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Development and Optimization of Viable Human Platforms through 3D Printing

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Development and Optimization of Viable Human Platforms through 3D Printing

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Abstract

3D printing technology offers a unique method for creating cell cultures in a manner far more conducive to accurate representation of human tissues and systems. Here we print cellular structures capable of forming vascular networks and exhibiting qualities of natural tissues and human systems. This allows for cheaper and readily available sources for further study of biological and pharmaceutical agents.

Introduction & Background

Cell culturing involves the techniques and protocols by which cellular lines may kept alive outside a natural environment to be utilized for further studies. Currently, cell cultures typically are made in a two-dimensional (2D) environment inside of a culture flask. 2D cultures form a monolayer of cells, and though they can be useful in assessing biological/chemical warfare agents as well as other pharmaceuticals, they are otherwise very limited in their ability to fully represent the human body and be fully scalable to human tissue systems. Three-dimensional (3D) cell cultures would be far more useful in assessing the toxicity and potency of various agents and drugs due to allow for multiple angles of entry into the cultures for these drugs. These cultures would be more representative of natural, *in vivo* human tissue systems and organs. 3D printing, otherwise known as additive manufacturing, is a technology used primarily in small-scale fabrication using polymers or composite materials. 3D bioprinting, a very new way of culturing cells, allows for the formation of *in vitro* cellular geometries and networks by printing cells in a “bioink” comprised of required nutrients. When reconfigured as not to subject the cells to mechanical stress, 3D printers can easily extrude viable cell cultures for further research. Fibrinogen and Thrombin, used naturally in blood clotting, have been shown to show promise in forming a fibrin support network for cellular mixtures (Lee, YB et. al 2010 and Norotte, C et. al 2009). When cells are printed in a gel support network, they can be induced to form networks resembling natural human tissues.

Methods

All printing was carried out within a Class II, A2 biological safety cabinet (BSC) using a MakerBot® Replicator® 3D printer (Figure 1). G Code was used as the language for controlling the 3D printer, all commands using G Code and subsequent geometries were novel structures with original source code. 3DTin software was employed in visualizing printed structures and geometries in an interactive, user-friendly program to build 3D structures. Replicator G was used as the software G Code interface for the MakerBot 3D printer.

