Vanderbilt University

Department of Biomedical Engineering

BME 3861 Final Manuscript

Exploration of Commercially Available 3D Bioprinter Capabilities in Artificial Tissue Development Using the CELLINK BioX6

Eric C. Spivey, Madeline E. Colley, James K. Metz

Exploration of Commercially Available 3D Bioprinter Capabilities in Artificial Tissue Development Using the CELLINK BioX6

Eric C. Spivey, Madeline E. Colley, James K. Metz

Novel methods of artificial tissue development using 3D bioprinters have shown promising results in generating structures that can support neural cell growth, but these printers feature a variety of highly specific modifications and are not easily reproducible. 3D bioprinters now on the market may be able to generate these models without the need for modification, but this has yet to be validated. Our study utilizes the CELLINK BioX6. The BioX6 is a commercially available microextrusion 3D bioprinter that features a pneumatic or screw dispensing system. Trials using the BioX6 and GelMA bioinks loaded with HeLa cells were used to characterize the functionality of the printer, including bioink printability window, spatial resolution, and cell viability. Continued exploration of the CELLINK BioX6 will reveal the possibilities of commercially available bioprinters and lay the foundation for more streamlined, accessible, and reproducible methods of tissue engineering.

INTRODUCTION

Methods for modeling the human nervous system have taken on a variety of different forms and two main approaches to generating these models have arisen. *In vivo* models using brain slices of animal brains have been a primary source for defining key features of human neurophysiology, pharmacology, and pathology[1]. These models have produced data that accurately mimics the variability and complexity of the nervous system. However, they are expensive and have limited relevance in studies focused on determining the physiological circumstances surrounding human-specific diseases like Alzheimer's or Parkinson's[1]. Furthermore, *in vivo* models that make use of human cells are susceptible to perturbations within the blood-brain barrier (BBB)[2].

In vitro models, like organ-on-chip devices, have been developed to bridge the gap between computer models and biopsies[2]. These organoid devices integrate true-to-form brain structures via pluripotent stem cells into high tech artificial microfluidic control systems; but fail to account for the influence of the blood-brain barrier and are not easily reproducible[1]. Current efforts to generate organoid models have made use of microphysiological systems (MPS) that mimic the microenvironment of cells in the brain tissue crucial to supporting cell growth and do account for the complex relationship of the blood-brain barrier[3]. One of these MPS models is VIIBRE's neurovascular unit (NVU) that is a multicompartment microfluidic bioreactor[4]. This technology allows for the insertion of volumes of cell-laden structures into select

multi-planar layers, effectively encapsulating these structures in a 3D 'gateway' like the blood-brain barrier[3]. The bioreactor also has the capability to open and close allowing for analyses including imaging mass spectrometry[3]. One critical component of the MPS that still needs development is the successful incorporation of a neural circuit too complex to be model via traditional 2D cell cultures. The aim of this research is to develop the technical foundation for protocols utilizing hydrogel structures to print cells in 3D space and mimic neural circuitry found in brain tissue. These structures will allow for the placement of specific neural and glial cell types from the immortalized murine cell line Neuro-2a within a spatially selective location and support the extension of axons and dendrites between these cell populations to generate circuits via synapses.

Three dimensional bioprinters currently on the market can produce structures that can sustain cell growth; however, the methodology for developing these structures has not yet been established[3]. Because of the complexity of the neurovascular tissue, fabricating structures with enough precision is challenging, and determining the capabilities and limitations of commercial bioprinters is pivotal in confirming the feasibility of this approach. Current studies using 3D bioprinters to generate structures embedded with neural cells have been useful in modeling neural tissue[2]. These printers, however, have been modified and do not accurately represent the capabilities of commercial printers that often sacrifice high precision for ease of use[4,5]. Many of these prints also utilize additional structural bioinks such as polyethylene glycol diacrylate (PEGDA) as biocompatible scaffolding[4,5]. Our study explores the use of commercial 3D bioprinters in generating structures using gelatin methacrylate (GelMA). The printing methods developed focus on generating structures with enough spatial resolution for future applications in brain tissue development. Our findings have provided insights into the capabilities of the Cellink BioX6 and an understanding of how specific alterations to printing parameters and limitations may impact the use of commercially available 3D bioprinters in tissue engineering applications.

