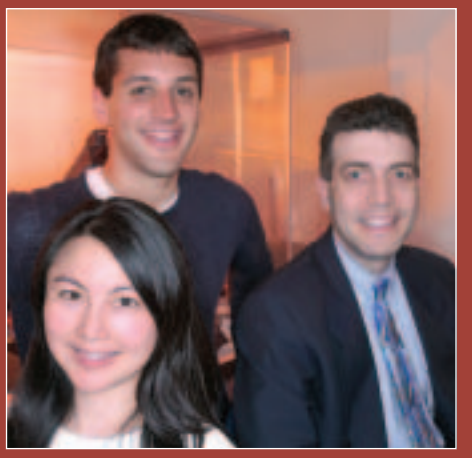
(pictured right) Jeffrey Borenstein
(pictured bottom) Wing Cheung
Lauren Hartman
Mohammad Kaazempur-Mofrad
Kevin King
Alec Sevy
Michael Shin
(pictured left) Eli Weinberg
Joseph Vacanti



Living 3-Dimensional Microfabricated Constructs for the Replacement of Vital Organ Function

ABSTRACT

We report the first demonstration of high-resolution, 3-dimensional constructs of living tissue suitable for transplantation for vital organ replacement devices. This work invokes a microfluidic approach in which the blood supply of the liver or kidney is simulated using computational fluid dynamics; the vascular supply is then fabricated by replica molding of thin sheets of biopolymers stacked to form 3-dimensional channel networks. These vascular networks are lined with endothelial cells in capillary-like structures that permeate the polymer construct with a rich bed of blood vessels. Organ function is achieved by arranging organ-specific cells in compartments adjacent to the blood vessels, which are separated by nanoporous polymer membranes. Initial results demonstrate that rat hepatocytes are sustained and retain their function for periods of weeks when oxygenated medium is perfused through the engineered capillary networks, a promising first step toward the ultimate goal of organ replacement.

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INTRODUCTION

Tissue loss and failure represent a crisis in health care, with tens of billions of dollars in medical costs, nearly ten million surgical procedures annually, and a steadily worsening shortage of donor organs. Currently, several therapeutic approaches are being pursued, including the development of mechanical devices for the replacement of organ function, xenotransplantation (typically through the use of pig organs), and the rapidly emerging field of tissue engineering. One solution for the treatment of tissue loss and organ failure is through the use of replacement organs grown using autologous cells on biodegradable polymer constructs, the approach invoked in this paper. To date, this approach has shown clinical success in skin, bone, cartilage, and a wide range of tubular structures in the digestive, excretory, and circulatory systems.

One of the principal approaches used to generate replacement organs in the laboratory is to seed and expand autologous cells on microporous polymer constructs engineered to replicate the structure and function of the target organ.[1] The ultimate potential of this approach has been limited by the inability to generate thick, complex tissues because of oxygen transport limitations within the growing tissue. Nature solves this diffusion problem through the construction of a vast network of blood vessels that permeate the organ at size scales ranging from 1 cm down to 10 μm.

In tissue engineering applications, the microcirculation may be reproduced through the controlled introduction of angiogenesis-inducing growth factors[2] or by the construction of templates with subsequent microfluidic seeding of endothelial cells. For the latter, two barriers have limited progress in the development of vascularized structures: (1) insufficient dimensional resolution (~300-μm limit) in existing polymer processing methods, and (2) the absence of suitable models for the design of the microcirculation. To address the first barrier, this research team pioneered the use of microfabrication technology for tissue-engineered organs.[3] Through replica molding techniques,[4] we

have demonstrated high-resolution machining in both biocompatible and biodegradable substrates.[5]-[6] Other groups are now pursuing similar approaches for vital organs such as the liver.[7]-[8] The second barrier impeding progress has been addressed by the use of computational fluid dynamic models that mimic critical fluidic properties while accounting for the complex nature of blood flow.[9]

MODELING OF THE MICROCIRCULATION

There exists a substantial body of literature detailing the morphometry of blood vessel networks of the heart and liver.[10]-[11] The computational fluid dynamic model used to produce the vascular networks for tissue engineered molds is designed to reproduce the structural properties of the vasculature, as above, as well as the dynamic properties, including shear stress, pressure drop, velocity, and hematocrit distribution.

