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Bioreactor Cultivation of Anatomically Shaped Human Bone Grafts

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Abstract

In this chapter, we describe a method for engineering bone grafts in vitro with the specific geometry of the temporomandibular joint (TMJ) condyle. The anatomical geometry of the bone grafts was segmented from computed tomography (CT) scans, converted to G-code, and used to machine decellularized trabecular bone scaffolds into the identical shape of the condyle. These scaffolds were seeded with human bone marrow-derived mesenchymal stem cells (MSCs) using spinner flasks and cultivated for up to 5 weeks in vitro using a custom-designed perfusion bioreactor system. The flow patterns through the complex geometry were modeled using the FloWorks module of SolidWorks to optimize bioreactor design. The perfused scaffolds exhibited significantly higher cellular content, better matrix production, and increased bone mineral deposition relative to non-perfused (static) controls after 5 weeks of in vitro cultivation. This technology is broadly applicable for creating patient-specific bone grafts of varying shapes and sizes.

Keywords

Scaffold; Bone; Anatomical; Craniofacial; MSCs

1 Introduction

Bone tissue engineering has the potential to generate defect-specific functional bone grafts for skeletal reconstruction. A successful bone graft would ideally match the mechanical and physiological properties of the implant location and should provide a platform for healing. Autograft is considered to be the gold standard of bone grafting due to its biocompatibility (prevention of immunogenic responses), osteogenicity (containment of osteoblasts and/or osteoprogenitors), osteoconductivity (recruitment of bone-forming cells), osteoinductivity (induction of osteoblastic differentiation), and native mechanical properties. However, several drawbacks related to its use—including donor-site morbidity and lack of defect-specific size and shape—highlight the need to develop more effective and practical tissue engineering techniques.

Early studies showed that bone tissue may be formed in vitro by growing osteoblastic cells on 3D scaffolds with culture medium containing osteoinductive factors. Subsequent studies demonstrated the potential to induce stem cells from embryonic and adult tissues to differentiate into osteoblasts and form bone tissue in response to biological and mechanical stimuli (1–3). The cells within the engineered bone grafts express osteogenic genes and mineralized extracellular matrix. The increased mineral content and bone-like architectures can increase the mechanical properties of the scaffold and result in tissue constructs with compressive properties approaching those of native bone tissue (4–6). The development of bioreactor systems capable of providing effective nutrient transfer to cells embedded within a scaffold has enabled the formation of large (centimeter-scaled) tissue engineered bone grafts in vitro with homogeneously distributed tissue (7–9). With such advancements, our group has demonstrated the ability to engineer anatomically shaped bone grafts from human adult stem cells with clinically relevant sizes by employing 3-dimensional (3D) scaffold fabrication techniques and an advanced cultivation bioreactor system. Engineering bone tissue in these specially designed cultivation systems offers the potential to provide an alternative source of a functional bone graft in lieu of autografts.

A method to successfully engineer anatomically shaped human bone grafts is described in extensive detail in this chapter, including adult stem cell cultivation, scaffold preparation and fabrication, cell seeding, bioreactor assembly, and bone graft cultivation (see Fig. 1). This process covers the technique for fabricating an anatomically shaped scaffold from a trabecular bone block and the design of a perfusion bioreactor system which delivers sufficient nutrients to the cells throughout the scaffold. The construct anatomy and reconstruction processes were adapted from computer-aided surgical planning which is a common process in surgical procedure for complex skeletal anatomy and fabrication of alloplastic grafts. In brief, the anatomically shaped graft was designed by segmenting out the region of interest from the 3D-reconstructed CT images of the patient's skull to replicate the specific geometry. The scaffold was prepared from decellularized, bovine trabecular bone and was machined using a 4-axis CNC milling machine. The perfusion bioreactor culture chamber was fabricated to match the exact shape of the bone graft to ensure a tight seal around the scaffold and ensure medium perfusion through the interstitial spaces within the construct rather than around the periphery. The perfusion pattern through the complex geometry was modeled using the FloWorks flow simulation module of SolidWorks and was used to optimize the bioreactor design. The assembly of the culture system and troubleshooting for commonly occurred malfunctions are explicitly described and the anticipated results discussed.

2 Materials

2.1 Reagents

2.1.1 Cell Culture—

- High-glucose Dulbecco's modified eagle medium (GIBCO, cat. no. 11995) (sterile).

- Fetal bovine serum, non-heat-inactivated (Atlanta Biological, cat. no. S11550) (sterile).
- Penicillin–streptomycin solution, 100× (Cellgro, cat. no. 30-002-CI) (sterile).
- Recombinant human FGF-basic/FGF-2 (Pepro Tech, cat. no. 100-18B) (sterile).
- β -Glycerophosphate disodium salt hydrate (Sigma, cat. no. G9891) (non-sterile).
- Dexamethasone (Sigma, cat. no. D1756) (non-sterile).
- L-Ascorbic acid-2-phosphate sesquimagnesium salt hydrate (Sigma, cat. no. A8960) (non-sterile).

2.1.2 Decellularization—

- 10× Phosphate buffered saline (PBS), pH 7.4 (Quality Biological, cat. no. 119069131) (sterile).
- Ethylenediaminetetraacetic acid (EDTA) (Sigma, cat. no. E6758) (non-sterile).
- Trizma[®] base (Tris) (Sigma, cat. no. T6791) (non-sterile).
- Sodium dodecyl sulfate (SDS) (Sigma, cat. no. L4390) (non-sterile).
- DNase I grade II (Roche Applied Sciences, cat. no. 10104159001) (non-sterile).
- RNase A (Roche Applied Sciences, cat. no. 10109142001) (non-sterile).