EXPERIMENTAL METHODS

Hydrogel design

The hydrogel structure was designed as a simple cube using SOLIDWORKS (Dassault Systémes). The height of the cube was set to three times the nominal inner diameter of the nozzle tip used to extrude the bioink. The height for prints using 22G nozzles and 25G nozzles were 1.652mm and 1.040mm, respectively. The length and width of the cube were set to 10mm for both nozzle sizes. Heartware (Cellink) and Slic3r (Alessandro Ranellucci) were used to process the CAD model for printing. DNAStudio (Cellink) was used to establish printing parameters which were consistent across prints using both nozzle sizes and throughout the duration of each print apart from extrusion pressure.

Layer Height	0.25 mm
Print Pressure (22G)	18 kPa
Print Pressure (25G)	25 kPa
Print head Temp	24.5 ^{o}C
Print Speed	5 mm/s
Photocrosslinking Time	1 s
Printbed Temp	22 ^{o}C
Infill Pattern	Grid
Infill Density	40 %

Figure 1. Printing parameters used for all sample prints using GelMA bioink.

Preparation of GelMA/HeLa bioink

A 0.05% photoinitiator stock solution was made by dissolving solid lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) in DPBS buffer and mixing thoroughly at room temperature. A 10% GelMA solution was prepared by dissolving 300 mg of GelMA in 3mL of the 0.05% LAP solution and mixing. The GelMA solution was covered in aluminum foil and allowed to sit until air bubbles disappeared and the solution was clear. 5mg of fluorescence acrylate (FA) were dissolved in 1mL of dimethyl sulfoxide (DMSO). Approximately 3uL of the FA/DMSO solution was added to the GelMA and mixed thoroughly while shielded from light.

3mL of the final GelMA solution was loaded into a UV protective, 3mL syringe. A different 3mL syringe was loaded with 300uL of cell suspension (80k cells/mL). The two syringes were connected using a lure lock and the solutions were pushed back and forth gently 15 times to ensure thorough mixing. The entirety of the GelMA-HeLa mixture was loaded into the UV protective syringe and stored in an incubator until printing.

Pre-printing preparation and bioprinting procedures

The GelMA-HeLa bioink was loaded into a thermally-controlled printhead set to 30 ^{o}C and allowed to equilibrate at that temperature for 30 minutes. After 30 minutes, the mixture was tested for equilibration by flipping it upside down and observing air bubbles float to the top. The bioink was reloaded into the thermally-controlled printhead, set to 24.5 ^{o}C, and allowed to thicken for 20 minutes before printing. The 3D printer was autocalibrated three-dimensionally by situating the nozzle tip in contact with the desired print location, locking the syringe slider, and moving the printbed 0.2mm down to allow room for extruded bioink. A 405nm printhead tool was used to photocrosslink the GelMA prints in a layer-by-layer fashion. After printing, the samples were submerged in culture media in preparation for CMC-gelatin embedding.

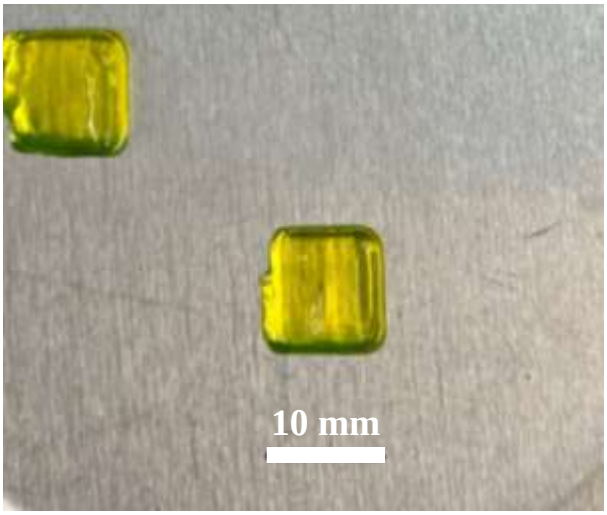

Figure 2. Printed GelMA samples spiked with fluorescein-acrylate.

