Bio-Pick, Place, and Perfuse: A New Instrument for Three-Dimensional Tissue Engineering

Andrew M. Blakely, MD,¹⁻³ Kali L. Manning, BS,^{1,2}
Anubhav Tripathi, PhD,^{2,4} and Jeffrey R. Morgan, PhD^{1,2}

A grand challenge of tissue engineering is the fabrication of large constructs with a high density of living cells. By adapting the principles of pick-and-place machines used in the high-speed assembly of electronics, we have developed an innovative instrument, the Bio-Pick, Place, and Perfuse (Bio-P3), which picks up large complex multicellular building parts, transports them to a build area, and precisely places the parts at desired locations while perfusing the parts. These assembled parts subsequently fuse to form a larger contiguous tissue construct. Multicellular microtissues were formed by seeding cells into nonadhesive micro-molds, wherein cells self-assembled scaffold-free parts in the shape of spheroids, toroids, and honeycombs. After removal from the molds, the parts were gripped, transported (using an x, y, z controller), and released using the Bio-P3 with little to no effect on cell viability or part structure. As many as 16 toroids were stacked over a 170 μm diameter post where they fused over the course of 48 h to form a single tissue. Larger honeycomb parts were also gripped and stacked onto a build head that, like the gripper head, provided fluid suction to hold and perfuse the parts during assembly. Scaffold-free building parts help to address several of the engineering and biological challenges to large tissue biofabrication, and the Bio-P3 described in this article is a novel instrument for the controlled gripping, placing, stacking, and perfusing of living building parts for solid organ fabrication.

Introduction

A GRAND CHALLENGE of tissue engineering has been to construct large living structures with high cell density akin to native organs and to sustain the viability of the structures while reproducing *in vivo* function. Scaffold-based tissue engineering methods usually involve either decellularizing a native organ and reseeded the residual extracellular matrix with the desired cells or seeding a biocompatible fabricated scaffold with cells.¹⁻³ Scaffold-free methods, including pellet culture and hanging drops, are generally limited by the small scale of the produced tissue construct.⁴ Cell sheets, while larger in scale, have had only limited success in specific applications such as vascular tissue engineering.⁵

There has been significant tissue engineering work with relatively thin planar structures, such as the skin or the trachea. These structures are neither vascularized nor perfused *in vitro*, and so they must rely on diffusion to maintain cell viability. When transplanted, ingrowth of host vascular tissue sustains long-term cell viability.⁶ Some clinical success has been reported with more complex tissues such as blood vessel, trachea, skin, and urinary bladder.⁷⁻¹¹ However, no

method has been able to successfully produce a large-scale, high-density tissue mass with a perfusion network thus far.

The potential clinical impact of engineered tissues in treating disease is vast. Liver failure, for example, leads to 1 million deaths per year worldwide, the 10th most common overall cause of death.¹² This represents enormous direct and indirect healthcare costs. The supply of donor organs is far too small to meet the need, and many patients die while waiting on the transplant list. As the obesity epidemic increases, nonalcoholic fatty liver disease leading to liver failure will become an even more important clinical issue.¹³

Current liver assist devices are inadequate in meeting this growing need. They are largely split into artificial methods, based on modified renal hemodialysis, and bio-artificial methods, which utilize bioreactors with hepatocytes to provide a more *in vivo* method of serum filtration. However, success with these devices has been limited, mostly evidenced in acute fulminant liver failure from acetaminophen overdose as opposed to the chronic liver failure as seen in cirrhosis.^{14,15} Engineered solid organs represent an opportunity to meet this clinical need, but current tissue engineering methods have thus far been unable to create a reliable supply of such organs.

¹Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, Rhode Island.

²Center for Biomedical Engineering, Brown University, Providence, Rhode Island.

³Department of Surgery, Rhode Island Hospital, Brown University, Providence, Rhode Island.

⁴School of Engineering, Brown University, Providence, Rhode Island.

Here, we describe a novel device, the Bio-Pick, Place, and Perfuse (Bio-P3). By adapting the principles of the pick and place devices used in the high-speed assembly of multi-component electronics, we have developed an innovative device that is able to pick up complex microtissue building blocks, transport them to a build area, and precisely place the microtissues at the desired destination. In this way, piece by piece, a larger tissue construct can be assembled layer by layer, while incorporating a perfusion network to maintain microtissue viability and allow the microtissue fusion process to proceed. The device has exciting potential applications in solid organ tissue engineering as a platform for applications such as organ function replacement, solid tumor modeling, or three-dimensional drug testing.

Materials and Methods

Micro-molded hydrogels and microtissue formation

Agarose gels were cast from 3D Petri Dish[®] micro-molds (Microtissues, Inc., Providence, RI). Powder UltraPure[™] Agarose (Invitrogen, Carlsbad, CA) was sterilized by autoclaving and then dissolved via heating in sterile water to 2% (weight/volume). Molten agarose was pipetted into each micro-mold and air bubbles were removed by agitation with a sterile spatula. After setting, gels were separated from the micro-mold using a spatula, transferred to 12- or 24-well tissue culture plates, and equilibrated for at least 4 h with several changes of culture medium. Micro-molds with three different recess geometries were used to produce agarose gels to create spheroid, toroid, or honeycomb microtissues. Round recesses for spheroids were 800 μm in diameter and contained 81 recesses per gel. Toroid recesses were 1400 μm in diameter with a central agarose peg of 600 μm and surrounding 400 μm trough and contained 36 features per gel. Honeycomb recesses (one per gel) were 3.4 mm in maximum diameter, with a central peg and surrounding orbital of six pegs, each peg 600 μm in diameter and a surrounding trough width 400 μm .