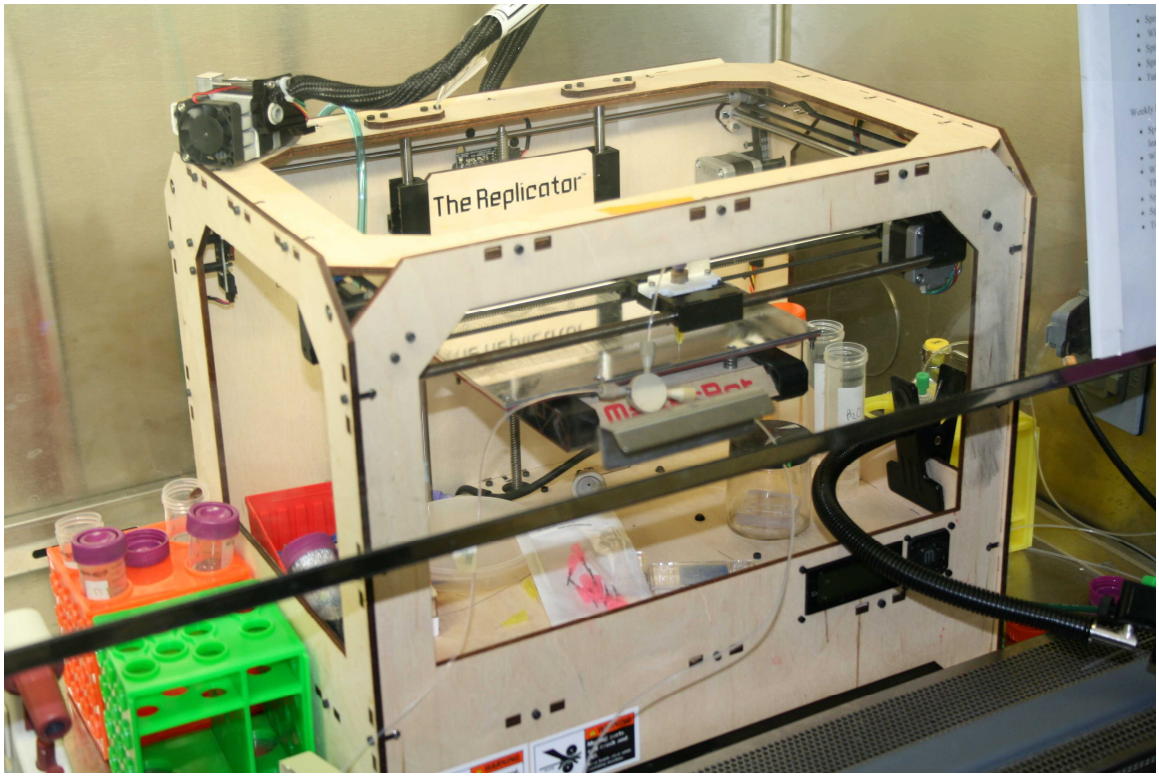

Figure 1. MakerBot® Replicator® 3D printer within the BSC

The printer was set up within the BSC as to ensure sterility of the printed cells. Microfluidic tubing was used as the channel through which cells were flowed. A water pump was used in order to provide a pressure source to dispense cells out of the printing needles and aspirate cells. Multiple lines were used in the printing configuration for both the solutions passed through the lines (Figure2) to keep fluidics unclogged. Stopcock valves were used so that one line could be loaded at a time. Fibrinogen from

bovine plasma (Sigma) was dissolved DPBS (Dulbecco's Phosphate-buffered saline, Life technologies) at a concentration of 25 mg/mL. 50 U/mL thrombin from bovine plasma (Sigma) was frozen and diluted ten fold to 5 U/mL in 40 mM $CaCl_2$ (Sigma).

