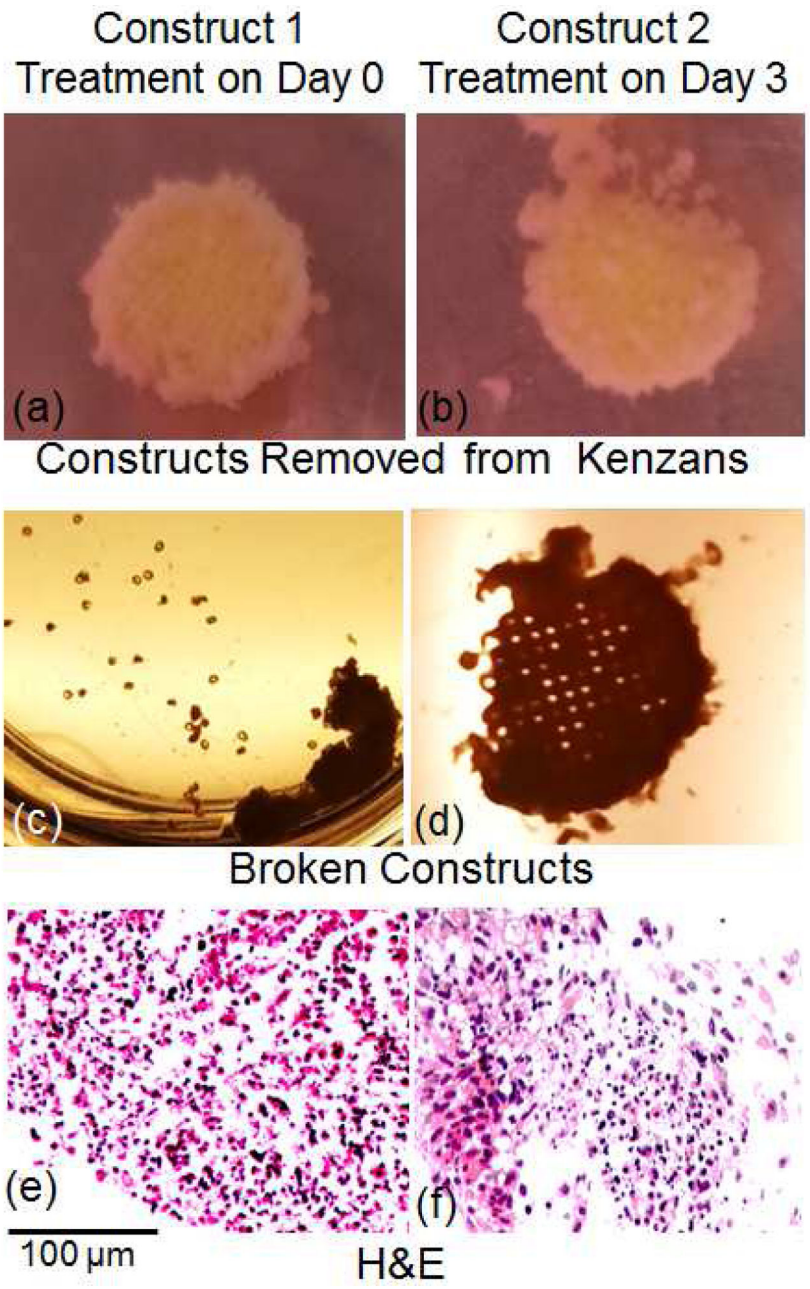

Figure 4: Trial 3 Construct Outcomes Following Osteogenic Differentiation 0 and 3 Days After Bioprinting.

(a and c) Constructs treated with osteogenic differentiation medium immediately following bioprinting disintegrated upon Kenzan removal, even after 2 weeks of culture on the Kenzan. (b and d) Constructs treated with differentiation medium 3 days after bioprinting, cultured on-Kenzan for 2 weeks, and then cultured off-Kenzan for 1 week showed increased integrity, yet still showed construct breakage and spheroid-level disintegration. Continued culture off-Kenzan resulted in a warped and misshapen construct that did not maintain original dimensions. Micropores left by the Kenzan needles are also visible. These pores provide Haversian canallike microchannels through which nutrients can be transported to the interior

of the construct. (e) Hematoxylin and eosin stain from tissue fragments of construct 1. (f) Hematoxylin and eosin stain from tissue fragments of construct 2.