Rat hepatoma (H35), human ovarian granulosa (KGN), and human breast cancer (MCF-7) cells were expanded in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO). Cultures were maintained in a 37°C, 10% CO₂ atmosphere. Cells were trypsinized, counted, and resuspended to the desired cell density for each experiment. A cell suspension was pipetted into the seeding chamber above the recesses of each micro-molded spheroid (190 μL), toroid (190 μL), or honeycomb (20 μL) agarose gel. Gels were seeded at a concentration of 1250 cells per spheroid feature, 25,000–40,000 cells per toroid feature, and 250,000 cells per honeycomb feature. Samples were then incubated for ~20 min to allow cells to settle into recesses before 1–2 mL of medium was slowly added to each well. Medium was exchanged every other day. Spheroids and toroids were kept in the micro-molds for 18–24 h and honeycombs for 48 h.

Live-Dead staining

For Live-Dead staining, microtissues were incubated with a mixture of 2 mL phosphate-buffered saline with 4 μM of

ethidium homodimer-1 and 1 μM calcein AM (Invitrogen) for 75 min at 37°C. Spheroids and toroids were stained for Live/Dead within 4–6 h of manipulation. Microtissue viability was assessed via fluorescent imaging using a Zeiss Axio Observer Z1 equipped with an AxioCam MRm camera with AxioVision Software (Carl Zeiss Micro-Imaging, Thornwood, NY) and an X-Cite 120 fluorescence illumination system (EXFO Photonic Solutions, Mississauga, Ontario, Canada).

Fabrication of Bio-Pick, Place, and Perfuse instrument

The gripper and build heads of the Bio-P3 instrument were fabricated using Millicell cell culture inserts (EMD Millipore, Billerica, MA) (12 mm diameter, 10 mm height), which have a polycarbonate membrane with track-etched 3- μm (gripper) or 8- μm (build) pores. The manufacturer's website has high resolution images of these pores. The three small feet on the bottom of the cylindrical insert were removed. A 4-mm hole was drilled into the side of the insert and a polypropylene elbow joint fitting (gripper) or a straight fitting (build) (2.4 mm inner diameter) (Cole-Parmer Instrument Co., Chicago, IL) was connected using plastic cement (The Testor Corp., Rockford, IL). The open end of the insert was sealed with a standard 12 mm circular micro cover glass (VWR Scientific, Inc., West Chester, PA) using epoxy (ITW Devcon, Danvers, MA).

The physical plant of the Bio-P3 consisted of an L-shaped polystyrene construct, with a gripper membrane on one end and a male-male fitting connected to tubing run through a Multistaltic[®] peristaltic pump (Haake Buchler Instruments, Inc., Saddle Brook, NJ) on the other end, capable of reversible flow rates up to 2 mL/min. The polystyrene tubing was held by a manual x, y, z micromanipulator (Narishige, Nikon, Tokyo, Japan), which was placed off to the side of the microscope base. Top-view microscopy was performed using an Olympus Stereo Zoom 60 microscope (Olympus America, Center Valley, PA), which provided an improved focal length over inverted microscopes. External light was provided by a Schott KL 1500 LED dual light box (Schott North America, Inc., Elmsford, NY). A custom-made build box constructed of 5 mm thick acrylic measuring ~9×7×3 cm was used for microtissue manipulation. The acrylic was bonded using Sci-Grip[®] Weld-On 16 acrylic cement (IPS Corp., Gardena, CA). Integrated into the box was the build head, whose assembly was similar to the gripper head, with the primary differences being the use of a membrane with track-etched 8 μm pores and a straight fitting instead of an elbow fitting. Flow through the build head was achieved using a Gilson Minipuls[®] 2 peristaltic pump (Gilson, Inc., Middleton, WI), capable of flow rates up to 7.5 mL/min, so that the direction of flow through the gripper and build heads could be independent and opposite, facilitating transfer of microtissues from gripper head to build head. The build box included a removable central divider made of acrylic to separate the box into a microtissue holding pen on one side and the build area with the stationary build head on the other side. The bottom of the holding pen was coated with a thin layer of agarose, and agarose molds containing microtissues were directly inverted into the holding pen to release the microtissues for subsequent manipulation. The box included tubing fittings

for the build head and for return flow from each peristaltic pump, minimizing loss of media over time. Cleaning of the box between experiments was performed by washing with a 2% chlorhexidine solution.