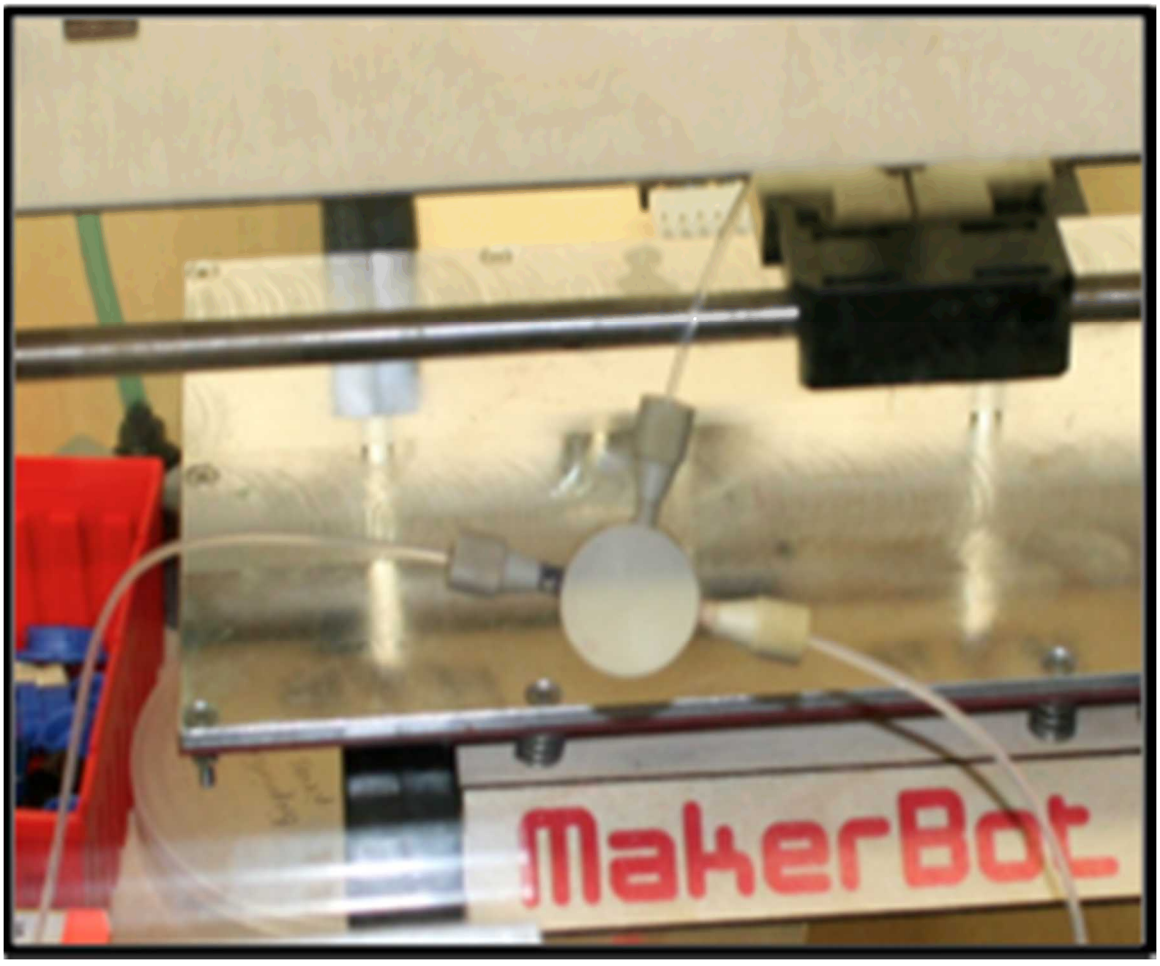

Figure 2. Mixing junction through which Fibrinogen and Thrombin are combined

Printer needles through which mixtures were extruded varied in size, with gauge widths as low as 0.1 mm and high as 0.4 mm. All needles used Luer Lock adapters to attach to the printer configuration, and were soaked in ethanol when not in use. Prior to printing, all solutions required sterile filtration, using both low protein affinity and standard sterile filters with a pore size of 0.2 µm. Water used in the pump and subsequently passed through tubing also required sterile filtering. The 3D printer stage was leveled before use. Media for cultures included EndoGRO (EMD Millipore) and EGM-2 (Lonza) for endothelial cells, and FGM-2 (Lonza) for fibroblast cells. alamarBlue®

assay was used to assess growth rate in cells, fluorescent dextran (Sigma) was used to observe permeability of cell membranes in 12 mm transwells with 0.4 µm pores (Sigma).