The algorithm used for this work is a fractal computational model[9] that accounts for the non-Newtonian rheology and multiphase nature of blood, both of which lead to several important phenomena, particularly for the microcirculation. Simple approximations, such as the Hagen-Poiseuille relation, which relates flow to the inverse fourth power of the radius of a vessel, are not readily applicable to modeling the vasculature due to phenomena such as the Fahraeus-Lindquist effect. Flow behavior is governed by viscosity. The viscosity of blood varies with both the vessel diameter and the hematocrit (volume fraction of red blood cells). The nonlinear dynamics of blood flow for the mixed phase system was solved using a two-step algorithm. This algorithm further attempts to emulate the adaptation and remodeling of microvascular structure in response to deviation from homeostatic shear stress levels, allowing for dynamic evolution of the design of the microvascular network.

Application of the 2-dimensional model to the design and construction of polymer scaffolds for organ replacement is relatively straightforward, since the product of the computational model may be transferred directly to a mask layout, with a single inlet and a single outlet for introduction of cells, growth medium, oxygen, and blood. An example of such a network is shown in Figure 1. The most challenging aspect of 2-dimensional network fabrication is the potential need for variable channel depth as a function of their cross section. A simple approximation that greatly simplifies the fabrication process is to vary the width but not the depth of each generation of channels, thereby producing a rectangular rather than cylindrical vessel geometry.

Figure 1. Schematic of a sample 2-dimensional network design for organ vasculature.

For a 3-dimensional scaffold, the approach is not as straightforward, since replica molding processes impose certain restrictions on the design, such as the requirement for 90-deg vertical channels along the z-axis. Again, the simplest approximation is to stack a series of identical 2-dimensional layers with a simple through-connector at both the inlet and outlet ends of the design.

Thus far, the description of the design has focused on the vasculature, but the tissue engineering scaffold incorporates organ-specific cells (parenchymal cells) and function within the vascularized matrix. Ultimately, the parenchymal cells will form specific structures and geometries as well, such as the sinusoids of the liver. For this early work, however, the parenchymal cells are introduced through separate inlet channels into large compartments arranged adjacent to the engineered blood vessels with a nanoporous membrane separating the vasculature from the parenchymal compartment.

SCAFFOLD FABRICATION

3-dimensional organoids (sections of a vital organ such as the liver) were built using the stacked sets of 2-dimensional templates as described in the design discussion above. A simple blood vessel network, such as that shown in Figure 1, was used as a template for a large stack of polymer films integrated with a simple inlet and outlet channel at opposite corners. Each vascular channel network was arranged across from a polymer film containing large compartments for the parenchymal cells, separated only by a nanoporous polymer membrane.

Master molds for the polymer replica molding process were fabricated using deep reactive ion etching, isotropic plasma etching, and SU-8 photolithography. Most of the experiments described here used isotropic plasma etching, and the process was optimized to provide rounded features at both the top and bottom of the mold features. Polymers cast using the replica molding process included both polydimethylsiloxane (PDMS) and poly-lactic-co-glycolic acid (PLGA). The former yields a biocompatible but nondegradable elastomer. The latter produces a biodegradable construct that will dissolve completely within a period of weeks. To construct PDMS films, the polymer (Sylgard 184, Dow Corning) was cast in mold wafers etched in the inverse image of the channels. These films tend to be quite thick (on the order of several hundred microns), but the processes for molding and bonding PDMS are straightforward and well-described in the literature.[12] Processes for generating ultra-high-resolution scaffolds in a biodegradable substrate did not exist prior to this overall program, and were developed by one of us (K.R. King). The preferred method for producing high-resolution PLGA films was melt processing from a PDMS mold (which is produced from a Si mold); an example of the resolution obtained using this technique is shown in Figure 2. These films were joined using a fusion bonding technique, producing permanent structures without leaking or occlusion. Very thin layers of PLGA (<100 μm) were produced, and the bonded films show no measurable deviation from the mold geometry.