2.1.3 DNA Extraction and Quantitation—

- Trizma[®] base (Tris) (Sigma, cat. no. T6791) (non-sterile).
- Ethylenediaminetetraacetic acid (EDTA) (Sigma, cat. no. E6758) (non-sterile).
- Triton[™]-X 100 (Sigma, cat. no. T8787) (non-sterile).
- Proteinase K (Sigma, cat. no. P2308) (non-sterile).
- Quant-iT[™]PicoGreen[®] dsDNA Assay Kit (Invitrogen, cat. no. P7589).

2.2 Reagent Setup

2.2.1 Stock Solutions—

- Human Basic Fibroblast Growth Factor (bFGF): Prepare at least 10 mL buffer for reconstitution consisting of 5 mM Tris and 0.1 % (wt/vol) BSA, sterile filter and store at 4 °C until use. Add 50 μ g of lyophilized FGF-2 and centrifuge vial at 12,000 $\times g$ for 1 min. Reconstitute with 1 mL of buffer. Transfer contents to a 15 mL conical tube and add 9 mL of buffer. Split into 50 μ L aliquots and store at –20 °C. Thawed aliquots can be stored at 4 °C for up to 1 week.
- β -Glycerophosphate: 200 mM β -glycerophosphate dissolved in high-glucose DMEM, sterile filtered, aliquoted, and stored at –20 °C.
- Dexamethasone: 1 mM dexamethasone dissolved in 100 % ethanol, sterile filtered, split into 500 μ L aliquots, and stored at –20 °C.

- Ascorbic Acid-2-Phosphate: 5 mM ascorbic acid dissolved in high-glucose DMEM, sterile filtered, split into 1 mL aliquots, and stored at -20°C .

2.2.2 Cell Culture Media—

- Expansion medium: 10 % (vol/vol) FBS, 1 % (vol/vol) pen—strep, and 0.1 ng/mL bFGF. Expansion media should be stored at 4°C . Once supplemented with bFGF, medium can be used for up to 1 week.
- Osteogenic differentiation media: 10 % (vol/vol) FBS, 1 % (vol/vol) pen—strep, 10 mM sodium- β -glycerophosphate, 1 nM dexamethasone, 50 μM ascorbic acid-2-phosphate with the remaining volume made up with high-glucose DMEM. Osteogenic media should be made fresh weekly and stored at 4°C .

2.2.3 Bone Decellularization Solutions—

- Detergent wash 1: 0.1 % EDTA (wt/vol) in 450 mL deionized water and 50 mL $10\times$ PBS. Stored at room temperature.
- Detergent wash 2: 0.1 % EDTA (wt/vol) and 10 mM Tris in 500 mL deionized water. Stored at room temperature.
- Detergent wash 3: 10 mM Tris and 0.5 % SDS (wt/vol) in 500 mL deionized water. Stored at room temperature.
- Enzymatic wash: 50 units/mL DNase, 1 unit/mL RNase, and 10 mM Tris in 450 mL deionized water. Stored at 4°C for up to 1 month.

2.2.4 DNA Extraction and Quantitation—

- DNA extraction solution (TEX + proteinase K): 10 mM Tris, 1 mM EDTA, 0.1 % Triton-X, and 0.1 mg/mL proteinase K stored at -20°C .
- DNA quantitation: Following protocol for Quant-iT™ Pico-Green® dsDNA Assay Kit with a cell solution with known cell number to determine the amount of DNA per cell.

2.3 Equipment

- Transparent vacuum desiccator (Thermo, cat. no. 53110250).
- 16-G Luer-Lok™ needles (Fisher, cat. no. 1482618A) (sterile).
- 4 oz. screw-top polypropylene histology container (Fisher, cat. no. 22026310) (sterile).
- Incubator.
- Biological safety cabinet.

2.4 Bone Segmentation

- Bandsaw appropriate for segmenting bone and cutting bioreactor components. (Optional) Water-cooled bone bandsaw (Mar-Med, cat. no. 80) reduces dust and splatter.
- Bandsaw blades: it can be useful to have multiple as they tend to break. (Optional) Diamond bandsaw blade (Mar-Med, cat. no. 75) will not cut hands and easily slices bone.

2.5 Bioreactor

- Screw-top polypropylene histology containers (Fisher, cat. no. 22026311) (non-sterile).
- Three-prong swivel ring stand clamps (Fisher, cat. no. 02300209).
- Disposable 20 mL Luer-Lok™ syringes (Fisher, cat. no. 148232B) (sterile).
- 100 mL Glass media bottles with cap (Fisher, cat. no. FB800100) (non-sterile).
- 20" Ring stand (Fisher, cat. no. 14675BQ).
- 26-G Luer-Lok™ needles (Fisher, cat. no. 1482610) (sterile).
- 23-G, 1" length Luer-Lok™ needles (Fisher, cat. no. 14826A) (sterile).