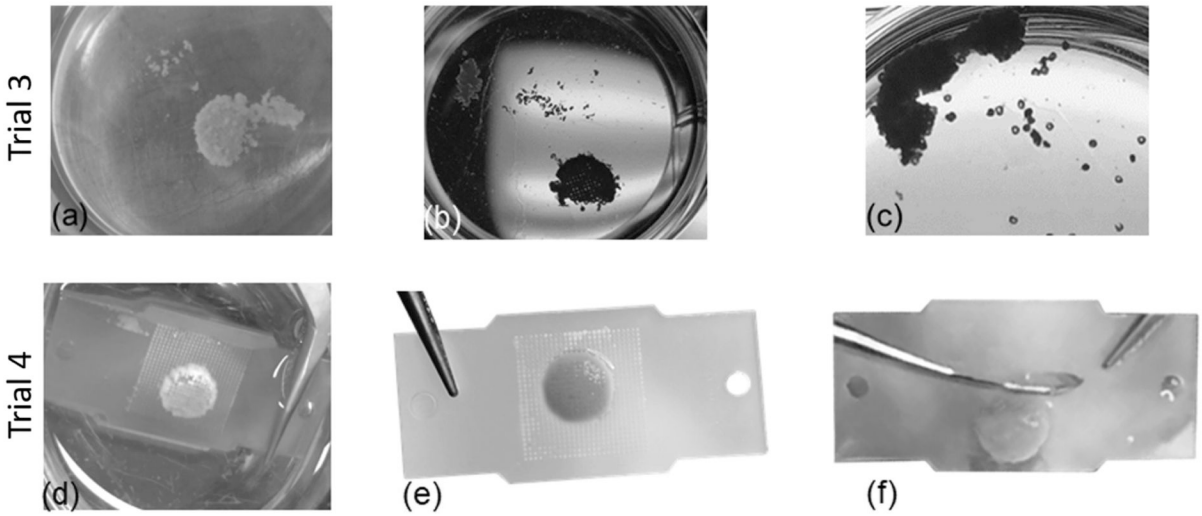

Figure 5: Trial 3 and 4 Constructs Outcomes

(a through c) Trial 3 constructs resulted in constructs with poorly-fused spheroids. (d through f) Trial 4 constructs maintained integrity and could withstand handling.

Table 1.

Bioprinting Trials, Corresponding Metrics, and Outcomes.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Trial #	**Passage #**	**Cells/well**	**Media**	**Method**	**Total #of plates**	**#of Nozzle Changes**	**Total print Time (Hours)**	**Average Print Time (Hours) per Construct**	**#of Spheroids Printed Per Hour**	**#of Constructs printed**	**Layers/ Construct**	**Maturation Time on Kenzan (Weeks)**	**Maturation Time Off Kenzan**	**Outcome**
1	24	20,000	Control	Gravity	10	40	10	5	65	2	2	1	2 Weeks	No Structural Rigidity, Misshapen, Disintegrated
2	30	20,000	Osteogenic	Centrifuge	14	13	7.5	3.75	173	2	4	2	1 Week, Petri Dish Contained 2% Agarose	No Structural Rigidity, Misshapen, Disintegrated
3	35	40,000	Osteogenic (Day 0) Osteogenic (Day 3)	Centrifuge	14	2	7	3.5	185	2	4	3	2 hours Petri Dish Contained 2% Agarose	Osteogenic Medium Day 0: Disintegrated Osteogenic Medium Day 3: Kept Integrity
4	37	40,000	Osteogenic (Day 3) Osteogenic (Day 7)	Centrifuge	14	3	5.5	2.75	236	2	4	3	0 Weeks	Kept Integrity, Easier to Handle
4	37	40,000	Osteogenic (Day 3) Osteogenic (Day 7)	Centrifuge	14	0	5.5	2.75	236	2	4	3	1 Weeks	Kept Integrity, Easier to Handle
4	37	40,000	Osteogenic (Day 3) Osteogenic (Day 7)	Centrifuge	14	2	5	2.5	260	2	4	3	2 Weeks	Kept Integrity, Easier to Handle