Peristaltic Pump to Automate Media Flow for Tissue-Engineered Muscle Repair (TEMR) Construct

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Peristaltic Pump to Automate Media Flow for Tissue-Engineered Muscle Repair (TEMR) Construct

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Abstract

Volumetric Muscle Loss (VML) injuries result in functional loss in the muscle where more tissue is lost than the body can regenerate or repair. The Christ Lab at UVA has developed a Tissue Engineered Muscle Repair construct (TEMR) to help with treatment of VML injuries. However, contamination can easily be introduced during the TEMR biomanufacturing process following media changes, since the bioreactor used to incubate the TEMRs must be opened every forty-eight hours. To address this issue, we developed a perfusion system using two peristaltic pumps derived from an open-source design to change media in the bioreactor automatically while maintaining a closed system. We adapted a rotor to provide more torque than was inherent in the original design, which relied on static friction, increasing the range of possible flow rates. After calibrating motor speed to flow rate, we tested three parameters that impact media perfusion into and out of the bioreactor: 1) flow rate, 2) volume pumped in/out at once, and 3) pause time between pumping stages. Using a dye diffusion technique where dyed water simulated fresh media, we found that the peristaltic pumps were able to create a homogenous distribution of dye throughout the bioreactor during a half-media change, and that pause time does not significantly affect the concentration of dye in the wastewater. Furthermore, the results showed a flow rate of 1.5 mL/min with 3 mL of media exchange resulting in the lowest dye concentration in waste. These findings indicate that peristaltic pumps are a valid and efficient way to perfuse fresh media and discard depleted media.

Keywords: Volumetric Muscle Loss, peristaltic pump, dye perfusion, tissue engineering, closed-loop system

Introduction

Every year, approximately 65.8 million Americans suffer from musculoskeletal injuries of which the treatment costs are estimated to exceed 176 billion dollars annually.[1] Severe musculoskeletal injuries often lead to volumetric muscle loss (VML). Volumetric muscle loss is the loss of skeletal muscle, due to trauma or surgery, that results in significant functional impairment[2] (Figure 1).[3] Because of the substantial loss, it is beyond the inherent regenerative capacity of the body.[4] Furthermore, of the 65.8 million Americans affected by VML, many are veterans who have sustained extremity injuries from combat which have been estimated to require the most resources for initial treatment and are the leading cause for disability in veterans, resulting in large disability benefit costs.[5] Current surgical procedures include methods such as autologous free flap grafting, scar tissue debridement, or minced skeletal tissue transfer, which are utilized to reconstruct the tissue defects. However, these methods are limited by the availability of tissue for surgery and by the need for skilled surgeons with expertise in this area to perform the surgery.[4] Hence, as a possible treatment for VML injuries, the Christ Lab at UVA has developed a Tissue-Engineered Muscle Repair construct (TEMR), which seeds muscle progenitor cells (MPCs) onto a bladder acellular matrix

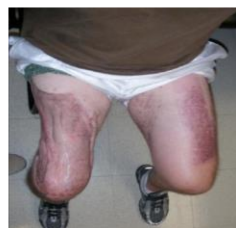


Figure 1: Volumetric Muscle Loss (VML) patient. Courtesy of Dr. George Christ.

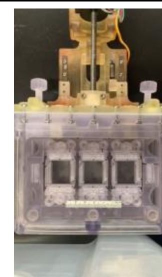


Figure 2: Bioreactor setup with cassettes and motor.

(BAM) before incubation in a bioreactor to prepare the TEMRs for surgical implantation.[6] The bioreactor design includes cassettes to hold the TEMR membranes and repeatedly stretch them to stimulate muscle fiber alignment (Figure 2). However, the bioreactor must be open for media changes, introducing the potential for biocontamination. By making the system more closed-loop, the bioreactor system will be easier to automate in the future for more efficient TEMR production.

Significance

The significance of this study was to create a closed system to reduce risk of contamination and allow for easier automation.

Contamination risk is higher when a sterile field is not in a closed system. This is because airborne microbes can enter an open-loop system and contaminate it and because of human interaction despite the current sterile culture protocol.[7] Contamination is detrimental when creating TEMRs for future human implants. As the media is exchanged manually, the bioreactor necessitates opening for the replacement of depleted media with fresh media, thereby potentially augmenting the risk of contamination. This risk is a major issue for the current bioreactor design.