2.6 Milling

- Starrett 0.2" tip edge-finder (McMaster-Carr, cat. no. 20535a653).
- 3/16" dia. solid carbide ball endmill (MSC Industrial Supply, cat. no. 07766645).
- 3/16" dia. screw machine length twist drill bit (McMaster-Carr, cat. no. 2908A39).
- 1 ft long, 25 mm dia. white Delrin® acetal plastic rod (McMaster-Carr, cat. no. 8572 K61).
- Anti-fog, anti-scratch safety glasses (MSC Industrial Supply, cat. no. 89972509).
- Shop-Vac 6-gal 3-HP wet/dry vacuum (Aubuchon Hardware, cat. no. 118208).
- (Optional) 8" Black oxide hand file (McMaster-Carr, cat. no. 42405A45).
- (Optional) High-pressure precision compressed air can (McMaster-Carr, cat. no. 8431 K22).
- (Optional) USB jog dial (LittleMachineShop.com, cat. no. 3414).

2.7 Software

- Mimics Innovation Suite (Materialise).
- SolidWorks (Dassault Systèmes).
- Mastercam (Mastercam).
- Mach3 (ArtSoft USA).

3 Methods

3.1 3D Reconstruction from CT

1. Open Materialise Mimics and select File > Open. Navigate to desired DICOM directory file and import it. Follow the software prompts to setup the file and orient the slices.
2. Select *Segmentation > Thresholding* and select the *Bone* preset from the dropdown menu. Click *OK*.
3. Scan through the slices to make sure the mask is correctly selecting bone. Adjust the threshold cutoffs from the previous menu if necessary.
4. The *Multiple Slice Edit* and *Edit Mask* tools (accessed from the *Segmentation* menu) can add or remove areas of the mask and separate areas into new masks if necessary.
5. Select *Segmentation > Calculate 3D*, choose *Optimal* under *Quality* and click *OK* to generate a 3D model of the selected mask. Depending on the computer used to run Mimics, you may need to adjust the *Quality* settings.
6. To isolate a specific area of the scan as a model (in this case, the temporomandibular joint), select *Segmentation > Edit Mask in 3D*. Mimics will display a white bounding box on each mask viewpoint. Adjust this bounding box to crop the mask rendering. In the 3D window, draw around the area to be isolated. It will change color.
7. Select the *Separate* button to create a new mask from the selected area. A new model can be generated from this cropped mask in the same way as in step 5.
8. (Optional) Right clicking on the model name in the *3D Objects* tab and selecting properties
9. The *Smoothing*, *Triangle Reduction*, and *Wrap* features (accessed from the *Tools* menu) are useful for cleaning up the geometry of the model. Avoid overusing these features as they modify the geometry, decreasing the model's correlation to the original scan data.
10. Select *Export > Binary STL*. Under the 3D tab, select the model to be exported and click the *Add* button. Select *Finish* to generate a .stl file of the model.

3.2 CNC Toolpath Generation (Fig. 2)

1. Open SolidWorks and import the STL file from previous step.
2. Insert a reference plane on the base of the TMJ, coplanar with the axial segmentation plane of the anatomy (Fig. 2a).
3. Insert a sketch and draw a circle of diameter sufficient to enclose the entire silhouette of the TMJ, with its center coincident with the centroid of the silhouette (Fig. 2b).

4. Exit the sketch and create an extruded base feature extending at least 1 cmin the inferior to the base of the condyle (in anatomic orientation) (Fig. 2c).
5. Orient the modified TMJ such that the axis of the cylindrical extruded base is coaxial with the x -axis of the world coordinate system, with the TMJ anatomy along the positive direction (Fig. 2c).
6. Insert a sketch on either the top or front reference plane. Sketch a single point along the x -axis approximately 1 mm to the right of the most positive extent of the geometry.
7. Exit the sketch and move the modified TMJ such that the sketched point is coincident with the origin of the world coordinate system.
8. Measure and record the distance from the origin to the start of the cylindrical feature, as well as the diameter of the cylinder. These parameters are required for later fabrication steps.
9. Export the modified TMJ in IGES format and open in Mastercam.
10. Select the milling machine type from the menu that is associated with the CNC controller installed on your machine.
11. In order to machine geometry accurate to the 3D model, maximum rigidity during the milling process is desired. To achieve this, the toolpaths are subdivided into sections progressing from the material region furthest from the fixture to the regions closest to the fixture. For the human TMJ, using two sections has proven sufficient. The subsequent steps define these regions and the toolpaths associated with them:
12. Orient the view to the right side, looking at the YZ plane. Sketch a circle centered on the x -axis at a negative x value equal to half the axial anatomic length and with a diameter approximately double the bulk diameter of the TMJ.
13. Extrude the circle in the positive x direction at least 5 mm further than the most positive point on the 3D model to create a cylinder defining a tool containment boundary.
14. Create a rotary 4-axis toolpath using a 3/16" ball endmill at a feed rate of 9 IPM (inches per minute) and 3000 RPM. Use the TMJ surface to generate the toolpath, and use the cylinder from the previous step to establish the tool containment boundary. For this initial toolpath, leave approximately 0.020" on the drive surface. The 4-axis toolpath should be setup as an axial cut with 10° increments for the full 360° rotation.
15. Duplicate the 4-axis toolpath created in the previous step, and mill the remaining material (remove the 0.020" offset and set to zero). Modify the toolpath to have an axial cut with 2° increments.
16. Orient the view to the right side, looking at the YZ plane. Sketch a circle centered on the x -axis at a negative x value equal to half the axial anatomic length plus 1/4"

(a value at least the size of the cutting tool) and with a diameter approximately double the bulk diameter of the TMJ.