CMC-gelatin embedding and cyrosectioning

A 5% and 10% carboxymethocellulose-gelatin (CMC-gelatin) embedding mixture was prepared in the same fashion as Nelson et al[6]. Printed samples were placed in molds, covered in the CMC-gelatin embedding mixture, and flash-frozen at -80 ^{o}C. The frozen samples were loaded onto a cutting chock set to -21 ^{o}C. Tissue samples were sectioned at 10 um, collected on an 8Ω ITO glass slide, and stored at -80 ^{o}C until matrix application and imaging.

Tissue Preparation and Mass Spectrometric Imaging

Sublimation of the samples were performed using a SubliMATE device (unpublished, Eric C. Spivey). The SubliMATE was prepared by adding dry ice chips to an ethanol alcohol (EtOH) bath until minimal sublimation of chips was observed.

The sample slides were inserted into the holder indentations of the sample holder and secured with copper tape. 20 mg of dry 2,5-dihydroxybenzoic acid (DHB) were dissolved in 2mL of acetone so that a total of ~1.33 $\mu g/mm^2$ of DHB matrix could be deposited onto the sample. The solution was pipetted in three drops of 0.67mL at points forming and equilateral triangle around the center of the matrix pan and allowed to dry for 3 minutes. The matrix pan was placed on the heat plate of the SubliMATE device and the vacuum was started. Once the vacuum was established, coolant was placed in the bath and the stopcock was opened to keep the bath cold. Once the vacuum fell below 50 mTorr, the thermocouple was turned on and the induction heater was set to 320°F and turned on. The device was allowed to run for 10 minutes ensuring that the ice bath was kept stocked and ensuring sufficient melt to cover the bath surface. After 10 minutes and with the vacuum still running, the induction heater was turned off and warm water was poured into the bath until all the ice was melted and the SubliMATE top returned to room temperature. The vacuum pumped was turned off and the vacuum was disrupted by opening the leak valve.

Sublimated tissue samples were imaged for lipids using techniques based on Angel et al and Stoeckli et al.[7,8] Bruker Rapiflex imaging experiments were performed in negative ion mode with 200 laser shots per trigger. The pitch was set to 5µm. Results were normalized by total ion count.

RESULTS and DISCUSSION

Imaging Analysis

Cell-laden GelMA samples displayed unanticipated large pores in GelMA structure in all sectioned samples (Figure 3). This may be an impact of suboptimal printing conditions or an impact of bioink properties. Equilibration of the GelMA mixture was intended to generate a more fluid bioink that allowed for confluent structures. Smaller periods of UV light were used to photocrosslink the GelMA structure after each layer. UV photocrosslinking time was purposefully kept short to prevent damage to cells; however, it may have limited the structural compactness of generated prints.

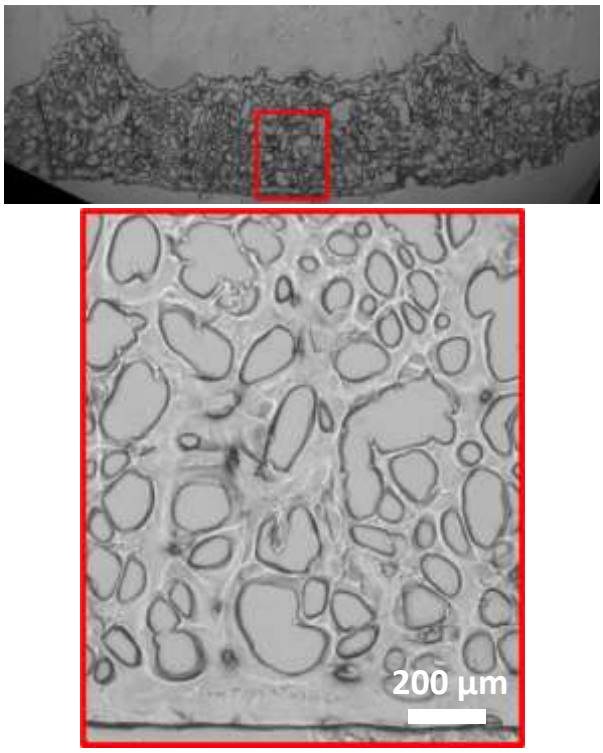

Figure 3. Microscopic images of cell-laden GelMA samples. Cells are visible as small, dark pores or punctate bodies.