An automated system is ideal because of its potential to
improve the efficiency of the TEMR biomanufacturing
process for future use in VML patients. Numerous benefits stem from the implementation of automated systems including the reduction of manual intervention by humans, reduction of human errors, lowering of cost, improving product quality of scaffolds, and accelerating TEMR biomanufacturing for use in clinical applications.[8] With the development of the closed-system bioreactor, the limitations for the availability of tissue and risk of contamination would be significantly lowered.

Innovation

Our design approach for this project was to build and calibrate the peristaltic pumps to circulate media into the bioreactor and then test the effect of the flow rate on the cell viability in the closed environment for prevention of contamination and for easier automation. We planned to take the new data from each of these aims and assess it in comparison to the original design without media perfusion. The comparison would then be used to determine if the results have stabilized or increased cell viability from the original design and to see if the results were close to what we want with the circulation of media.

However, due to contamination of our tissue scaffolds in the middle of the spring semester, we were unable to restart 3D printing TEMRs with C2C12 cells which necessitated us pivoting from our original plan for testing into another direction for testing perfusion of media. Instead of using live cells, we used dye to simulate the perfusion of "fresh media" being pumped into the bioreactor. In lieu of measuring what the best flow rates were based on cell viability, we measured the amount of "fresh media" (dye) that ended up in our waste container to determine what the best set of permutations were of pause time, volume pumped in/out, and flow rate. We defined "best" as the permutation that resulted in the least amount of dye in the waste container set up at the end. The rationale for this approach
was to enhance nutrient exchange and cellular health,
by ensuring that cells were exposed to the fresh media, without loss (i.e. media pumped out within the same media change). Hence, this would allow for future experimentation on TEMRs with C2C12s

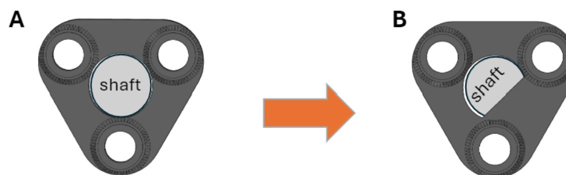


Figure 1: Object problem (A) and object solution (B). A) The initial rotary design for securing the top shaft lacked sufficient static friction, necessitating modification to ensure that the shaft was not reliant on static friction to prevent slippage. B) Altered design with shelf to keep the shaft from slipping.

to test the best flow rate for these cells. We hypothesized that longer pause times, larger volumes pumped in and out at once, and faster flow rate would lead to the least dye waste media perfusion. Additionally, we hypothesized that if there were a homogenous dye distribution throughout the bioreactor during the half-media change, the cells would be supplied with enough fresh nutrients to ensure metabolic health, leading to confirmation that this perfusion system is a valid substitution for manual media changes.

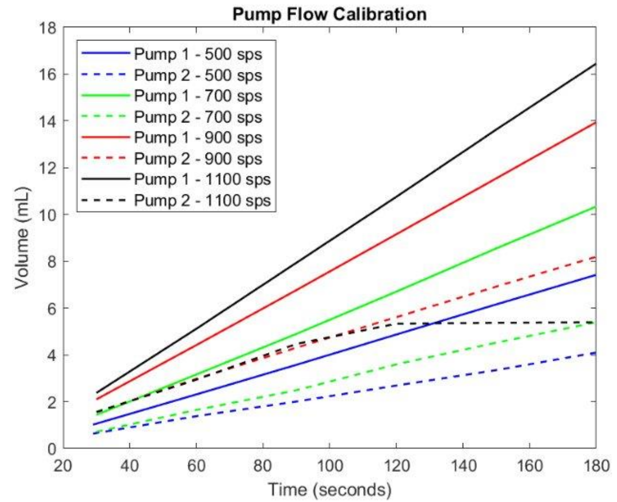
Results

Peristaltic Pump Manufacturing and Design Iteration

Figure 2: 3D SOLIDWORKS rendering of peristaltic pump.

An open-source peristaltic pump design published in Nature was used for our pumps.[9] However, we developed our own circuit and code (Supplementary Figures 1 and 2) due to the following reasons: 1) difficulties in replicating the functionality of the open-source design, 2) the need to connect two pumps to the same Arduino, and 3)different code requirements. We 3D-printed parts available from the open-source design within ABS material and assembled the pump with the other necessary parts, including an Arduino, power source, ball bearings, steel shafts, bolts, and tubing (Figure 3).