17. Extrude the circle in the negative $\times$ direction at least 5 mm further than the most negative point on the anatomic surface to create a cylinder defining a second tool containment boundary.
18. Duplicate the existing toolpaths (the original 4-axis toolpath and its modified duplicate) and modify each of the two new toolpaths to use a containment boundary defined by the cylinder created in the previous step.
19. Generate G-code based on the entire set of toolpaths using a post-processor appropriate for your milling machine. Save the text file generated in an appropriate format (e.g., .txt or .nc) and transfer to your milling machine's controller.

3.3 Bioreactor Design and Optimization

3.3.1 Bioreactor Design—

1. In SolidWorks, open the STL file of the TMJ anatomy.
2. As a separate part, model a syringe needle with a standard gauge. In this case, 26G needles were chosen.
3. Create an assembly of the scaffold, and add multiple needles to serve as perfusion ports. We chose three ports arrayed uniformly in the radial orientation, and anatomically targeted in the axial direction.
4. The needle ports are designed so that media exits the scaffold through the needles, and enters the scaffold axially from the lowermost section of the geometry.
5. The needles are targeted in such a way as to allow the entire scaffold to be perfused, and are placed at extremities of the scaffold (medial, lateral, and superior extents).
6. As a separate part, model a syringe casing that is of sufficient size to enclose the entire scaffold, plus an additional 3 cm in length. Do not model the plunger—only the casing plus syringe port is needed. Add a 3/16" hole in the radial direction that cuts through the entire case (both sides). This hole should be approximately 1 cm from the top of the syringe casing (the end opposite of the Luer-Lok port).

3.3.2 Mold Assembly—

1. As a separate part, model a tube with the same inner and outer diameters of the syringe casing, and with a length at least three times the length of the TMJ. Add two 3/16" holes along the length of the tube that cut radially through the entire tube, and are spaced approximately twice the length of the TMJ, and centered along the length of the tube (Fig. 3a).
2. As a separate part, duplicate the TMJ scaffold with the 1 cm long cylindrical base (from step 14) with a diameter equal to the inner diameter of the syringe tube. Add a 3/16" hole in the radial direction in the center of this cylindrical feature

3. As a separate part, model a cylinder of 2" length and 3/16" diameter.
4. Create an assembly from two of these cylinders, the scaffold with cylindrical feature, and the tube created in previous steps. Mate one of the 3/16" holes in the tube with the same size hole in the modified scaffold and mate this combination with one of the cylinders. Create a cylindrical mate to align the modified scaffold within the center of the tube, such that the TMJ geometry is contained within the tube. Align the remaining 3/16" diameter cylinder with the remaining hole in the tube (Fig. 3b).
5. The assembly created in the previous steps represents a mold in which to cast a PDMS component, which will be used to surround the TMJ bone scaffold. For purposes of modeling flow rates during perfusion, this PDMS component will need to be modeled in SolidWorks.
6. Insert a new part into the mold assembly and create a cylinder with diameter equal to the inside diameter of the tube and of a length to match the tube. Perform a Boolean subtract operation on this assembly to create the PDMS component. The result should show a TMJ shaped cavity within a cylinder with a second cylindrical hole above the TMJ running in the radial direction.

3.3.3 Bioreactor Assembly—

1. Open the bioreactor assembly and insert the PDMS component, the syringe casing, and one 3/16" cylindrical component, which will be used as an alignment rod (Fig. 4a).
2. Align and mate the scaffold within the PDMS cavity. Next, align the PDMS cavity to be internal and concentric with the syringe casing. Align the 3/16" hole in the PDMS cavity with the 3/16" hole in the syringe casing and mate the 3/16" diameter alignment rod to pass through these holes (Fig. 4b).
3. Create a new part and model the housing that supports and surrounds the syringe casing assembly, leaving the top open to the alignment rod. Cut a channel in the top of the housing for the alignment rod. Cut holes in a radial orientation aligned with each perfusion needle (Fig. 4a–c).
4. Create a new part and model a cap that surrounds the top of the syringe casing and extends slightly below the alignment rod. Cut a channel in the cap for the alignment rod. For the inner diameter of the cap that interfaces with the syringe casing, decrease the diameter by approximately 0.5 mm to achieve a press fit during the assembly process (Fig. 4a–c).

3.3.4 Fluid Modeling—

1. Create a new fluid simulation on the bioreactor assembly.
2. Edit the PDMS part and remove the material where the internal diameter of the needle intersects the PDMS. There should be a channel from the outside of the PDMS to the internal TMJ cavity where each needle is placed.

3. Modify each needle and the bottom of the syringe casing (the port) and model a plug on each opening to close the tube.
4. The faces of these plugs are used to specify boundary conditions for the fluid modeling. In the fluid simulation setup create an inlet flow rate of 1 mL/min at the syringe port. For each needle port opening, model this boundary interface as open to ambient pressure
5. In the fluid model, define the scaffold as a porous medium. (A value of 0.7 represents 70 % porosity.) The fluid viscosity should be adjusted to represent the media perfused through the scaffold. In our modeling, we used water as the fluid.
6. Run the fluid model and create a velocity map on several cross sections of the scaffold (Fig. 5a, b). The velocity will always increase near the needle port exits. There will also be variations in flow direction and velocity due to the complex geometry of the graft. However, flow should be optimized to be fairly uniform throughout the bulk of the scaffold. To adjust the perfusion through the scaffold, needles can be repositioned, more can be added, or their sizes can be changed to achieve the desired flow throughout the scaffold.
7. Once the flow model has been refined, save the final geometry and fabricate the needle guide based on the model's results (Fig. 5c).

