3D Bio-Printing of Muscle Tissue

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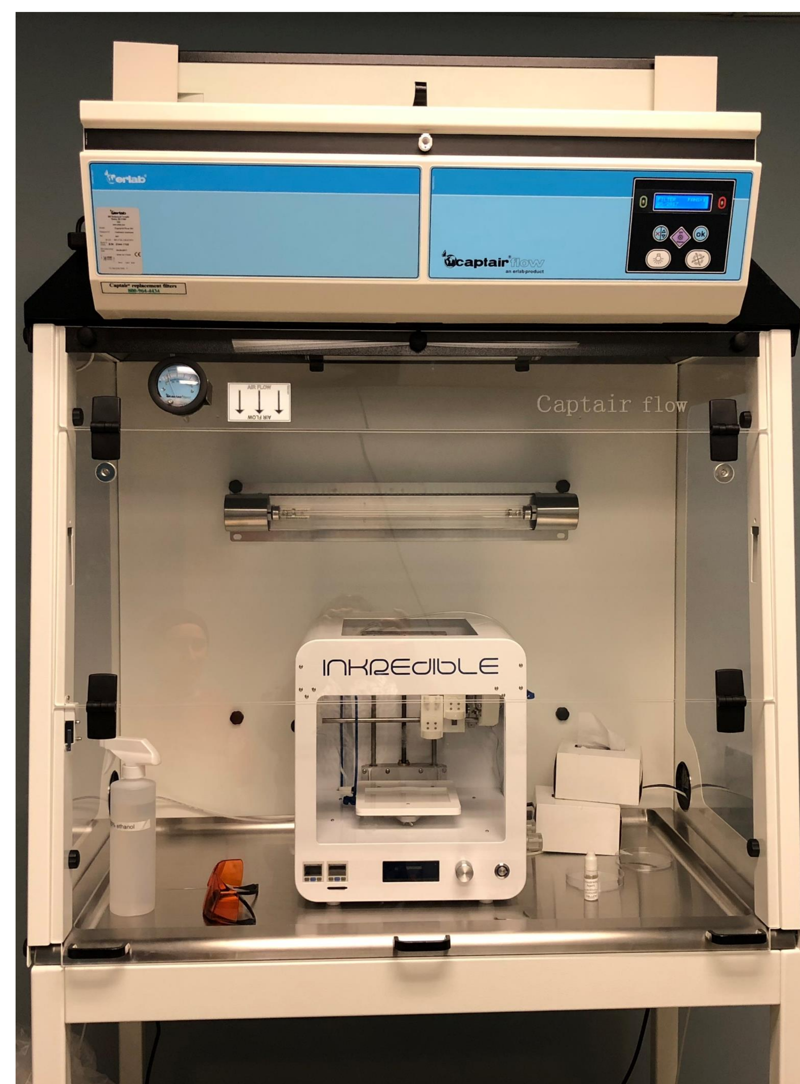
Abstract

Myoblasts, Myogenic stem cells, are capable of differentiating into functioning contractile myocytes (muscle fibers). In skeletal muscle, myocytes are aligned in parallel arrays as fascicles. This allows for contraction in only one direction, all myocytes working together collectively to provide contractile force in a functional manner. This geometry cannot be achieved by traditional culturing methods. Bio-Printing offers a method of achieving this geometry, aligning cells in parallel arrays in culture. Using the Inkredible® bio-printer to print out grid like structures containing myoblasts allows us to control the tissue geometry. After cross linking the matrix, we feed and grow the cells inducing them to form muscle tissue with parallel arrays of muscle fibers. In order to confirm we have live muscle tissue we utilize a number of assays: nuclear staining to observe the present nuclear morphology by microscopy, scanning electron microscopy to observe structure of the matrix, qPCR to access biological function and muscle specific gene expression.



M1: 3D Printing System

M2: Printed tissue grid

Methods

- Grew stem cells to sub-confluency and harvested the cells to mix with the auger based bio gel.
- The printer was set to approximately 18 psi head pressure to the bio ink cartridge, with the pump attached to a separate power source than the printer to avoid interference. Then, we set the stage at the start point and calibrated the axis.
- The cells were then printed in a bio gel mix in a grid (tissue) pattern using Inkredible® bio-printer using the "tissue model" 3D printing program.
- Then the tissue was flooded with chemical cross linker which was activated using UV radiation and fed with Fetal calf serum (10%) for 7-10 days.
- After culturing, some grids were fixed in formalin for microscopy and the others were pooled made into a single pellet for RNA extraction
- The tissues were then placed under an Olympus dissecting microscope fitted with an "Idea Inc." digital camera calibrated with a stage micrometer. Images were captured using "Spot" digital imaging capturing software.
- The average bar width and standard deviation was measured manually on the computer monitor to identify the symmetry of the tissue bars. We analyzed 6 images for a total of 22 measurements and averaged the bar width and determined standard deviation.
- Day one of RNA extraction was completed with the addition of Amersco's Ribozol(RNA extraction reagent). This was followed by vortex mixing the sample using Scientific Industries Vortex-Genie 2 Laboratory and centrifuged the samples using BioExpress SpinMate 24 micro-centrifuge.
- After the addition of chloroform and upper clear aqueous phase contains RNA was removed and transferred to another 1.5mL micro-centrifuge tube and froze overnight at -20°C
- Day 2, the samples were thawed out and centrifuged for 10mins at 13,330 RPM. A whitish pellet of RNA was present at bottom of tube.
- Isolate RNA by 1,000μL of 75% ethanol to tubes.
- After samples were centrifuged again, ethanol was removed and RNA pellets visible for air dry.
- Re-suspend the pellet in 200μL of nuclease free water and stored at -80 °C.
- Analyze RNA samples through qPCR