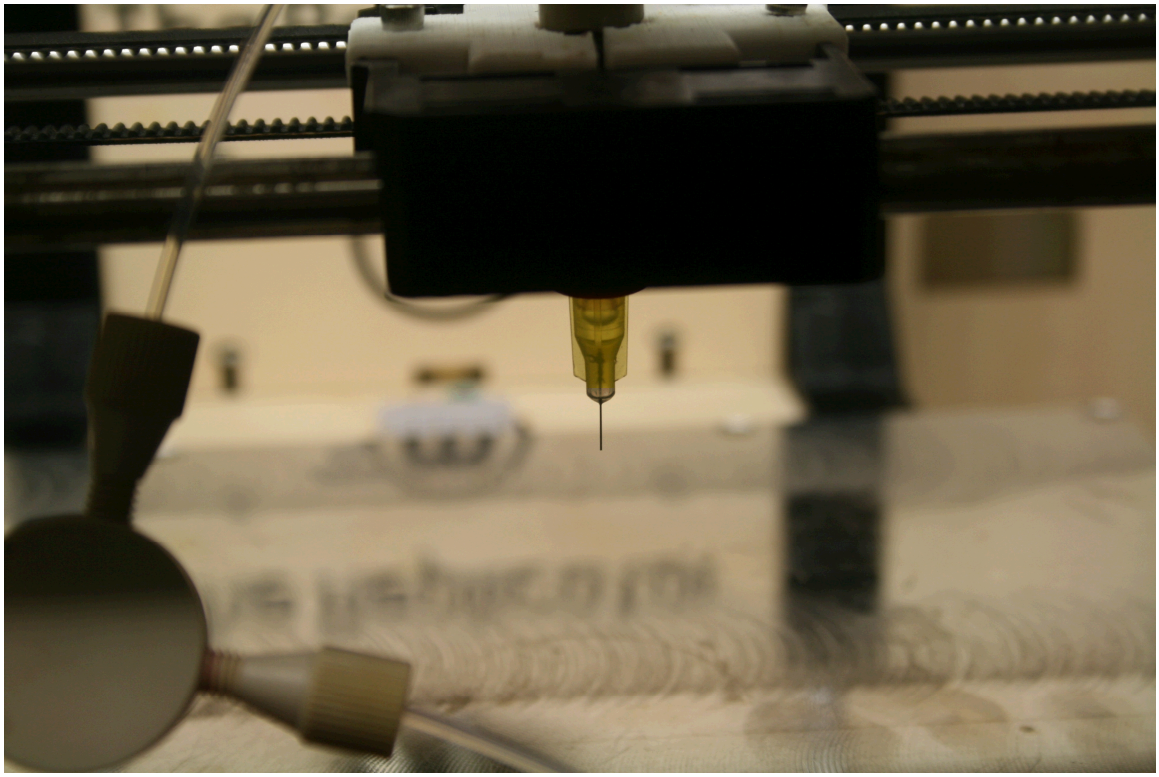

Figure 3. 0.1 mm (100 µm) gauge needle used in extruding cells

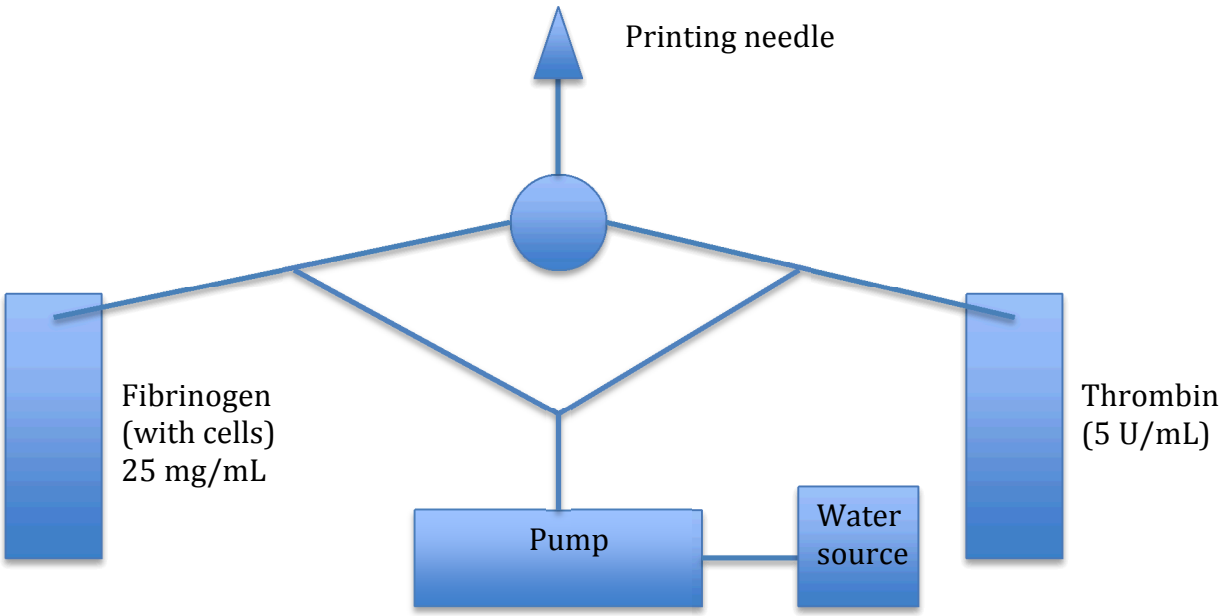

Figure 4. Flow layout for 3D bioprinting configuration

Experimental Procedure

Human Umbilical Vein Endothelial Cells (HUVECs) and Normal Human Lung Fibroblasts (NHLFs) were co-cultured in separate 75 cm^2 culture flasks (Corning) for one week. The cells were lifted using Trypsin-EDTA (Life Technologies), and placed into Fibrinogen at a density of roughly 200,000 cells/mL.. The two lines were loaded separately by pump aspiration with 150 µL of either fibrinogen or thrombin. The stage was raised to a flushed level with the needle, then lowered 20 mm. The ladder design was chosen for size and ease of culturing; G Code was used to map out the design (Figure 5). Once the printer was primed and both valves were opened, the needle was primed with instantly mixed fibrinogen and thrombin at a rate of 100 µL/sec until the mixture was visibly right up to the tip of the needle.. The stage was then raised 20 mm to return the needle to a flushed position, and the program was initiated to print the ladder structure (Figure 6). The gel was printed at a rate of 5 µL/sec onto 60mm petri dishes.