Operation of Bio-Pick, Place, and Perfuse instrument

The Bio-P3 was operated as follows. Microtissues (spheroids, toroids, or honeycombs) released from their micro-molds were brought into view using the microscope's x, y stage. The gripper head, submerged in cell culture medium, was lowered down onto the microtissue. Proximity of microtissue and the membrane of the gripper head were evident when both were in focus. The peristaltic pump was run at 1 mL/min to grip tissues. After gripping, the bio-gripper head was raised, with both the microtissue of interest and the membrane moving out of focus.

To place the microtissues, the microscope's x, y stage was adjusted so that the intended target was brought into position under the bio-gripper head with the gripped microtissue. The head was lowered, and when it reached the appropriate distance in the z -dimension, flow across the membrane was reversed to facilitate release of the microtissue. The build head's peristaltic pump was run at 1.5 mL/min to encourage microtissue deposition and stable placement during subsequent build box manipulation. Gripper heads were exchanged with each experiment to mitigate the effect of membrane clogging from debris.

FIG. 1. Schematic of Bio-Pick, Place, and Perfuse instrument. Step 1. Gripper head is lowered and uses liquid suction to pick up a living part from the holding pen. Step 2. Gripper head with gripped part is raised and the x, y controllable stage moves build head into position under gripper. Step 3. Gripper head is lowered, suction is reversed, and living part is deposited onto stack of living parts. Peristaltic pumps attached to gripper head, build head, and box provide controllable suction to heads, perfusion of proto-organ and recirculation of cell culture medium. Optimally aligned stacking of subunits allows stack flow through microtissue lumens. Color images available online at www.liebertpub.com/tec

Results

Fabrication of the Bio-Pick, Place, and Perfuse instrument

The key steps and general design elements of the Bio-P3 instrument are described in Figure 1. The device must be able to pick up a multi-cellular living building part, move it into location (via x, y, z control), and precisely place this part onto a stack of living building parts. The gripping mechanism must be reversible, must not harm the cells, and must not disrupt the structure of the building part, and all processes must occur within cell culture medium.

To build a manually operated Bio-P3, we fabricated a custom acrylic build box, custom gripper and build heads connected to two peristaltic pumps and mounted the apparatus onto the x, y stage of a stereo microscope (Fig. 2). The gripper/build heads were fabricated by capping the top of cell culture inserts and adding side ports so that controllable fluid suction (driven by peristaltic pumps) could be drawn

FIG. 2. Bio-Pick, Place, and Perfuse (Bio-P3) instrument. Photograph of Bio-P3 instrument key components. The build box filled with cell culture medium sits on the x, y controllable stage of a stereo microscope. The gripper head in the center of the build box is connected to a piece of L-shaped white polystyrene tubing. The gripper head is positioned (x, y) and raised and lowered (z) via a micro-manipulator holding the stiff polystyrene tubing. The gripper head, the build box, and the build head are connected to tubing run through two peristaltic pumps. The pumps provide controllable suction to grip and release living building parts and recirculate the cell culture medium in the build box. Close up photo of build (left) and gripper (right) heads. Scale bar 1 cm. Each head consists of a 12-mm diameter cell culture insert with membrane, with a hole drilled in the side to attach either straight or elbow fittings, capped by a 12 mm glass slip cover. Build heads have membranes with track-etched 8- μ m pores, while gripper heads have 3- μ m pores. Scale bar 5 mm. Close-up photo of holding pen for living building blocks, with released honeycombs. Scale bar 5 mm. Color images available online at www.liebertpub.com/tec

through their porous membranes. The porous membranes (effective membrane diameter $D=0.88$ cm, area $A=\pi D^2/4=0.6$ cm², thickness $t=10$ μ m; pore size $d=3$ or 8 μ m and pore density $n=2\times 10^6$ or 1×10^5 pores/cm², respectively) are transparent when wet, enabling visualization of a microtissue when gripped. To computationally estimate fluid flow, the membranes were assumed for purposes of simplification to consist of parallel cylindrical pores of uniform cross section.¹⁶ Permeability of the membrane was $\sim K=n\pi d^4/128=4.0\times 10^{-10}$ cm² for 3- μ m pore size membranes and 1.0×10^{-9} cm² for 8- μ m pore size membranes. The pressure drop across the membrane at low flow rates ($Q=1$ cm³/min) were estimated to be $\Delta p=Q\mu t/K/A$, where water viscosity $\mu=0.001$ Pa*s. Therefore, the pressure drop for 3- μ m pore size membranes is $\Delta p=69.4$ Pa, versus $\Delta p=27.8$ Pa for 8- μ m pore size membranes. The minimum flow rate to consistently grip a tissue of interest was ~ 1 mL/min. The

gripper and build heads are modular pieces that can be easily exchanged and custom designed for microtissues of varying sizes and shapes. The build box is separated into a build area and a holding pen for building parts. Via the controlled action of the peristaltic pumps and the controlled movement of the x , y stage and the x , y , z controller of the gripper head, a part is gripped in the holding pen, moved into place, and deposited onto the build head.

Gripped microtissues are viable

To determine whether gripping altered the viability of microtissues, H35 spheroids (1250 cells/spheroid) were gripped, moved, and dispensed into Petri dishes coated with agarose. Nongripped control spheroids were kept in parallel dishes and subjected to all the same treatments except gripping. Spheroids were then stained for viability (Fig. 3).