Additionally, pores in the GelMA structure may be associated with volumes once occupied by absorbed cell culture media after printing.

Because hydrogels, like GelMA, have high water absorption and retention capabilities, these pores may have been generated by pockets of absorbed water between printing and sectioning. Furthermore, while the BioX6 was able to extrude filaments ~0.216mm in

diameter, these structures are generated in a layer fashion (Figure 2). This layer form of printing can compromise the integrity of a confluent structure by generating interfaces between lines of layered material.[5,9] These

interfaces could have allowed for openings in the structure for air or other material to infiltrate and produce pockets in the overall structure. Other studies have focused on reducing the impact of layer-by-layer interfaces by using alternative 3D printing methods. Microextrusion continuous projection printing (uCPP) allows for quick printing of structures and decreases layer interfaces via quick photopolymerization.[5,9] These printers, however, can require unique technological know-how and often feature unspecific methods and printer alterations tailored to their study. Koffler et al. developed and in-house software to control the 3D printer used to make quick spinal cord scaffolds[5]. Similarly, Joung et al. used a custom-built extrusion-based printer using quantum dot-based light-emitting diodes.[4,10] While these printers have shown promising results in generating structures suitable in tissue

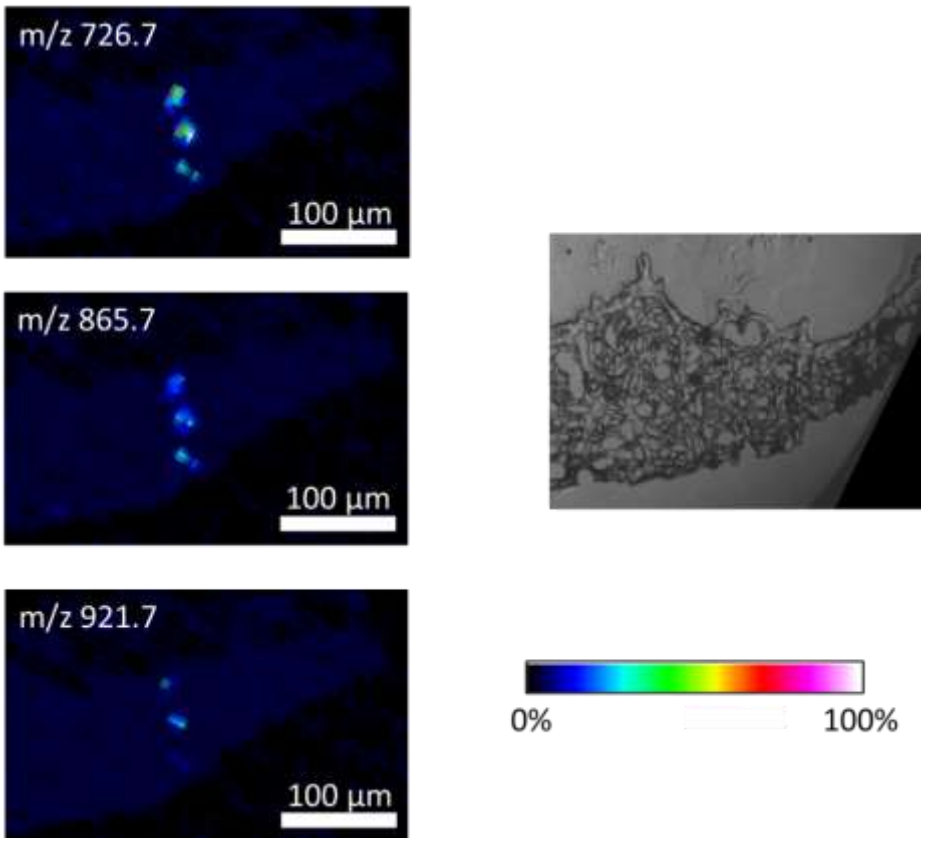

Figure 4. *Imaging using Bruker Rapiflex at 5 um resolution. Cells display strong lipid signal absent from surrounding GelMA.*