(**** start.gcode for The Replicator, single head ****)
M103 (RPM off)
M73 P0 (enable build progress)
G21 (set units to mm)
G90 (set positioning to absolute)
(**** begin homing ****)
G92 X0 Y0 Z0 (set X, Y and Z to 0)
(**** end homing ****)
G1 X0 Y0 Z0 F1000.0 (move to waiting position)
G130 X0 Y0 A0 B0 (Lower stepper Vrefs while heating)
M6 T0 (wait for toolhead, and HBP to reach temperature)
G130 X127 Y127 A127 B127 (Set Stepper motor Vref to defaults)
G0 X0 Y0 (Position Nozzle)

```
M108 R10.0    (Set Extruder Speed)
G4 P1500     (Create Anchor)
(**** end of start.gcode ****)
G90 ; use absolute coordinates
G21 ; set units to millimeters

M101
G4 P100
G1 X0 Y20 F500
G1 X10 Y20 F500
G1 X10 Y0 F500
G1 X0 Y0 F500
M103

G1 X0 Y6 F500
M101
G4 P100
G1 X10 Y6 F500
M103

G1 X10 Y14 F500
M101
G4 P100
G1 X0 Y14 F500
M103
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Figure 5. G Code used to print ladder structures in Replicator G program

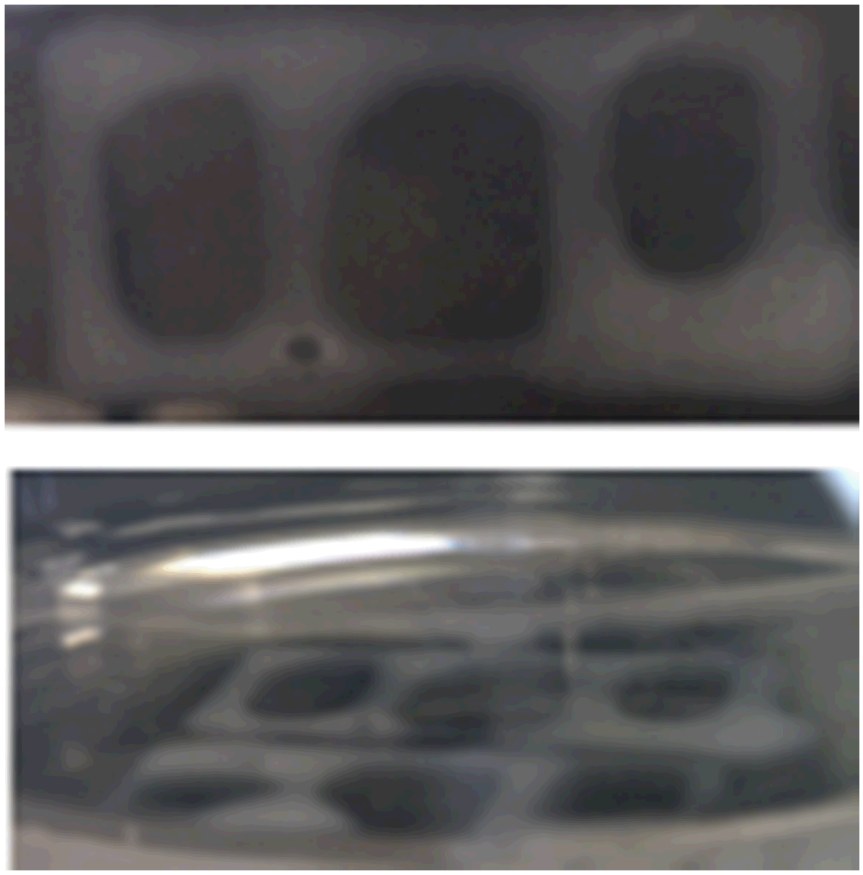


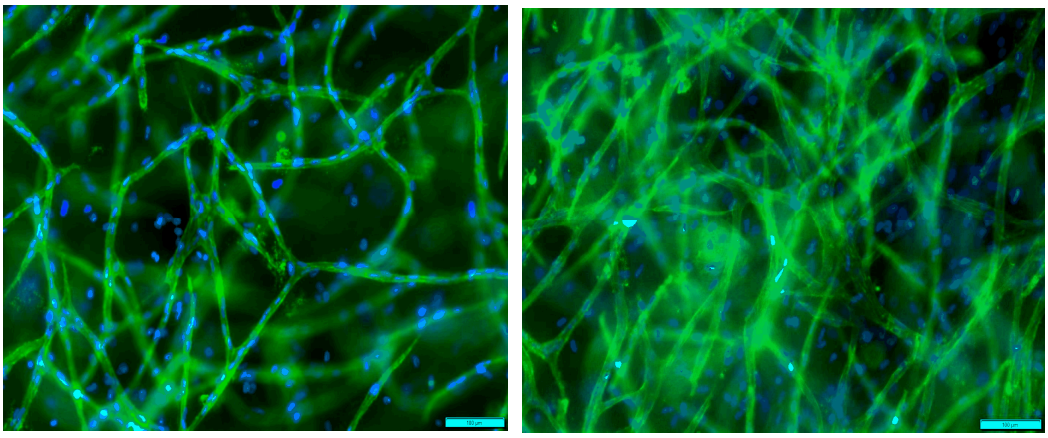
Figure 6. 3D Printed Ladder geometries

Gelling occurred very quickly, almost always within five minutes of extrusion onto surface. Several ladders were printed in order to use as much of the cell solution as possible. The dishes were covered, and further cultured for a week in both EndoGRO and EGM-2 media to induce network formation. Cells were given fresh media every other day in order to provide adequate nutrients and conditions for proper network formation. Upon contraction of gels (top of Figure 5), cells underwent fixation and immunofluorescent staining for CD31 endothelial cell marker using anti-mouse CD31 antibody (Alexa Fluor®). In addition to this, we printed gels using the same method but using two cell types, HCMECs (Human Cranial Microvascular Endothelial Cells) and HUVECs, and incubated both for a week in EndoGRO and EGM-2, respectively, before fixation and staining. This was done in order to observe the efficacy of the fibrin gel. Additional bioprinting was done in support for the Blood Brain Barrier (BBB) portion of the *in vitro* Chip-Based Human Investigational Platform (iCHIP) project. We used

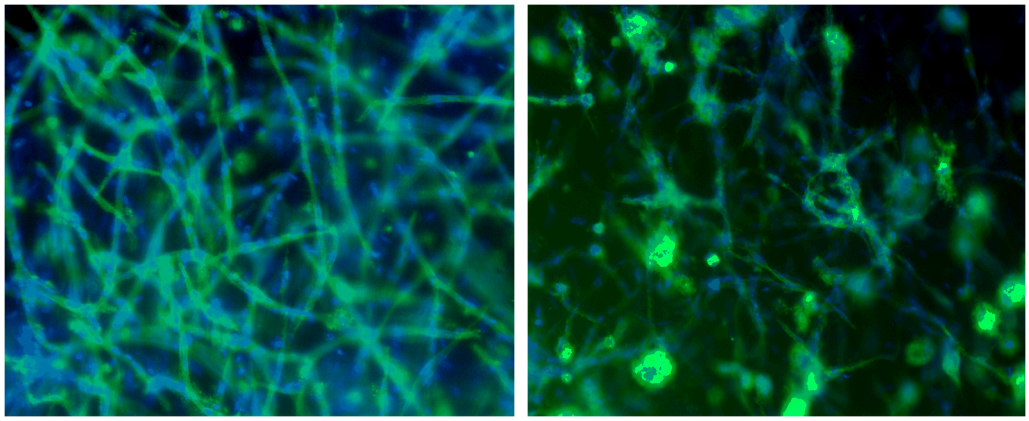
bioprinting as a means to create cellular structures that mimic the BBB in an *in vitro* physiological system capable of assessing biological agents (Weksler et. al 2013). To further assess differences in the two types of cells used both in iCHIP and to create networks, we conducted permeability and proliferation studies by seeding HUVECs and HCMECs onto transwells and in EGM-2 and EndoGRO media, respectively. Additional trials of multiple layers of printed ladders was carried out to investigate feasibility of printing cells in layers. Finally, for additional support on iCHIP, we used a printer program in two dimensions in an attempt to coat very small biochemical sensor electrodes using a 3D printer.

Discussion

Stained gels showed vastly extensive HUVEC/NHLF co-culture network formation after one week of culturing in various types of media (Figures 7, 8). Cells grown in EGM-2 media showed very distinct networks, perhaps due to the presence of vascular endothelial growth factor (VEGF) in EGM-2, a growth factor not present in EndoGRO. Cells grown in EndoGRO showed vascular network formation as well, but these networks appeared fainter and not as rigid as those grown in EGM-2 conditions.



Figures 7, 8. GFP/DAPI staining of vascular self-assembled networks of HUVEC/NHLF co-culture in EGM-2 (left) and EndoGRO (right) media