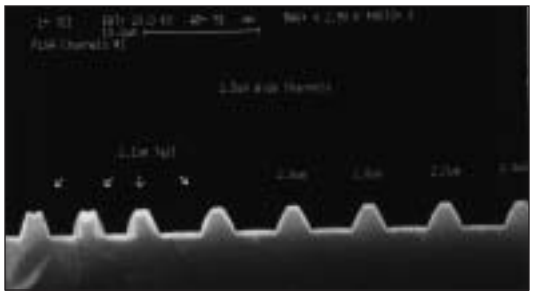

Figure 2. Scanning electron microscope image of PLGA film with 2-µm features, built using replica molding from a PDMS template.

3-dimensional vascular beds are realized by the integration of multiple 2-dimensional films using the vertical inlet and outlet ports, as described earlier. As many as 10 layers of PLGA films have been integrated to form a 3-dimensional scaffold; Figure 3 illustrates a cross section of a 6-layer PLGA scaffold. Infusion of fluorescent dye into these networks confirms that each of the network layers is open and that the channels are all open to flow.

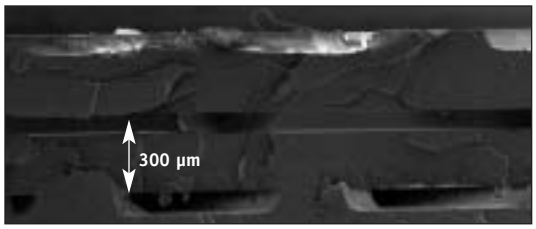

Figure 3. Cross section of multilayer stack of PLGA polymer films bonded together to form a 3-dimensional vascular supply.

Formation of the parenchymal compartments is readily achieved using the same replica molding processes as are used for the vascular component. Integration is achieved by forming separate vertical inlet and outlet channels at opposite corners of the structure. These ports do not communicate with the vascular bed except through the nanoporous membrane separating each vascular and parenchymal compartment. The material of choice for the nanoporous membrane depends on the particular requirements of the experiment. For all the PDMS scaffolds, a commercially-available polycarbonate membrane (Millipore, Inc.) was sandwiched between each vascular and parenchymal compartment. These films, like the surrounding scaffold matrix, are not degradable, but are biocompatible and may be fastened using a small quantity of liquid PDMS. For PLGA scaffolds, a nanoporous PLGA membrane was fusion bonded between each vascular and parenchymal film layer. Principally, the nanoporous membrane serves as a path for the transport of oxygen and nutrients from the oxygenated capillaries to the adjacent parenchymal compartments. Leakage of other blood components from the vascular to the parenchymal compartment will be controlled by intracellular and cell-cell junction transport phenomena once the vascular compartments are endothelialized.

Figure 4 shows a nanoporous film covering vessels in the right side of the network.

Figure 4. Image of nanoporous film covering vessels in right half of network.

CELL CULTURE RESULTS

To date, two types of cell culture experiments have been pursued. In the first set of experiments, 3-dimensional vascular networks constructed from PLGA have been seeded with endothelial cells for periods of several weeks in order to demonstrate long-term cell viability in a microfluidic device. Either rat lung microvascular endothelial cells or cells drawn from sheep carotid arteries have been used to seed PLGA constructs using the network structure shown in Figure 1. Culture media consisted of Dulbecco's Modified Eagle Medium (DMEM) high-glucose supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. Culturing was done at 37°C with 5% CO_2. Oxygen was introduced through the culture medium, perfusing throughout the vascular bed. Prior to endothelialization, the PLGA scaffold was lined with collagen to promote cell attachment. The cell suspension was injected in the absence of dynamic flow for a fixed period to allow for cell attachment without flow-induced shearing from the walls. Cell culture was characterized using direct imaging through the clear scaffold structure, as well as with staining for CD31 and von Willebrand factor.

Endothelial cell culture in these devices demonstrated viability for periods of up to 4 weeks. Confluent single layers of endothelial cells were seen across vast areas of the 3-dimensional network. Figure 5 shows that the time required to achieve confluency in a microfluidic network is much longer than in a typical culture flask, perhaps due to the long distances across which proliferating cells must traverse during culture in a microfluidic network.