Upon initial testing, we realized that the pump struggled to produce sufficient torque for a flow rate over 0.25 mL/min since the rotor was only held onto the steel shaft via static friction (Figure 4A). To mitigate this problem, we edited the CAD files to add a shelf to the rotor design to provide a surface for the shaft, which would be shaved down to fit into the newly semi-circular through hole, to transmit force to the rotor (Figure 4B).



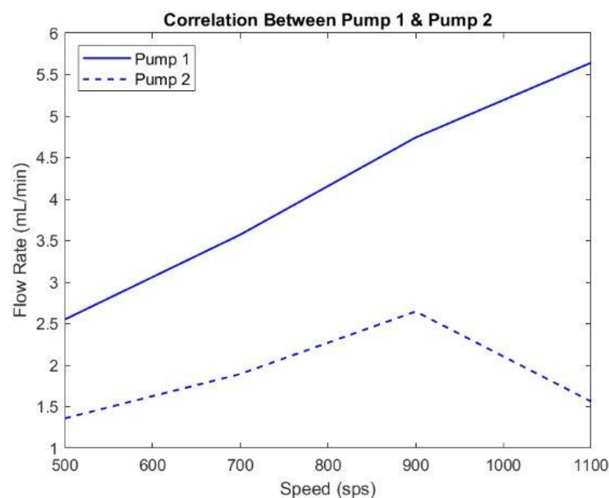


Figure 5: A) Calibration shows the volume pumped by pumps 1 (fresh media) and 2 (waste) in steps per second (sps) over 180 seconds. Pump 2 failed partway through testing at 1100 sps, resulting in a nonlinear relationship. B) Correlation between pump 1 (fresh media) and 2 (waste) speed in sps and flow rate (mL/min). Calibration equations were made using data points from 500-900 sps due to pump 2 failing at 1100 sps.

Peristaltic Pump Calibration

To determine the relationship between motor speed and flow rate for each of the pumps, the pumps were set at 500, 700, 900, and 1100 steps per second (sps) to pump water into a beaker on a scale. The mass was taken every 30 seconds and the resulting flow rate (Figure 5A) used to create a linear equation relating motor speed to flow rate (Figure 5B).

TEMR Production Contamination and Pivot

To test whether our perfusion system would impact the quality of the TEMRs produced, we began the procedure to make these cellular constructs. We took two sets of three bladder acellular matrices (BAMs) – one set of three as a control with media changed manually and one set of three which would have the media perfused via our peristaltic pumps – and bioprinted C2C12 cells in a 2% w/v hyaluronic acid (HA) hydrogel onto the matrices. These were to be incubated for ten days before transferring to the bioreactor. However, by day three of the culture, they had become contaminated in the incubator. Due to the extensive procedure for manufacturing TEMRs lasting approximately a month and the limited availability of the BAMs, there was insufficient time and resources to redo the experiment. Therefore, we pivoted to a dye testing experiment to optimize the perfusion parameters of flow rate, volume, and pause time.

Dye Diffusion Testing

Optimization of the pump-mediated media exchange into and out of the bioreactor is necessary for several reasons. 1) The flow rate capable of the peristaltic pumps in use is too slow to pump all the media out of the bioreactor before pumping fresh media back in. Even for a half media change at the approximate maximum flow rate of the pumps, pumping out 120 mL at 3 mL/min would take 40 minutes, and another 40 minutes of pumping media back in. This would result in 80 minutes of the cells on the membranes sitting out of media, which would likely have detrimental effects. 2) The membranes form pockets of air underneath them when media is added after it drops low enough for air to enter under them. These pockets of air (or bubbles) must be removed manually via a bent needle attached to a syringe. Therefore, if too large a volume of media is removed at once, the bubbles will reform and require opening of the bioreactor to manually remove them. 3) Cell culture media is expensive, and if the fresh and depleted media mix during media perfusion, fresh media may be pumped out of the bioreactor immediately after it is pumped in. This chance is heightened if the inlet and outlet are closer together, or if the fresh media has not had a chance to diffuse throughout the bioreactor. Minimization of the waste of fresh media is necessary to reduce the cost of manufacturing.