engineering applications, they are not easily reproducible or commercially available. Cells in our printed structures could be distinguished from larger GelMA pores and denoted as smaller dark areas or punctate bodies. Cells were visibly dispersed throughout the sectioned area. These images suggest that the BioX6 has the capability to embed cells in spatially distinct areas of the printed structure.

Imaging using matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) displayed significant lipid signals from embedded cells (Figure 4.) Signals from the fluorescein- acrylate spiked GelMA regions could be contrasted with lipid signals from the cells to display regions of high lipid concentration.

Characterization of BioX6 printing capabilities

Proper selection of bioinks is a crucial aspect in all tissue engineering applications. Bioinks must have enough viscosity to print in distinct filaments while having biocompatible properties sufficient for cell growth and proliferation.[9] These, and other variables, combined determine an ink's printability window which remains a major limitation in 3D bioprinting cell-laden structures.[9]

We printed cell-laden structures in all experiments using in-lab formulated GelMA, spiked with fluorescein-acrylate, and loaded with HeLa cells. GelMA is a soft hydrogel with many biocompatible properties, however, it is thermally sensitive. The optimal temperature dependent printability window of the GelMA bioink was determined during exploratory printing trials. The GelMA bioink was extremely sensitive to temperature and when heated to just ~1 ^{o}C above 24.5 ^{o}C became thin and did not extrude as distinct filaments on the print surface. Similarly, GelMA bioinks cooled ~1 ^{o}C below 24.5 ^{o}C became viscous and would not extrude at all. The thermally controlled printhead was able to maintain a set temperature but fluctuations in this temperature by $\pm$ 5 ^{o}C caused small perturbations in extrusion volumes and limited the filament deposition resolution. Our print model remained simple in order to reduce the impact of these effects in imaging experiments.

Potential light contamination of bioink loaded in the nozzle tip posed additional issues during printing. Frequent replacement of the extruding nozzle was performed to mitigate the effects of mid-print clogging; however, this introduced additional structural impediments. Replaced nozzles could not be perfectly fitted to the position calibrated for the previous nozzle; therefore, each nozzle had to be individually re-calibrated to prevent potential collision of the nozzle with the printing surface.

All GelMA bioink printing experiments utilized the BioX6's pneumatic dispensing system. These dispensing systems use air to apply pressure to the extruding syringe pump.[9] This extrusion method can fail if the material requires more force for extrusion than established. While this was limiting for experiments where continuous filament deposition was desired, it can act as a safeguard for maintaining biocompatible printing conditions. Cells embedded in bioinks may not be robust enough to endure extensive or prolonged force. For all HeLa embedded printing experiments, we maintained a constant pneumatic pressure viable for the cells and changed other printing window variables in order to optimize bioink printability while maintaining pressures suitable for HeLa cell viability.

CONCLUSION

Tissue engineering using 3D printers have shown promising results in generating structures that can be highly specific and quickly produced. Our printed hydrogel structures have unveiled many of the capabilities and limitations of the CELLINK BioX6 and have provided preliminary data towards generating cell-laden tissue for tissue engineering applications. Because of the complexity and number of variables involved in

these experiments, determining the relationships between bioink printability window, spatial resolution, and cell viability is challenging and new approaches to generating these structures need to be considered. Our exploration into the technicalities of printing with commercially available printers has provided more defined and reproducible methodology for 3D bioprinting cell-laden structures for future tissue engineering applications.