FIG. 3. Gripping and releasing do not harm the structure of microtissues or cell viability. H35 spheroids were gripped, moved 2 cm, and released. Fluorescent images of live (green)/dead (red) staining shows spheroid structure is intact and no difference in viability between gripped (bottom row) (G1–G6) and not gripped (top row) (C1–C6) spheroids. Scale bar 100 μ m. Likewise, toroids of KGN cells (25,000 cells, left panel) (30,000 cells, right panel) were gripped, released, and stained for live/dead. Top row control nongripped (C1–C2), bottom row (G1–G2) gripped. Scale bars 200 μ m. Color images available online at www.liebertpub.com/tec

From these images, there was no significant difference in the viability between gripped and nongripped spheroids.

To determine whether larger, more complex structures could be gripped, toroids of KGN cells (25,000, 30,000, and 35,000 cells/toroid) were made. The toroids were dispensed into cell culture dishes coated with agarose, gripped, and deposited into a recipient agarose-coated cell culture dish. Nongripped control toroids and gripped toroids were stained for viability (Fig. 3). There was no breakage of the toroid structure and there were no differences in viability between gripped and control toroids at any of the seeding densities tested (35,000 cells/toroid images not shown).

Large sheets of toroids can be formed and gripped

To determine whether the Bio-P3 could safely manipulate even larger, more complex microtissues, sheets of fused toroids were created. After 15 h of self-assembly, toroids (30,000 or 40,000 KGN cells/toroid) were released from their micro-molds into culture dishes coated with agarose. After the toroids settled, the dishes were tilted, causing the toroids to collect on one side of the dish and physically contact each other. Twenty-four hours later, the toroids had fused into a contiguous sheet of toroids. Gripping, moving and releasing these sheets did not fracture the sheet nor alter its viability (Fig. 4). The same flow rate (1 mL/min) was used to grip the sheet of toroids as individual microtissues. During the fusion process, the lumens of the toroids narrowed, less so for the toroids of 30,000 cells than those of 40,000 cells (40,000 cells/toroid images not shown). The dead cells and/or debris seen within the lumens may have been extruded from or trapped by the toroids.

Toroids can be stacked

To test the ability of the Bio-P3 to stack toroids, KGN toroids (35,000 cells/toroid) were gripped and then released over small diameter capillary tubes (170 μ m outer diameter, 100 μ m inner diameter) embedded in and protruding upward from agarose. One at a time, toroids were gripped and transported so that their lumens were aligned in the *x*, *y* plane with the outer diameter of the capillary tubes. The *z* distance between the bio-gripper's membrane and the end of the capillary tube was approximated by observing the end of capillary tube catching the toroid as the tube was moved in the *x*, *y* plane relative to the toroid. Upon alignment, the

toroid was released by reversal of flow through the membrane. Careful approximation of the membrane and the capillary tube in the *z* direction minimized occurrences of the toroid not successfully being released onto the capillary tube (Fig. 5). The procedure was repeated until taller stacks were made, containing up to 16 toroids. Manual stacking of each toroid required less than 5 min. Dishes containing stacked toroids were incubated in a 37°C incubator with 10% CO₂ and removed for side view imaging using inverted microscopy and a prism to assess fusion of the toroid stack. Temperature and CO₂ control were later incorporated into the instrument for stacking of honeycombs. The sequence of side view images was taken over a period of 72 h. The tallest stack of toroids, 16 in total, measured over 3.5 mm in height. Over time, the toroids fused, as shown by the closing of small gaps and the melding and flattening of the round edges of the toroids, which indirectly demonstrated microtissue viability. In addition, the stack of toroids contracted their lumens and appeared to attach to the capillary tube. In addition to demonstrating the fusion of stacked parts and the precision of placement of parts, these experiments show that guide posts might also be useful for positioning of parts. However, other types of guide posts would need to be devised, since glass capillary tubes might limit nutrient availability in the long term.

Honeycombs can be gripped and stacked

To determine whether the Bio-P3 could handle even larger and more complex building parts, we prepared multicellular honeycombs (Fig. 6). Micro-molded agarose gels with a single honeycomb recess were seeded with 250,000 MCF-7 cells per honeycomb. Forty-eight hours later, the cells had self-assembled a honeycomb shaped microtissue that remained intact after harvesting from the agarose gel. Honeycomb microtissues were introduced into the holding pen of the Bio-P3 instrument. They were subsequently gripped one at a time, moved into position and deposited onto the build head (Fig. 7). A stack of four honeycombs was assembled over ~15 min, with fair alignment of lumens through the four tissues seen on immediate postplacement imaging. This most directly demonstrates the potential of the Bio-P3 device, to assemble a large (>2 mm in smallest dimension), multi-lumen, high-density (~1 million cells total) tissue construct.