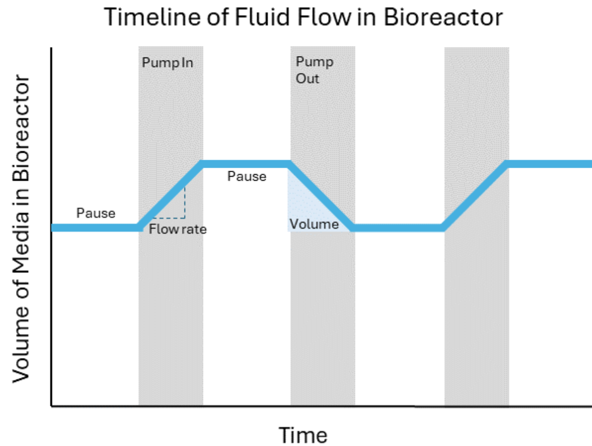


Figure 6: Schematic of the change in volume of media in the bioreactor as fluid is pumped in and out. Pause time between fluid being pumped in and out, fluid flow rate, and volume pumped are parameters that need to be optimized for full diffusion but minimal waste of fresh media.

Therefore, we designed a protocol for media perfusion that would simultaneously mitigate the limitations noted above and allow optimization of media exchange. A small volume of media would be pumped in (pumping in media first to ensure that air is not allowed to enter under the membrane and form a bubble), a 10-30 second pause allows time for passive diffusion, media is pumped out at the same flow rate it was pumped in, and another pause allows time for diffusion (Figure 6).

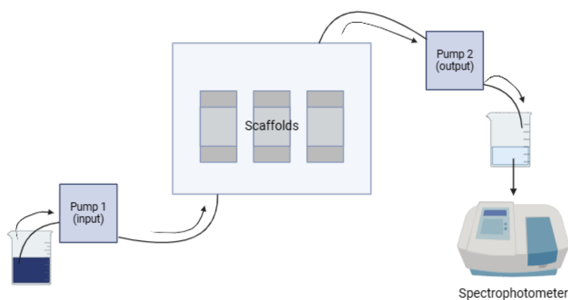


Figure 7: Schematic of dye testing setup. The bioreactor contains the three scaffolds and holders, filled with ~220 mL of "media." Pump 1 flows "fresh media" containing blue dye into the bioreactor and pump 2 removes "waste media" from the bioreactor. Samples from the "waste" are taken and a concentration of dye found using a spectrophotometer.

Diffusion of blue food coloring dye was used to simulate the diffusion of fresh media into and throughout the bioreactor. A beaker of "fresh media" consisting of a 1:500 dilution of dye in water was pumped according to the parameters for the experimental group into the water-filled bioreactor. The same volume of water was pumped out into a "waste" beaker. Samples from this beaker were analyzed via a spectrophotometer to determine the concentration of dye (Figure 7).

This allowed for the optimization of parameters to minimize the waste of fresh media, proxied by blue food dye. Small sponges were also placed in two corners of the bioreactor to see that the dye diffused fully, although due to the sponges being compressed against the side of the bioreactor, the dye distribution on the sponges was speckled and therefore unable to be quantified reliably. Still, visually the dye qualitatively reached all corners of the bioreactor. The parafilm we used to stand in for the bladder acellular matrices did not form air pockets similarly to the real membranes, so no data was able to be collected on bubble formation. The outlet tube was moved from its original place next to the inlet to the top right corner of the bioreactor, at the same level as the water in the bioreactor, to both allow for better diffusion and to provide a safeguard to prevent the media level from dropping too low if there was a malfunction where the outlet pump was running at a higher effective flow rate.

A three-way ANOVA with a post-hoc Tukey's Honest Significant Difference (HSD) test was performed with an alpha of 0.05 (Figure 8). It was found that the experimental parameters that produced the best results were a 1.5 mL/min flow rate of 3 mL separated by 30s of pause time resulting in the lowest dye concentration in waste. Additionally, statistical analysis showed that pause length did not affect the variance in the samples, while Volume, Flow Rate, and the combination of the Volume and Flow Rate did have a significant effect.