3.4 Bioreactor Fabrication

3.4.1 Component Construction—

1. Alignment rod: cut a 3/16" stainless steel, alloy 316 rod to length using a bandsaw and de-burr the edges.
2. Syringe casing: cut a 30 mL syringe to length using a bandsaw and drill a 3/16" hole at specified location using a vertical milling machine. You may also wish to pre-drill holes for needles to enter the casing more easily. For this, you will need a hexagonal collet block, and a collet of appropriate size to hold the syringe casing. This allows the user to drill small pilot holes at 60° angular increments on the length. For other angles, a fourth axis is needed, which can be manually operated or automated.
3. PDMS mold: see next section.
4. Cap: this component is made from polyetherimide and machined to specifications using a lathe. The component is then secured on a milling machine to cut the channel for the alignment rod.
5. Housing: the concentric cylindrical cuts of this component are made first on a lathe in acrylic or polycarbonate plastic. The syringe-case-port end of the housing should have an additional 1" diameter extrusion machined about 0.5" length to serve as a fixing feature for holding in a collet block or fourth axis collet for machining on a vertical milling machine. On the milling machine, drill the holes needed for the perfusion needles, as well as the channel for the alignment rod.

3.4.2 PDMS Mold Pouring—

1. Remove the plunger from a 20 mL syringe. Cut off the Luer-Lok[®] end 2" from the tip. The Luer-Lok[®] end will be used in the bioreactor, while the remainder will serve as the mold casing.
2. Using a 3/16" twist drill bit, drill two holes, 1.5" apart from each other, through both sides of the syringe.
3. Fit the drilled syringe casing over the TMJ mold machined from acetal plastic and insert a metal rod through each hole.
4. Weigh out 12.36 g of the PDMS base mixture, tare the balance, and add 1.236 g of the curing agent. Mix components well (Note 1).
5. Transfer the mixture to a vacuum desiccator and degas until there are no bubbles visible in the mixture (30–60 min).
6. Remove mixture from the vacuum and pour the PDMS into the syringe casing smoothly and quickly to avoid incorporating more air. Return to the vacuum desiccator until bubbles are gone (up to 1 h).
7. Meanwhile, preheat the oven to 80 °C. Transfer mold to oven and bake for 30–45 min.
8. To remove the PDMS from its casing, the syringe must be cut open. Using wire cutters, carefully cut into the plastic and gradually peel and cut it away from the PDMS. Next, make a small incision along the length of the TMJ and pull the PDMS off of the acetal mold.

3.5 Bone Blank Preparation

1. Using a sharp surgical blade, separate the femur and tibia of the knee of 2–4 month old calves by severing the ligaments. Remove the meniscus and clean as much connective tissue and fat from around the joint as possible. The distal femoral condyle generally yields the more uniform trabecular bone than the tibia.
2. Using a bandsaw, cut through the femur about 10 cm above the condyle and discard the diaphysis.
3. Shave off a small slice of bone (about 1 cm thickness) along the length of the section to reveal the growth plate. Slice the remainder into a block with dimensions roughly approximating 8 × 4 × 4 cm with an adequately large section of trabecular bone not split by the growth plate. The same process can be repeated to varying degrees of success (due to smaller size) with the tibial condyle.
4. Depending on the lathe being used, you may need to cut away the thickness on the end of the block to facilitate firm grasping by the lathe chuck jaws.

¹Both the base mixture and curing agent have low viscosities. Pour slowly to avoid overshooting.

5. Clean the block with a high velocity water jet. It will not be possible to remove all marrow from the inside of the block, but washing at this stage reduces splatter while turning.
6. Secure the block in the lathe chuck, tightening enough to keep the block snug, but taking care not to crush or warp the block. Center the block axially within the chuck so it does not wobble heavily while spinning (Note 2).
7. Turn on the lathe and begin cutting at the widest portion of the bone, sweeping across the bone horizontally. After each pass, increase the cutting depth by 1 mm. Stop cutting once the bone cylinder reaches a diameter of 25 mm.
8. Clean the cylinder with a high velocity water jet, wrap in aluminum foil, and freeze at -20°C to store.
9. Cleaned bone blank cylinders may be stored in aluminum foil at -20°C for up to 3 months.

3.6 TMJ Scaffold Decellularization

1. Place scaffolds in detergent solution 1 and place on rocker for 1 h at room temperature ($18-25^{\circ}\text{C}$).
2. Repeat previous step, but in detergent solution 2 for at least 12 h at 4°C .
3. Remove scaffolds from detergent and transfer to PBS for 1 h at room temperature ($18-25^{\circ}\text{C}$).
4. Transfer scaffolds to detergent solution 3 and place on rocker for at least 24 h at room temperature ($18-25^{\circ}\text{C}$).
5. Place scaffolds in fresh PBS and agitate for 1 min. Drain PBS and replace with fresh PBS. Continue washing until bubbles do not form in the PBS when agitated (this is a sign that all detergent has been removed).
6. Transfer scaffolds to enzymatic wash solution and place on rocker for 5 h at room temperature ($18-25^{\circ}\text{C}$).
7. Rinse scaffolds extensively first with PBS and then with deionized water.
8. Prior to use in culture, place in 100 % ethanol overnight on the rocker at room temperature ($18-25^{\circ}\text{C}$) (Note 3).