REFERENCES

1. Geerts, H, Wikswo, J, vander Graaf, P H, Bai, J P F, Gaiteri, C, Bennett, D, Swalley, S E, Schuck, E, et al., Quantitative systems pharmacology for neuroscience drug discovery and development: Current status, opportunities and challenges. CPT Pharmacometrics Syst. Pharmacol., , 9:5-20. 2020. https://doi.org/10.1002/psp4.12478
2. Brown, J A, Pensabene, V, Markov, D A, Allwardt, V, Neely, MD, Shi, M, Britt, C M, Hoilett, O S, et al. Recreating blood-brain barrier physiology and structure on a chip: A novel neurovascular microfluidic bioreactor. Biomicrofluidics, 9:054124. 2015. PMCID: PMC4627929
3. Watson, D E, Hunziker, R, and Wikswo, J P. Fitting tissue chips and microphysiological systems into the grand scheme of medicine, biology, pharmacology, and toxicology. Experimental Biology and Medicine 242:1559-1572. 2017. PMCID: PMC5661772
4. Joung, D, Truong, V, Neitzke, C C, Guo, S Z, Walsh, P J, Monat, J R, Meng, F B, Park, S H, et al. 3D Printed Stem-Cell Derived Neural Progenitors Generate Spinal Cord Scaffolds. Adv. Funct. Mater., 28 1801850. 2018
5. Koffler J, Zhu W, Qu X, et al. Biomimetic 3D-printed scaffolds for spinal cord injury repair. *Nat Med*. 2019;25(2):263-269. doi:10.1038/s41591-018-0296-z
6. Nelson, K. A., Daniels, G. J., Fournie, J. W., & Hemmer, M. J. (2013). Optimization of whole-body zebrafish sectioning methods for mass spectrometry imaging. *Journal of biomolecular techniques : JBT*, *24*(3), 119–127. https://doi.org/10.7171/jbt.13-2403-002
7. Angel, P. M., Spraggins, J. M., Baldwin, H. S., & Caprioli, R. (2012). Enhanced sensitivity for high spatial resolution lipid analysis by negative ion mode matrix assisted laser desorption ionization imaging mass spectrometry. *Analytical chemistry*, *84*(3), 1557–1564. https://doi.org/10.1021/ac202383m
8. Stoeckli, M., Chaurand, P., Hallahan, D. *et al.* Imaging mass spectrometry: A new technology for the analysis of protein expression in mammalian tissues. *Nat Med* **7,** 493–496 (2001). https://doi.org/10.1038/86573

9. Zhang, J., Wehrle, E., Rubert, M., & Müller, R. (2021). 3D Bioprinting of Human Tissues: Biofabrication, Bioinks, and Bioreactors. *International journal of molecular sciences*, *22*(8), 3971. https://doi.org/10.3390/ijms22083971
10. Kong YL, Tamargo IA, Kim H, Johnson BN, Gupta MK, Koh TW, Chin HA, Steingart DA, Rand BP, McAlpine MC. *Nano Lett.* 2014;14:7017.
11. Wikswo, J P, Spivey, E C, Schaffer, D K, Reiserer, R S, Seale, K T, and Block, F E, Multicompartment microfluidic bioreactors, cylindrical rotary valves and applications of same, Patent Application No. PCT/US2019/034285, 2019
12. PT. (2019). Safety Data Sheet: CELLINK START, Version: 3. Boston, MA. https://www.cellink.com/wp-content/uploads/2019/03/START-SDS-12-July-2019.pdf
13. MG, JB. (2021). Specification Sheet: GelMA Bioink LAP 0.25%, Version 4. Boston, MA. https://www.cellink.com/wp-content/uploads/2022/03/Specification-Sheet-GelMA-Bioink-LAP-0.25_08-Apr-2021-1-4.pdf
14. JB, VK. (2019). Photocrosslinking Optimization Protocol: GelMA Series bioinks, Version 3. Boston, MA. https://www.cellink.com/wp-content/uploads/2022/03/Photocrosslinking-Optimization-Protocol-GelMA-series-19-Aug-2019-17.pdf