Discussion

We determined that the peristaltic pumps were able to fully diffuse dye during a half-media change. Furthermore, a 1.5 mL/min flow rate of 3 mL separated by 30 seconds of pause time resulted in the lowest dye concentration in waste, and hence, group number 8 had the best results for our application of minimizing dye (fresh media) in the waste container. Lastly, we concluded that the

pause time from 10 to 30 seconds had no significant effect and could be reduced greatly to save time between pumping in fresh media and pumping out old media – up to 30 minutes to 1 hour every 48-hour media cycle change.

manual changes. This is essential to determine whether the design for automated perfusion of media is sterile, optimal, and feasible using the pair of peristaltic pumps designed. The next iteration of the bioreactor could be to change the CAD files to shift the placement of media inlet and outlet ports,

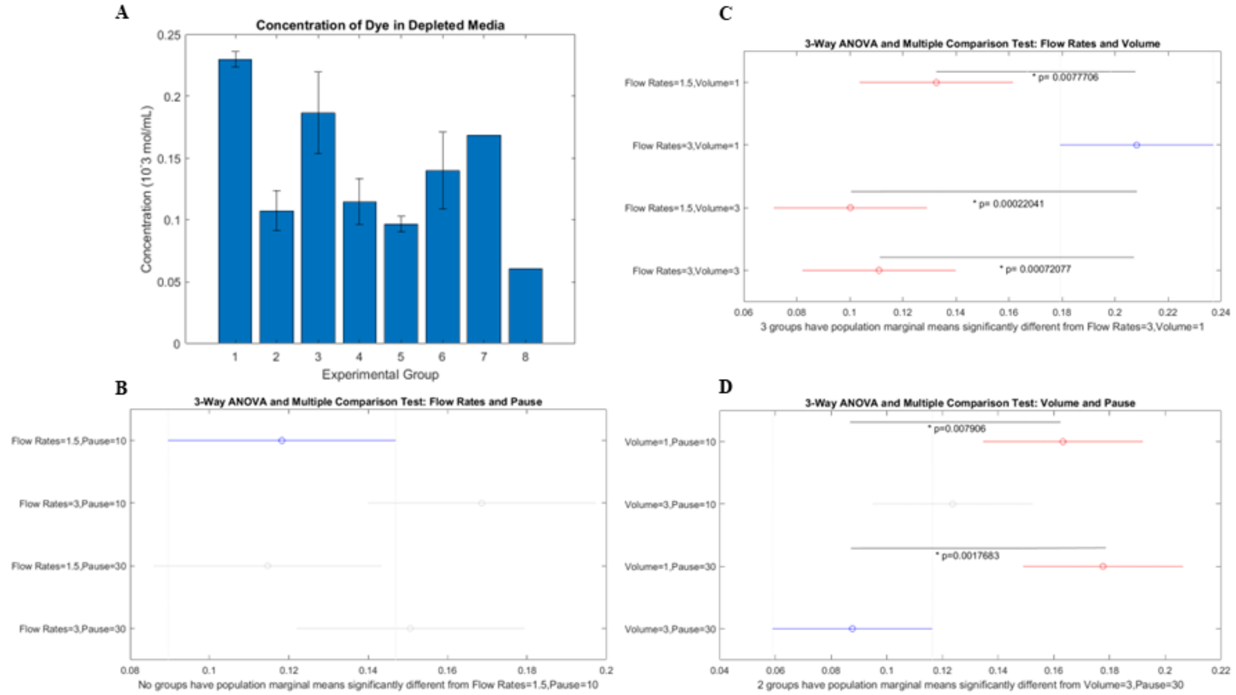


Figure 8: A) Dye concentration results, error bars are standard deviation. Significant differences not shown on this graph. Results of 3-Way ANOVA and Tukey's Honest Significant Difference test with α = 0.05. B) Comparison of Flow Rates and Pause had no significant differences while C) Flow Rates and Volume and D) Volume and Pause did have significant differences, indicated by the black lines with an asterisk and p-value noted.

The perfusion of dye was a rough approximation for how likely fresh media is to diffuse into the live cell TEMR setup, so further testing is needed to ensure there is no detriment to the quality of TEMRs produced. Additionally, this system will reduce the time spent by graduate students performing manual media changes and thereby allocate time and resources for other projects in the Christ lab.