Results

The fundamental geometric data represents the tissue structure **(Figure 1)** was determined by light microscopy. Each grid has a series of smaller bars within it. Measurements were taken of each bar width are in millimeters. The average bar width was 1.12mm with a standard deviation of .22mm, these results are consistent within the range which we believed to be acceptable. The grids were very fragile in nature and could easily break and need to be handled with care.

RNA was isolated for qPCR analysis. RNA is a product of the biological component of the tissue and is not a part of the biogel. After Ribozal extraction, the purified RNA was subjected to spectral analysis, Nanodrop®. **(Figure 2)** The sample ID 3D 400 1.2 showed the highest amount of nucleic acid concentration for RNA. This, 65.1 ng/μl, seems to be a very reasonable amount. This sample had the most tissue material when the tissues were processes for RNA extraction. Based on the 260/280 ratio, the sample appears to be near pure(purity=1.8). However, the 260/230 ratio of 0.13 is far below the expected value of 2. This indicates contamination from most likely Guanidine Thiocyanate. (Qiagen Technical Bulletin FAQ ID 2248)

Figure 1 Data

Image Number	Average Bar Width (mm)
1	1.28
2	1.18
3	1.11
4	.995
5	1.06
6	1.16

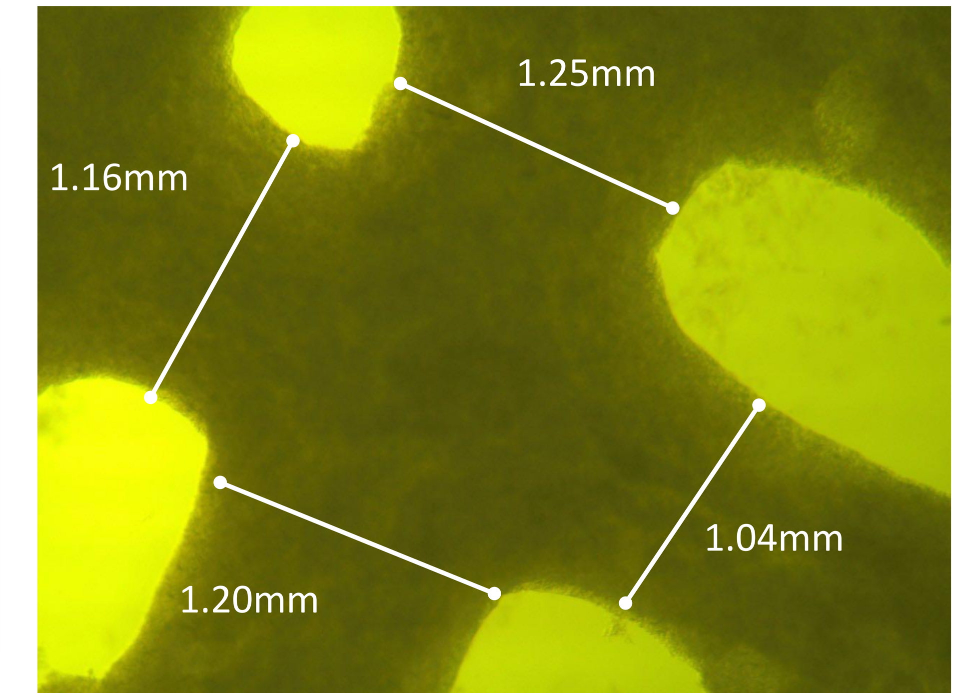


Figure 1: R1: These measurements were made using stage micrometer. What is indicated in the table is average width in millimeters of each image. The average bar width is 1.12mm with a standard deviation of .22mm

Figure 2 RNA Data

Sample ID	Nucleic Acid Conc.	Unit	260/280	260/230	Sample Type
3D 400 1.2	65.1	ng/μl	1.73	0.13	RNA
3D 400 2.2	4.6	ng/μl	2.66	0.01	RNA
3D 200 3.2	5.8	ng/μl	4.02	0.01	RNA
3D 200 4.2	4.4	ng/μl	2.5	0.01	RNA

Figure 2: This is an analytical table showing the spectral data (Nanodrop®) verifying that cells survived the printing and subsequent culture phase of tissue construction.

Discussion

To further confirm the viability of the cells embedded in the tissue future experiments will use the fluorescent DAN stain, DAPI, to confirm normal nuclear structure. Also, in future printing runs, the distribution and careful quantitation of each sample must be much more carefully controlled and recorded.
Lastly, before quantitative PCR(qPCR) analysis, the contamination indicated from 260/230 ratio needs to be "cleaned up." Then quantitative Polymerase chain reactions can be performed using select myo-specific primers.

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