FIG. 4. The gripping and releasing of fused toroids does not harm structure or cell viability. Fluorescent images of live/dead stains of KGN toroids (30,000 cells/toroid) (A, B). Toroids that had been allowed to fuse on one plane into a multi-toroid structure for 24 h were gripped, released, and stained. Gripping did not disrupt multi-toroid structure. Scale bar 500 μ m. Color images available online at www.liebertpub.com/tec

FIG. 5. Toroids that are gripped and stacked will fuse into a single tissue. The Bio-P3 was used to pick and place toroids comprised of 30,000 KGN cells on a capillary tube (170 μm outer diameter and 100 μm inner diameter). *Side view* images of stacks of 8 toroids (*top row 1*), 8 toroids (*middle row 2*), and 16 toroids (*bottom row 3*) at 0 (**a**), 12 (**b**), 24 (**c**), 48 (**d**), and 72 h (**e**) after stacking showing fusion into single tissues. Scale bar 500 μm .

FIG. 6. Preparation of honeycomb-shaped microtissues. Photos of micro-mold (**a**) and micro-molded agarose gel prepared from mold (**b**). After equilibration in cell culture medium, micro-molded agarose gel was seeded with 250,000 MCF-7 cells that self-assembled a multicellular honeycomb structure in the gel within 24 h (**c**). Brightfield image of multicellular honeycomb with seven lumens after release from the gel (**d**). Scale bar 500 μm . Color images available online at www.liebertpub.com/tec

FIG. 7. Progressive picking, placing, and stacking of honeycomb microtissues. Honeycomb microtissues (comprised of 250,000 MCF-7 cells) were picked, placed, and stacked onto the build head. Close-up overhead photos of a building sequence showing a stack of two (2), three (3), and four (4) honeycombs (*top*). Approximate time to stack four honeycombs was 15 min and each image was taken within about 1 min after each part was stacked. Close-up side view photo of a stack of three honeycombs on the build head (*bottom left*). Angled *top* view photo of stack of three honeycombs on build head (*bottom right*). Scale bars 1 mm. Color images available online at www.liebertpub.com/tec

Discussion

To date, the field of tissue engineering has been unable to reliably produce large-scale, thick (>2 mm) solid tissues with high cell density that maintain viability long-term *in vitro*. This remains one of the great unsolved challenges. Early successes were achieved with sheet-like constructs such as skin that survive *in vitro* as avascular structures and are vascularized after grafting.⁶ More recent successes have been shown with thin hollow constructs, such as the trachea or the urinary bladder, which are relatively thin tissues that also rely on diffusion to maintain viability *in vitro* and are vascularized after transplantation.^{9,11} However, solid organs, such as the liver or pancreas, are significantly thicker and have a high cell density and a greater metabolic demand. Diffusion alone will not maintain the viability of these thick tissue constructs *in vitro*. Hence, long-term *in vitro* viability requires a strategy to integrate or build a perfusion or vascular network.^{17,18}

The fabrication of solid organs is currently limited by many engineering and biological challenges. Numerous approaches have been developed to address one or more aspects of what is widely recognized as a very complex process, which is yet to be fully defined or adequately solved. Bio-printing, based on the principles of inkjet printing, deposits drops (x, y, z) of extracellular matrix along with cells or spheroids to build various structures including sheets and tubular structures layer by layer.^{19–21} Sub-millimeter-sized modules of cells in collagen gels have been packed into a column and the interstitial space seeded with endothelial cells and perfused.²² Cell-laden microgels of varying shapes have been fabricated into predetermined 3D architectures.²³ Microfluidic networks have been molded into cell-laden alginate or poly(ethylene glycol) diacrylate (PEGDA).^{24,25} Sacrificial materials, such as gelatin and carbohydrate strands, have been cast into cell-laden hydrogels and when dissolved, create channels for perfusion.^{26,27} These methods suffer from a high content of noncellular

materials to create a structure for cell seeding, which limits the ultimate cell density of the tissues.

The Bio-P3 instrument described here is a novel approach to solid organ tissue engineering. The device manipulates living building parts that have several important biological properties conducive to the fabrication of larger tissue constructs. First, the building parts are scaffold-free and are produced by the process of self-assembly, whereby cells aggregate to form 3D multicellular microtissues.^{28,29} Since no exogenous scaffold material is added, cell density is high and cell–cell interactions are maximized (e.g., gap junctions and cytoskeletal interactions). Individual building parts have cell densities comparable to native solid organs, such as the liver, a critical design criterion.^{30,31} Second, parts with two or more cell types can be self-assembled. In some instances, pairs of cell types may self-sort and/or undergo additional morphogenesis during self-assembly, allowing the assembly of tissues that more closely replicate organ cell composition.^{29,32} Cell lines were used to demonstrate the proof of concept of the Bio-P3, however, building parts of primary cells including cardiomyocytes, smooth muscle cells, fibroblasts, embryonic stem cells, neurons, and chondrocytes have also been produced using agarose micro-molds demonstrating that self-assembly of 3D microtissues is a fundamental property of many different cell types.^{33–38} For example, endothelial cells will form a capillary-like network within multicellular spheroids.⁴ Building parts can be self-assembled into complex geometries such as rods, toroids, and honeycombs.³² Lastly, as this and other studies have shown, multicellular parts will fuse with one another when brought into contact.³⁹ Fusion can be controlled through the use of secondary molds or guides and by the time of maturation of the building parts.⁴⁰