3.7 Scaffold Machining

1. Set Mach3 to millimeters using the Settings tab.
2. Fourth-axis machining is performed using a rotary table mounted on the mill, providing the A axis in addition to the XYZ axes. Place the rotary table on the mill

²Turning bone will splatter marrow over large distances. It is advisable to cover as much of the body, face, and surrounding area as possible when performing this step. Freezing the bone block immediately prior to lathing may also reduce splatter.

³Decellularized scaffolds should be lyophilized prior to storage. Clean, lyophilized scaffolds can be stored at 4°C indefinitely.

table with the chuck facing along the *X*-axis and fasten it loosely with the corresponding bolts.

3. Load a dial indicator into the collet of the mill. This indicator will be used to align the rotary table parallel with the axis of the mill.
4. Step the indicator up to a corner of the rotary table, using the dial to find the precise edge.
5. Jog the indicator along the edge of the rotary table to the opposite side. If the indicator shows pressure, lightly tap the rotary table away from the indicator with a rubber mallet until the dial returns to zero. If no pressure is shown, step the indicator up to the rotary table's exact edge and repeat this step.
6. Tighten the bolts securing the rotary table, but not completely. Repeat steps 4 and 5 and tighten down the bolts all the way.
7. Secure the bone cylinder in the rotary chuck, seeking to align it horizontally along the *X*-axis. Avoid heavily crushing the bone while tightening.
8. Now that the workpiece is secured, you will need to define the machining origin (0, 0, 0). This point will be located in the center of the bone cylinder at the end farthest from the rotary table. Load an edge-finder into the collet of the mill. Slightly displace the tip of the edge-finder from its central axis and start the spindle at a low RPM (300–600).
9. Step the edge-finder up to the edge of the cylinder that extends along the *X*-axis. As the edge-finder contacts the edge of the workpiece, the workpiece will gradually center the tip of the edge-finder. When the tip is spinning precisely in alignment, the machine is half the radius of the tip from the workpiece.
10. In Mach3, select the *Y*-axis coordinate readout and type in the distance from the central axis of the cylinder. This distance is the radius of the edge-finder tip (in millimeters), added to the radius of the cylinder.
11. To zero the *X*-axis, repeat step 9, using the face of the cylinder as the edge.
12. Select the *X*-axis coordinate readout in Mach3 and type in the distance from the face of the cylinder. This distance is the radius of the edge-finder (in millimeters). This number should be negative.
13. To zero the *Z*-axis, load the 3/16" ball endmill into the collet of the mill.
14. Step the *Z*-axis down until there is a slight drag on a sheet of copy paper slid between the workpiece surface and the cutting tool. This point is approximately 0.1 mm above the workpiece.
15. Select the *Z*-axis coordinate readout in Mach3 and type in the radius of the workpiece added to the thickness of the paper (0.1 mm). All zeros should now be defined (Note 4).
16. Press Cycle Start to begin the program (Note 5).

3.8 Scaffold Cell Seeding and Incubation

1. Scaffolds should be sterilized in 70 % EtOH overnight prior to cell seeding.
2. Remove the lid lining from one seeding container and loosely cap it with the lid. Autoclave the container and a magnetic stirrer bar of appropriate length.
3. To construct the seeding container, make holes in the lid of the plastic with a 16-G needle, three holes per scaffold to be seeded.
4. Expand MSCs to a cell count of 30 million and resuspend in 30 mL of expansion medium (1×10^6 cells/mL).
5. Under sterile conditions, securely attach the scaffold to the container lid needles.
6. While remaining sterile, add the stirrer bar to the seeding container and transfer the cell suspension to the container.
7. Invert the lid and submerge the scaffolds in the cell suspension. Place the container on a magnetic stirrer at 300 RPM.
8. Allow the scaffold to seed for 1 h in the incubator.
9. Remove the scaffold from the seeding container and transfer it to a 50 mL tube containing 10 mL of osteogenic medium. Allow the scaffolds to culture statically for 7 days in the incubator.
10. To determine seeding density, a scaffold is taken from the conical tube 1 day after seeding. DNA is extracted in 5 mL TEX with 0.1 mg/mL proteinase K overnight at 56 °C and the total DNA is quantified with Quant-it™ PicoGreen® assay kit. Cellular density can be determined based on a DNA extraction of a known cell number and total scaffold volume base on 3D image reconstruction.

3.9 Bioreactor Assembly

1. Place bioreactor components (syringe casing, PDMS gasket, alignment rod, cap, and needle guide), tubing (with attached needles and screws), media reservoir, and forceps into autoclave pouches and sterilize using the “liquid cycle,” 121 °C (see Fig. 6 for bioreactor components and assembled bioreactor).
2. Assembly requires two people: One who handles the sterile parts of the assembly, and one who prepares the components. The sterile person should put on sterile gloves before proceeding. Throughout the process, the non-sterile person will open autoclave pouches, handle the outsides of components, and position tools for the person doing the sterile assembly.
3. Remove the forceps from their autoclave pouch and place into a 50 mL conical tube filled with 70 % EtOH. This will keep the forceps clean while not in use.