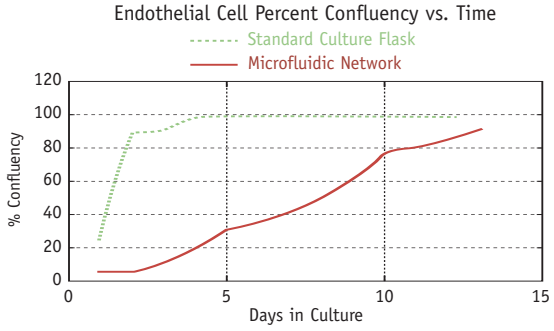

Figure 5. Comparison between the time required to achieve confluency in microfluidic culture vs. standard cell culture in a flask.

In the second set of experiments, hepatocytes were cultured in a parenchymal compartment adjacent to the vascular network, which was either seeded with endothelial cells or simply filled with oxygenated carrier fluid. In this case, vascular and parenchymal compartments were separated by the nanoporous membrane described earlier. Results shown here were obtained in PDMS constructs with a polycarbonate membrane between layers. Hep G2/C3a (human hepatoblastoma cell line) were obtained from culture flasks by trypsinization. Hep G2/C3a cells were seeded into the parenchymal compartment, resulting in devices containing 100,000 to 500,000 cells. Media flow was introduced at rates of 0.16 - 1 ml/h using a positive displacement pump. Control devices did not receive any flowing media. Again, all devices were incubated at 37°C with 5% CO_2. A standard live/dead assay from Molecular Probes was used to determine hepatocyte viability after 1 and 2 weeks. Hepatocytes in devices without oxygenated flow of media did not survive to the 1- or 2-week time points, while cells adjacent to oxygenated vascular networks did survive for the duration of the experiment (see Figure 6). Early results suggest that the optimal flow rate is 0.5 ml/h and the optimum seeding concentration is 0.5 to 1 million cells/ml. After 2 weeks, large aggregates of cells with apparent 3-dimensional conformation rather than simply flat sheets of hepatocytes are observed. Under optimal flow rate and seeding conditions, there is greater than 90% viability.

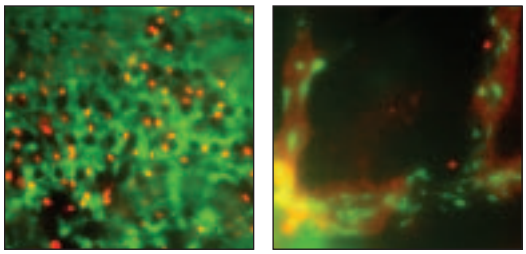

Figure 6. (Left) Stained image of hepatocytes in microfabricated 3-dimensional construct. Green stain shows that hepatocytes are kept alive for periods of 2 weeks by adjacent endothelialized capillary network. (Right) Closeup of hepatocytes kept alive for 1 week over vascular channels, with very few dead cells (roughly 97% cells still alive.)

CONCLUSIONS AND SUMMARY

This work has demonstrated the potential of microfabrication and microfluidic technology as a tool for the generation of vital organ constructs for transplantation. Previous methods for generating scaffolds for tissue engineering were limited by the resolution of existing polymer processing techniques, as well as by insufficient recognition of the crucial role played by the intrinsic microcirculation network. Here, the microcirculation is modeled using a powerful computational fluid dynamic approach that accounts for the complex nature of blood flow. Silicon micromachining and replica molding techniques are used to produce large 3-dimensional polymer scaffolds with precise geometries. These constructs are then seeded with endothelial and parenchymal cells, and preliminary results provide evidence for excellent viability in culture.

Future work will focus on the design and construction of organ-scale, 3-dimensional scaffolds co-cultured with endothelial and parenchymal cells, with the ultimate goal of building natural devices for vital organ replacement.

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BIOGRAPHIES

JEFFREY BORENSTEIN is currently Director of the Biomedical Engineering Center and is a Distinguished Member of the Technical Staff. He is also an Associate Director of the Center for the Integration of Medicine and Innovative Technology (CIMIT). Dr. Borenstein has 20 years of experience in microsystems technology and biomedical devices, and has developed high-performance microsensors, including accelerometers, gyroscopes, microphones, and chemical and biological sensors for both commercial and defense applications. Dr. Borenstein's current work is focused on the application of microsystems technologies toward pathogen detection, drug delivery, and cell-based devices and systems. Dr. Borenstein has a PhD in Physics.