Building parts can also help address some of the engineering challenges to solid organ fabrication. First, parts are typically self-assembled in 24 h using a process that is simple (i.e., adding monodispersed cells to a micro-mold) and amenable to automation and scale-up. Second, we have

a new quantitative understanding of directed self-assembly, the biological process that drives building part formation. When cells are seeded in a nonadhesive mold, they aggregate, exert forces, and form a 3D multicellular microtissue that undergoes predictable morphological changes in response to the size and shape of the obstacles in the mold.³² Thus, mold design determines both part shape and the morphological evolution of the part as it self-assembles. We recently reported a finite element simulation based on cell-derived tension that predicts part morphology.⁴¹ Moreover, the work or power exerted by cells as they self-assemble a part has been quantified for different cell types and cell mixtures.^{34,42} Third, as we have previously shown, self-assembled parts retain their viability and structure when harvested from molds for up to 10 days.³⁹ Released parts do undergo morphological changes with time, but these changes are predictable and can be factored into mold design.^{32,40} Fourth, the varied design possibilities for the shapes of parts are an aid to organ fabrication. The lumens in toroids and honeycombs ensure that cells are within the critical diffusion distance and that when stacked, lumens can help form a vascular-like network for convective nutrient transport. Parts can be designed to carry important architectural information in anticipation of assembling them into a tissue. Fifth, the size of parts significantly reduces build time. The honeycombs used in this study are comprised of 250,000 cells and are ~ 3 mm across, and even larger honeycombs have been self-assembled within the same 24 h period (6×10^6 cells/honeycomb, 2 cm across).⁴³ Compared to a single spheroid with about 1000 cells, a single large honeycomb part is equivalent to 6000 spheroids. The size of parts in the z dimension is limited due to diffusion, but since parts, such as honeycombs, are planar, the x , y dimensions can be quite large and we have yet to find the limit. Larger building parts mean fewer building steps. Lastly, the assembly of building parts is driven by fusion of parts and this process is complete in about 48 h.³⁹ Thus, within a week, significant numbers of parts can be self-assembled and fused into a large construct.

In this article, we have developed a proof-of-concept manual Bio-P3 instrument and we have demonstrated two fundamental principles of the device. First, the device is able to grip (Pick), transport, and release multicellular microtissues (spheroids, toroids, and honeycombs) with minimal effects on cell viability or building part structure. Low levels of fluid pulled through a porous membrane via the action of a peristaltic pump provided sufficient force to grip and secure a part for movement. Stopping flow with or without reversing flow facilitated release of the part from the membrane. When wet, the track-etched polycarbonate membranes are transparent and allow for visualization of parts as they are manipulated. The gripper and build heads were adapted from circular cell culture inserts and are modular units that can be replaced in the instrument. Larger heads or custom heads designed to grip parts of specific shapes can be easily fabricated.

The second principle demonstrated is the release and placement (Place) of building parts onto a specific target area. Toroids (outer diameter 1200 μ m, lumen diameter 600 μ m, mass ~ 5 μ g) were placed onto a fixed obstacle, a glass capillary tube (outer diameter 170 μ m).³⁴ In addition to serving as a well-defined target, the obstacle constrained the movement of toroids after they were released and was an aid to stacking as many as 16 toroids. Fixed obstacles serving as

guide posts may be part of a future strategy to help align much larger parts as they are stacked. Honeycomb building parts were also picked and placed onto the build head. The build head, connected to a second peristaltic pump, provides a gripping force to keep deposited parts in place. It also provides pulsatile fluid flow around parts and through the lumens of placed parts as a means to perfuse a growing stack of building parts. With this first manual instrument, we were able to stack four honeycomb building parts with some, but not perfect alignment of the lumens. From our photographs, we estimate that honeycomb parts are shifted by up to 200 μ m in the x , y plane and by a maximum of 60° in their rotational coordinates.

Numerous engineering and biological challenges still remain. With our manual instrument, we stacked 16 toroids within about 60 min, thus each pick and place cycle is about 4 min. Automation of our system would significantly reduce this cycle time. Precision part placement can be improved by more refined control of the gripping head and better definition of the operating parameters of part placement. Although we have described the changes in part morphology during self-assembly and after harvest from the molds, further work is needed to understand the morphological changes that occur when stacks of parts are fused, information useful for part design.^{32,41} We are currently refining the instrument and investigating long-term questions such as viability of the assembled construct and the morphology of its vascular network as a function of perfusion time. Some of the biological challenges include long-term perfusion of the growing construct, endothelialization of the construct, and the design of a vascular tree that can be built with stacks of parts. We have not yet tested the Bio-P3's ability to manipulate materials such as collagen or fibrin gels, which may also be useful for tissue fabrication. Significant challenges for the engineering of solid organs still remain and each organ has its own set of challenges. For example, some of the issues yet to be addressed for the liver include the production of large numbers of differentiated hepatocytes, the formation of sinusoids and canaliculi, and the formation of a vascular tree that can be integrated with the host. Overall, this first generation instrument offers a new strategy and is a significant step toward the large-scale biofabrication of solid organs with high cell densities that may someday be useful for the treatment of liver or pancreatic failure or for improved tumor or drug delivery models.