⁴It is generally best to “cut air” before running an unfamiliar program to make sure nothing unexpected is taking place in the code. To do this, jog the machine to a position safely above the part, set the Z-axis location to zero, turn off the spindle, and then run the program.

⁵Avoid sending the machine to (0, 0, 0) as the origin is now defined inside the part. Load the TMJ G-code file in Mach3 and check the tool path preview to ensure that the controller is configured properly.

4. Fill the lid of a large sterile petri dish with sterile PBS. If at any time, a component needs to be set down, it can be placed in this dish. In addition, the PBS will aid in inserting the PDMS gasket into the syringe casing.
5. Remove the PDMS mold from its pouch.
6. Remove the forceps from the ethanol and let dry for a few seconds. Since ethanol is toxic to cells, it is important to let it evaporate.
7. Using the forceps, remove the TMJ scaffold from the media and place it inside the PDMS gasket.
8. Remove the syringe casing from its pouch and slide the PDMS gasket into it, taking care to align the holes while inserting. Rolling the PDMS in the sterile PBS may help lubricate the gasket for insertion. This step requires a significant amount of force to fit the gasket into its casing.
9. Insert the alignment rod into the hole in the syringe casing. Leave enough of the rod on one side to properly clamp the bioreactor later.
10. Place the syringe casing into the needle alignment guide, lining up the alignment rod with the grooves on top of the guide.
11. Press fit the bioreactor cap on top of the assembly and set the bioreactor down onto the Petri dish.
12. Remove the media reservoir from its autoclave pouch. Place a sterile filter into the thick tubing to allow for air exchange. Fill the reservoir with 80 mL of osteogenic medium.
13. Remove the needle tubing one-at-a-time from the autoclave pouch and connect the open end of the tubing to the media reservoir.
14. Disconnect the tubing from the needle end and hand the needle to the non-sterile assistant. Thread the screw into the guide, taking care that the needle does not deflect up or down. Thread the screw all the way in, and verify that the needle penetrates the scaffold.
15. Reconnect the tubing. Repeat step 14 with the remaining two needle tubes.
16. Remove the pump tubing and connect the open end to the media reservoir. Place the thick section of the tubing into the peristaltic pump cassette. Be sure to line up the cassette arrow with the proper direction of flow. Attach the cassette to the pump, again verifying that the flow proceeds through the base of the bioreactor and out the needles. Do not connect to the bioreactor yet.
17. Turn on the pump at a low speed and wait until media almost reaches the other end of the pump tubing. At this point, stop the pump. This step ensures that air will not be pumped through the scaffold.
18. Tightly connect the other end of the pump tubing to the bioreactor's bottom port.
19. Connect the open end of the feeding tube to the media reservoir and a 10 mL syringe to the opposite end of the feeding tube.

20. Turn on the pump and watch the needle tubes for flow. If there is no flow through all three needle tubes, see Note 6.
21. Watch the timing of the drops of media into the reservoir, and use the flow restriction clamps to equalize the flow rate through each tube so the drops emerge into the reservoir at the same rate.

3.10 Bioreactor Culture

1. During culture, medium should be changed every 3 days. Remove the feeding syringe from the incubator and transfer it to the biological safety cabinet.
2. Use the syringe to remove and subsequently replace 40 mL of medium in the reservoir.
3. Wrap the bioreactor in fresh Parafilm and return to the incubator.

3.11 Anticipated Results

3.11.1 Cellular Density—The seeding efficiency calculated after 1 day after seeding was approximately 34 %, resulting in a cellular density of approximately 3.4×10^6 cells/mL of tissue. The cultivation of the scaffold in static culture for 1 week allowed the cells to become firmly attached to the scaffold prior to exposing cells to perfusion. Cells proliferated extensively over the first week of culture, as evidenced by an approximately 7.5-fold increase in DNA content. Cultivation in perfusion bioreactor further increased the DNA content by an approximately 75-fold increase relative to the initial seeding value as opposed to an only 37-fold increase in DNA content when cultured in static condition. As a result, the total cellular density in the scaffold under perfusion culture was approximately 250×10^6 cells/mL tissue. Histological analysis showed homogenous cell distribution throughout the construct (Fig. 7a).

3.11.2 Bone Matrix Deposition—Over the cultivation period, MSCs differentiated and deposited new tissue throughout the entire tissue volume. In addition to cellularity, histological sections demonstrated osteoid formation patterns throughout the constructs (Fig. 7b). SEM images showed pore spaces that were densely packed by cell and matrix structure (Fig. 7c). The increase in mineral content was also evident from the 3D reconstructions of μ CT images (Fig. 7d, e). After 5 weeks of cultivation, the bone volume in constructs grown in the perfusion bioreactors increased by approximately 11.1 %.

⁶Flow through the needle tubes may be prevented by a number of reasons:

- a. PDMS has clogged the needle and is preventing flow. In this case, remove the needle, disconnect it from its tubing, and use a fresh syringe to perfuse sterile PBS through the needle, unclogging it. Once the needle is unclogged, reinsert it into the hole in the TMJ chamber.
- b. Flow has not been properly equalized with the clamps, causing all of the media to flow through one tube. In this case, tighten the clamp on the tube with flow until flow is observed in the other tubes.
- c. Holes in the syringe casing from multiple needle insertion trials are leaking the media rather than directing flow through the needle tubing. Proper needle insertion technique should prevent this from occurring. If it does occur, the assembly may need to be stopped and a new syringe-mold complex built.