Figures 9, 10. Fibrin bioink encourages the self-assembly of both umbilical cord vein endothelial cells (left) and brain microvascular endothelial cells (right)

In comparing which cell types formed better vascular networks within the fibrin gel ladder structure, we found HUVECs form far better networks than HCMECs (Figures 9, 10). As verified by Figures 7 and 8, the presence of VEGF in the EGM-2 media seemed to make a large difference. The HUVEC line seems to be more conducive to vasculogenesis, as it formed networks in both EGM-2 and EndoGRO, despite a lack of VEGF in EndoGRO. This supports our use of HUVECs as a way to form vascular structures that closely mimic natural human endothelial structures and vessels. The permeability and growth assays showed interesting differences in cells types as well. Permeability studies (Chart 1), showed a clearly higher permeability in HUVEC cells. This seems consistent with HUVECs being derived from umbilical veins, whereas the more selectively permeable brain endothelial HCMECs show less dextran flowing through. In the alamarBlue® test (Chart 2), a clearly higher growth rate (% alamar blue reduced) was seen in HCMECs rather than HUVECs. We conclude that while HUVECs are more permeable and less selective than HCMECs, the latter grow at a faster rate than the former.

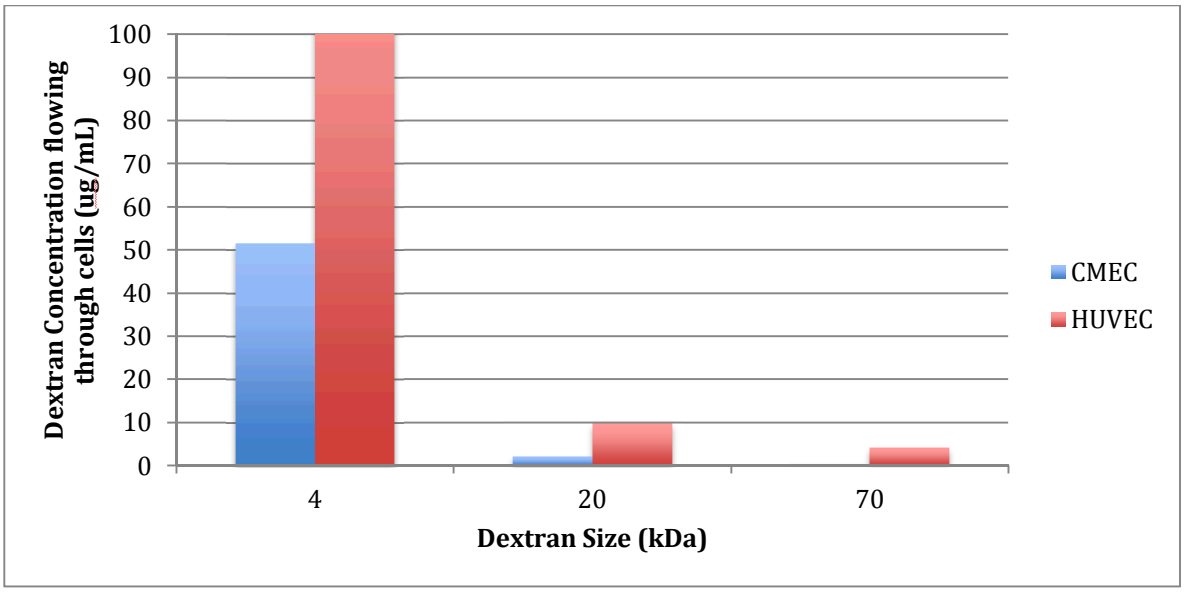


Chart 1. Permeability assay using FITC (495, 525 mm) and TRITC (557, 610 nm) fluorescent dextran

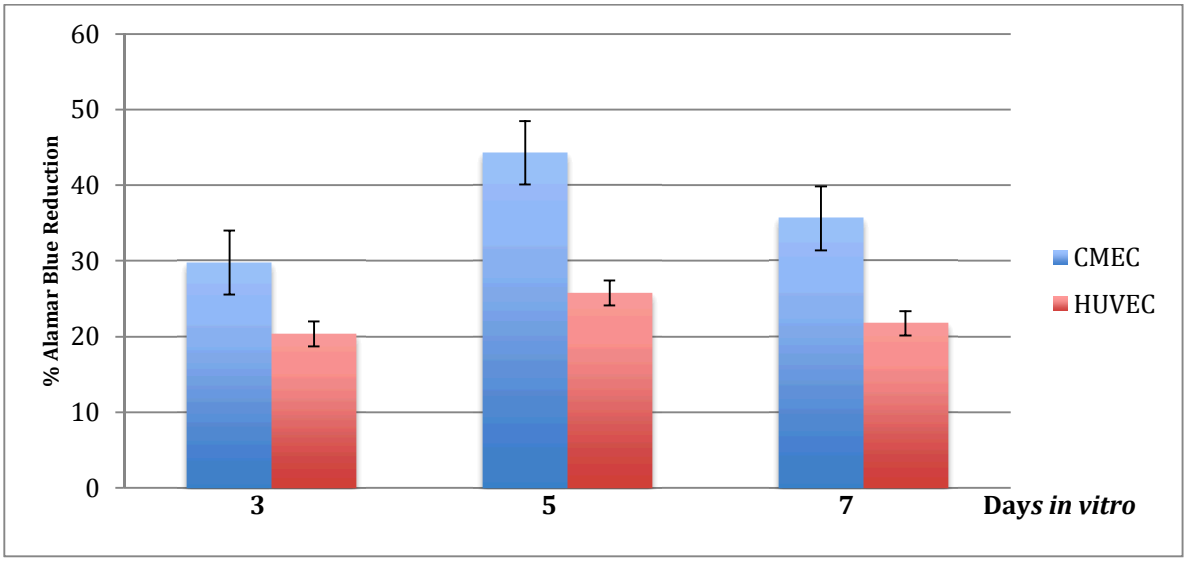


Chart 2. Alamar Blue growth assay using absorbance at 570, 600 nm wavelengths

Future Directions