Acknowledgments

Brown University has filed a patent on this work. This work was funded, in part, by NIH T32 grant GM065085-09 and NSF grant CBET-1428092.

Disclosure Statement

J.R.M. has an equity interest in Microtissues, Inc. This relationship has been reviewed and managed by Brown University in accordance with its conflict of interest policies. No other competing financial interests exist.

References

- Griffith, L.G., and Naughton, G. Tissue engineering—current challenges and expanding opportunities. *Science* **295**, 1009, 2002.

2. Uygun, B.E., Soto-Gutierrez, A., Yagi, H., Izamis, M.-L., Guzzardi, M.A., Shulman, C., *et al.* Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med* **16**, 814, 2010.
3. Hoque, M.E., Mao, H.Q., and Ramakrishna, S. Hybrid braided 3-D scaffold for bioartificial liver assist devices. *J Biomater Sci Polym Ed* **18**, 45, 2007.
4. Blakely, A.M., Schell, J.Y., Rago, A.P., Chai, P.R., Napolitano, A.P., and Morgan, J.R. Formation of multicellular microtissues and application in biofabrication. In: Forgacs, G., Sun, W., eds. *Biofabrication*. Philadelphia: Elsevier, Inc., 2013, pp. 149–66.
5. L'Heureux, N., Dusserre, N., Marini, A., Garrido, S., de la Fuente, L., and McAllister, T. Technology insight: the evolution of tissue-engineered vascular grafts—from research to clinical practice. *Nat Clin Pract Cardiovasc Med* **4**, 389, 2007.
6. Waymack, P., Duff, R.G., and Sabolinski, M. The effect of a tissue engineered bilayered living skin analog, over meshed split-thickness autografts on the healing of excised burn wounds. The Apligraf Burn Study Group. *Burns* **26**, 609, 2000.
7. L'Heureux, N., Dusserre, N., Konig, G., Victor, B., Keire, P., Wight, T.N., *et al.* Human tissue-engineered blood vessels for adult arterial revascularization. *Nat Med* **12**, 361, 2006.
8. L'Heureux, N., McAllister, T.N., and de la Fuente, L.M. Tissue-engineered blood vessel for adult arterial revascularization. *N Engl J Med* **357**, 1451, 2007.
9. Gonfiotti, A., Jaus, M.O., Barale, D., Baiguera, S., Comin, C., Lavorini, F., *et al.* The first tissue-engineered airway transplantation: 5-year follow-up results. *Lancet* **383**, 238, 2014.
10. Priya, S.G., Jungvid, H., and Kumar, A. Skin tissue engineering for tissue repair and regeneration. *Tissue Eng Part B Rev* **14**, 105, 2008.
11. Atala, A., Bauer, S.B., Soker, S., Yoo, J.J., and Retik, A.B. Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet* **367**, 1241, 2006.
12. Badylak, S.F., Taylor, D., and Uygun, K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Annu Rev Biomed Eng* **13**, 27, 2011.
13. Younossi, Z.M., Stepanova, M., Afendy, M., Fang, Y., Younossi, Y., Mir, H., *et al.* Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol* **9**, 524, 2011.
14. Leckie, P., Davenport, A., and Jalan, R. Extracorporeal liver support. *Blood Purif* **34**, 158, 2012.
15. Stutchfield, B.M., Simpson, K., and Wigmore, S.J. Systematic review and meta-analysis of survival following extracorporeal liver support. *Br J Surg* **98**, 623, 2011.
16. Truskey, G.A., Yuan, F., and Katz, D.F. *Transport Phenomena in Biological Systems*. 1st ed. Upper Saddle River: Prentice-Hall, Inc., 2004.
17. Ko, H.C.H., Milthorpe, B.K., and McFarland, C.D. Engineering thick tissues—the vascularisation problem. *Eur Cell Mater* **14**, 1, 2007.
18. Rouwkema, J., Rivron, N.C., and van Blitterswijk, C.A. Vascularization in tissue engineering. *Trends Biotechnol* **26**, 434, 2008.
19. Wilson, W.C., and Boland, T. Cell and organ printing 1: protein and cell printers. *Anat Rec A Discov Mol Cell Evol Biol* **272**, 491, 2003.
20. Jakab, K., Norotte, C., Marga, F., Murphy, K., Vunjak-Novakovic, G., and Forgacs, G. Tissue engineering by self-assembly and bio-printing of living cells. *Biofabrication* **2**, 22001, 2010.
21. Norotte, C., Marga, F.S., Niklason, L.E., and Forgacs, G. Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials* **30**, 5910, 2009.
22. McGuigan, A.P., and Sefton, M.V. Vascularized organoid engineered by modular assembly enables blood perfusion. *Proc Natl Acad Sci U S A* **103**, 11461, 2006.
23. Du, Y., Lo, E., Ali, S., and Khademhosseini, A. Directed assembly of cell-laden microgels for fabrication of 3D tissue constructs. *Proc Natl Acad Sci U S A* **105**, 9522, 2008.
24. Choi, N.W., Cabodi, M., Held, B., Gleghorn, J.P., Bonassar, L.J., and Stroock, A.D. Microfluidic scaffolds for tissue engineering. *Nat Mater* **6**, 908, 2007.
25. Cuchiara, M.P., Allen, A.C.B., Chen, T.M., Miller, J.S., and West, J.L. Multilayer microfluidic PEGDA hydrogels. *Biomaterials* **31**, 5491, 2010.
26. Golden, A.P., and Tien, J. Fabrication of microfluidic hydrogels using molded gelatin as a sacrificial element. *Lab Chip* **7**, 720, 2007.
27. Miller, J.S., Stevens, K.R., Yang, M.T., Baker, B.M., Nguyen, D.-H.T., Cohen, D.M., *et al.* Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nat Mater* **11**, 768, 2012.
28. Napolitano, A.P., Chai, P., Dean, D.M., and Morgan, J.R. Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels. *Tissue Eng* **13**, 2087, 2007.
29. Napolitano, A.P., Dean, D.M., Man, A.J., Youssef, J., Ho, D.N., Rago, A.P., *et al.* Scaffold-free three-dimensional cell culture utilizing micromolded nonadhesive hydrogels. *Biotechniques* **43**, 494, 2007.
30. Bianconi, E., Piovesan, A., Facchin, F., Beraudi, A., Casadei, R., Frabetti, F., *et al.* An estimation of the number of cells in the human body. *Ann Hum Biol* **40**, 463, 2013.
31. Heinemann, A., Wischhusen, F., Püschel, K., and Rogiers, X. Standard liver volume in the Caucasian population. *Liver Transpl Surg* **5**, 366, 1999.
32. Dean, D.M., Napolitano, A.P., Youssef, J., and Morgan, J.R. Rods, tori, and honeycombs: the directed self-assembly of microtissues with prescribed microscale geometries. *FASEB J* **21**, 4005, 2007.
33. Desroches, B.R., Zhang, P., Choi, B.-R., King, M.E., Maldonado, A.E., Li, W., *et al.* Functional scaffold-free 3-D cardiac microtissues: a novel model for the investigation of heart cells. *Am J Physiol Heart Circ Physiol* **302**, H2031, 2012.
34. Youssef, J., Nurse, A.K., Freund, L.B., and Morgan, J.R. Quantification of the forces driving self-assembly of three-dimensional microtissues. *Proc Natl Acad Sci U S A* **108**, 6993, 2011.
35. Kunstar, A., Otto, C., Karperien, M., van Blitterswijk, C., and van Apeldoorn, A. Raman microspectroscopy: a non-invasive analysis tool for monitoring of collagen-containing extracellular matrix formation in a medium-throughput culture system. *Tissue Eng Part C Methods* **17**, 737, 2011.
36. Gwyther, T.A., Hu, J.Z., Christakis, A.G., Skorinko, J.K., Shaw, S.M., Billiar, K.L., *et al.* Engineered vascular tissue fabricated from aggregated smooth muscle cells. *Cells Tissues Organs* **194**, 13, 2011.
37. Birenboim, R., Markus, A., and Goldstein, R.S. Simple generation of neurons from human embryonic stem cells using agarose multiwell dishes. *J Neurosci Methods* **214**, 9, 2013.