References

1. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc Natl Acad Sci USA*. 2002; 99:12600–12605. [PubMed: 12242339]
2. Marolt D, Marcos Campos I, Bhumiratana S, Koren A, Petridis P, Zhang G, Spitalnik P, Grayson W, Vunjak-Novakovic G. Engineering human bone from embryonic stem cells. *Proc Natl Acad Sci USA*. 2012; 109:8705–8709. [PubMed: 22586099]
3. Fröhlich M, Grayson WL, Marolt D, Gimble J, Velikonja NK, Vunjak-Novakovic G. Bone grafts engineered from adipose-derived stem cells in perfusion bioreactor culture. *Tiss Eng A*. 2010; 16:179–189.
4. Bhumiratana S, Grayson WL, Castañeda A, Rockwood DN, Gil ES, Kaplan DL, Vunjak-Novakovic G. Nucleation and growth of mineralized bone matrix on silk-hydroxyapatite composite scaffolds. *Biomaterials*. 2011; 32:2812–2820. [PubMed: 21262535]
5. Rockwood DN, Gil ES, Park SH, Kluge JA, Grayson W, Bhumiratana S, Rajkhowa R, Wang X, Kim SJ, Vunjak-Novakovic G, Kaplan DL. Ingrowth of human mesenchymal stem cells into porous silk particle reinforced silk composite scaffolds: An in vitro study. *Acta Biomater*. 2011; 7:144–151. [PubMed: 20656075]
6. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG. Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. *Proc Natl Acad Sci USA*. 2003; 100:14683–14688. [PubMed: 14657343]
7. Grayson WL, Marolt D, Bhumiratana S, Fröhlich M, Guo XE, Vunjak-Novakovic G. Optimizing the medium perfusion rate in bone tissue engineering bioreactors. *Biotechnol Bioeng*. 2011; 108:1159–1170. [PubMed: 21449028]
8. Ishaug SL, Crane GM, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J Biomed Mater Res*. 1997; 36:17–28. [PubMed: 9212385]
9. Grayson WL, Fröhlich M, Yeager K, Bhumiratana S, Canizzarro C, Wan LQ, Chan E, Liu X, Guo XE, Vunjak-Novakovic G. Engineering anatomically-shaped human bone grafts. *Proc Natl Acad Sci USA*. 2010; 107:3299–3304. [PubMed: 19820164]

Fig. 1.

Schematic of process for generating anatomically shaped human bone grafts. (*Step 1*) 3D reconstructive process used to generate a CAD-compatible 3D model from raw CT scan data. (*Step 2*) CAD model used to design and build customized bioreactors. (*Step 3*) The distal femoral condyles from calf knees are sectioned and decellularized into cylindrical blanks appropriate for milling. (*Step 4*) CNC milling is used to generate scaffolds in the desired anatomical shape. (*Step 5*) The scaffold is seeded with hMSCs and cultured in the bioreactor for 5 weeks

Fig. 2. TMJ preparation for CAM. (a) Imported TMJ. (b) Steps 13–14, adding a cylindrical support base. (c) Steps 15–16, setting the origin

Fig. 3. PDMS mold design. (a) *Step 1*, outer wall of mold. *Step 2*, TMJ component with alignment hole. *Step 3*, alignment pins. (b) *Step 4*, assembled mold cavity. (c) *Step 6*, CAD model of PDMS mold created via boolean subtraction

Fig. 4. Bioreactor assembly in solidworks. (a) Assembly components previously modeled and newly modeled in *step 3* (needle guide) and *step 4* (syringe casing cap). (b) *Step 2*, aligned TMJ, PDMS casting, and alignment pin. (c) Fully assembled bioreactor

Fig. 5. Modeling flow to optimize bioreactor design. (*Top left*) Color-coded velocity vectors of flow through scaffold. (*Bottom left*) Cross-section through the center of the scaffold gives spatial distribution of medium flow using color-coded scalar values. Computer-aided design of outlet port placement based on optimization of flow patterns throughout the scaffold

Fig. 6. Bioreactor assembly. (*Top*) Listing of all bioreactor components. This list should be consulted prior to sterilization and assembly to ensure that all components are present. (*Bottom left*) Exploded view of each bioreactor component. Parts can be sterilized partially assembled in this fashion in separate autoclave pouches. (*Bottom right*) Fully assembled TMJ bioreactor

Fig. 7.

Representative data of bone grafts cultured for 5 weeks in bioreactor. **(a)** Histological section of plastic embedded bone tissue section stained with Goldner's Trichrome. *Green* stain indicate bone scaffold. *Red* indicates new tissue formation. **(b)** Higher magnification image of histological section demonstrating new tissue formation in the interstitial spaces of the scaffold. *Dark red* stains indicate osteoid formation. **(c)** SEM image of central region of bone graft showing dense tissue formation following bioreactor cultivation (Scale bar = 1 mm). Micro-CT image of unseeded scaffold **(d)** and graft cultured in the bioreactor for 5 weeks **(e)** (scale bars = 5 mm)