Many times, the interface between the printer and software was often disrupted and required restarting the entire system, sometimes during a build, which caused premature gelling of fibrin and clogging of the printer lines. Future work should explore other 3D printers as well as the proper coding interfaces to run them. Leveling the printer stage involved some degree of subjectivity, and often times the stage was difficult to fully level. A better protocol for examining the stage to ensure a fully level surface to print on is a necessary hurdle. Getting the proper volumes of both fibrinogen and thrombin when loading the printer for extruding cells was also done by hand; we believe more work optimizing the proper rate and volume to prep the device for printing would allow for a more quantifiable means to create 3D cultures. Moreover, new geometries and bioinks should be explored for each given candidate cell line for printing in order to find which printed structure and media mixture provides optimal results for a specific cell line.

Conclusion

Using fibrin gel networks with HUVECs, we have demonstrated the ability to induce cellular vasculogenesis and angiogenesis and the building of realistic artificial human tissues. 3D printers, when reconfigured, are capable of extruding viable, sterile cellular mixtures for further culturing. As biological research across the world continues to grow, the need is clear for readily available, low cost tissue samples that accurately represent natural human systems. 3D printers used for biological applications could lead to a shift in the foundations of cell and tissue culturing (Figure 11). Fibrin gels provide support structures for inducing cells towards more realistic vascular networks.

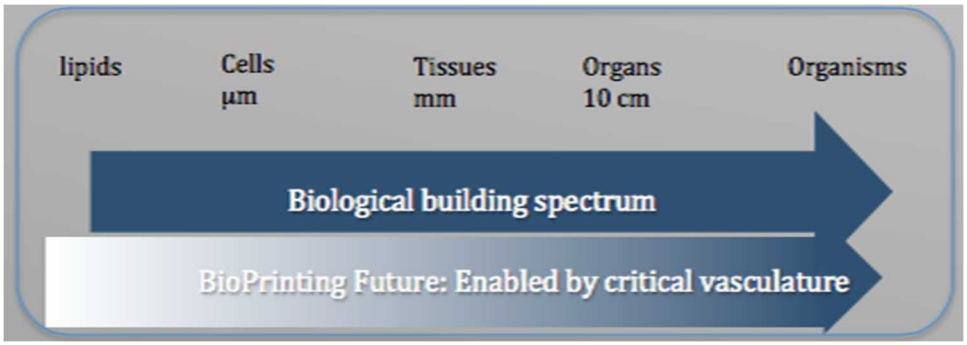


Figure 11. Future direction of bioprinting and constructing biological samples

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