38. Boutin, M.E., and Hoffman-Kim, D. Application and assessment of optical clearing methods for imaging of tissue-engineered neural stem cell spheres. *Tissue Eng Part C Methods* 2014 [Epub ahead of print]; DOI: 10.1089/ten.tec.2014.0296.
39. Livoti, C.M., and Morgan, J.R. Self-assembly and tissue fusion of toroid-shaped minimal building units. *Tissue Eng Part A* **16**, 2051, 2010.
40. Rago, A.P., Dean, D.M., and Morgan, J.R. Controlling cell position in complex heterotypic 3D microtissues by tissue fusion. *Biotechnol Bioeng* **102**, 1231, 2009.
41. Svoronos, A.A., Tejavibulya, N., Schell, J.Y., Shenoy, V.B., and Morgan, J.R. Micro-mold design controls the 3D morphological evolution of self-assembling multicellular microtissues. *Tissue Eng Part A* **20**, 1134, 2014.
42. Youssef, J., Chen, P., Shenoy, V.B., and Morgan, J.R. Mechanotransduction is enhanced by the synergistic action of heterotypic cell interactions and TGF- β 1. *FASEB J* **26**, 2522, 2012.
43. Tejavibulya, N., Youssef, J., Bao, B., Ferruccio, T.-M., and Morgan, J.R. Directed self-assembly of large scaffold-free multi-cellular honeycomb structures. *Biofabrication* **3**, 34110, 2011.

Address correspondence to:

Jeffrey R. Morgan, PhD

Center for Biomedical Engineering

Brown University

G-B 393

171 Meeting Street

Providence, RI 02912

E-mail: jeffrey_morgan@brown.edu

Received: July 22, 2014

Accepted: December 16, 2014

Online Publication Date: February 4, 2015