ELI WEINBERG is a Draper Fellow under the supervision of Dr. Jeffrey Borenstein at Draper Laboratory and Dr. Mohammad Kaazempur-Mofrad at MIT. His research interests are computational methods and simulation of the cardiovascular system. Eli is currently developing computational models of the heart mitral valve and of blood vessel network remodeling. He received a BS in Mechanical Engineering from MIT in 2002 and is currently pursuing an MS and PhD in that department.

KEVIN KING is interested in biological applications of microtechnology, and specifically living cell-based microdevices and microfluidics. He is currently a graduate student in the Harvard-MIT division of Health Science and Technology pursuing a PhD in Medical Engineering and Medical Physics. He received MS and BS degrees in Electrical Engineering from MIT and the University of Illinois at Urbana-Champaign, respectively.

WING CHEUNG is a Resident in General Surgery at Massachusetts General Hospital (MGH) and is currently the Kasanjian Research Fellow in Dr. Joseph Vacanti's Tissue Engineering Laboratory. With her experience in molecular and cell biology, she brings her surgical skills and insights from her surgery training to the tissue engineering project. She graduated from the California Institute of Technology with a degree in Biology and the Harvard Medical School/MIT HST program in 1999 and has been at the MGH since.

BIOGRAPHIES Cont.

LAUREN HARTMAN is currently a Research and Development Specialist at Transkaroytic Therapies, Inc. in Cambridge, Massachusetts. She has focused her research efforts on tissue engineering for organ replacement/augmentation. While a member of Dr. Vacanti's lab, she was involved in the engineering of a renal replacement device using microfabrication techniques. Ms. Hartman holds BS and MS degrees in Biomedical Engineering.

JOSEPH VACANTI is Chief of the Department of Pediatric Surgery, MGH; Surgeon-in-Chief, MGH for Children; John Homans Professor of Surgery at Harvard Medical School and MGH; Director of the Wellman Surgical Laboratories, the Laboratory for Tissue Engineering and Organ Fabrication, and Pediatric Transplantation. He has been in the field of tissue engineering since the early 1980s to solve the problem of organ shortages. His approach to developing tissue involves a scaffold made of an artificial, biodegradable polymer, seeding it with living cells, and bathing it in growth factors. The cells can come from living tissue or stem cells. The cells multiply, fill up the scaffold, and grow into a 3D tissue. Once implanted in the body, the cells recreate their proper tissue functions, blood vessels grow into the new tissue, the scaffold melts away, and lab-grown tissue becomes indistinguishable from its surroundings. He has authored numerous articles and publications, and has 50 patents or patents pending in the U.S., Canada, Europe, and Japan. Recent awards include recognition from the American Academy of Anti-Aging Medicine for contributions in the area of tissue replacement, the James Bartlett Brown Award from the Society of Plastic and Reconstructive Surgery, and the Clemson Award from the Society for Biomaterials. Recently, he was elected to the Institute of Medicine of the National Academy of Sciences. Dr. Vacanti received a BS summa cum laude from Creighton University; an MD with high distinction from University of Nebraska College of Medicine, and an MS from Harvard Medical School. He trained in General Surgery at MGH, in Pediatric Surgery at Children's Hospital, Boston, and Transplantation at the University of Pittsburgh.

ALEC SEVY is currently a medical student at Baylor College of Medicine. He worked in Dr. Vacanti's Tissue Engineering Lab while finishing a BA in Neurobiology at Harvard, and continued in the Lab for 3 years after graduation working on spinal cord, kidney, and liver tissue engineering projects.

MOHAMMAD KAAZEMPUR-MOFRAD is currently a Research Scientist in the Department of Mechanical Engineering and Division of Biological Engineering at MIT and a senior research fellow of the Tissue Engineering and Organ Microfabrication Laboratory at MGH. Dr. Kaazempur-Mofrad has a PhD in Mechanical Engineering from the University of Toronto.

MICHAEL SHIN is a Research Fellow in the Department of Surgery at the MGH and the Harvard Medical School in Boston, MA. He is working in the Laboratory for Tissue Engineering and Organ Fabrication, and his research focuses on cardiovascular, gastrointestinal, and orthopedic tissue engineering. He holds a BSc in Materials Science and Engineering from the Johns Hopkins University and received a PhD in Materials Science and Engineering from MIT.