Future directions could include testing how running the pumps with no pause (pause time = 0 seconds) would affect the diffusion of fresh media in the bioreactor. If there is still no significant effect due to changing the pause time to zero, this would greatly reduce the time needed to perform half-media changes with the peristaltic pumps. The next step would be to perform testing of the peristaltic pump setup on TEMRs to see if cells are just as viable with automated media changes as with

so they are not right next to each other to allow for a more even diffusion of fresh media. Our current setup plugged the output hole and fed the tubing for the waste pump (Pump 2) out from the top in a temporary setup. Furthermore, future work includes troubleshooting several design elements including: 1) finding a pump tubing that is more durable and less prone to break in between and during experiments, 2) fixing the bioreactor leak at the inlet connector and screw threads by adding a gasket, and 3) redesigning the CAD files so that there is less wear and tear on the connector threads. This continuation of work will not only improve on the current design iteration but also has the hope of leading to the automation of media that can be used not only in this application but also in other biomedical engineering projects involving tedious changes of media.

Materials and Methods

Peristaltic Pump Manufacturing

The 3D *SOLIDWORKS* files for the peristaltic pumps were printed in the Stacy Hall machine shop at the University of Virginia. The pumps were printed using Bambu Carbon X1 printers and ABS as the material. After printing the pumps (which took approximately 12-20 hours per pump), the plastic pieces for assembly were washed to remove an adhesive film, dried, and left processed using pliers to remove the supports that were present in the gear pieces and several other pieces.

The 3D printed parts were assembled with a NEMA 17 40mm 1.8-degree stepper motor, Hex Socket Machine Screws M3-0.5x16mm, 17x6x6 mm ball bearings, a 6mmx100mm steel shaft, zinc M3-0.5 nuts, 0.1875" ID x 0.3125" OD x 0.125" Width ball bearings, Hex Socket Machine Screws M3-0.5x40mm, an Easydriver Stepper Motor Driver, an Arduino Uno R3 board, wires, and a 12V power supply. The rod was cut in half and shaved down on two ends to create a notch to interface with the shelf on the 3D printed rotor and large gear. The two pumps were wired to the Arduino (Supplementary Figure 2) and powered through a 12V power supply.

Bioreactor Manufacturing

The bioreactor files for 3D printing were created in *SOLIDWORKS* as .sldprt files. The files were then transformed to .stl files to be sent through *FORMLABS*, a 3D printing technology developer and manufacturer. *FORMLABS* was used to create a model and maneuver the model with pseudo supports that will be printed with the part. Once the part went through this platform, it was sent to the printer. The bioreactor was printed in *FORMLABS* clear resin. After printing, the parts were cured by UV light and washed with isopropanol.

Calibration of Pumps

The following steps were performed in a separate experiment before the experiments with the perfusion of dye in the bioreactor system. To determine the volume of water pumped per second by each pump (1 and 2), the volume of water pumped per time was weighed periodically (in 30-second increments) and recorded. At the end of 3 minutes, the mass at each time increment was used to determine the flow rate of each pump by the following process. The density of water (ρ=997 kg/m^3) and the formula (volume = mass/density) were used to accurately determine the precise volumes at each time stamp for each pump. Then, by plotting the volume per time, the flow rates of each pump were calibrated to different steps per second (sps). This calibration then served as a reference for our experiment for us to determine whether the flow rates matched each pump.

Software and Hardware

The FEBE setup includes a black motor box that runs the bioreactor. This contained a DC power cord and a connecting wire attached to the bioreactor. These connections were attached before our experiments were run. Arduino Uno code (Supplementary Figure 1) was used to run both pumps with the corresponding independent variables that were changed according to each specific experiment. Additionally, *SOLIDWORKS* was used to make changes to the CAD files as seen for the rotary piece in Figure 4, and to make changes to the bioreactor design to simplify the inlet and outlet holes to be a 5 mm through hole.

Dye Testing

This process involved several steps to prepare and conduct experiments in the bioreactor setup. There was a total of eight different experiments with three independent variables – the flow rate (mL/min), the volume pumped in and out (mL), and a pause time (seconds). To run each experiment, the fully enclosed bioreactor environment (FEBE) bioreactor had to be cleaned of dust and debris using water and a washcloth. The bioreactor was then attached to a motor that actively stretched the scaffold holders within the environment. Next, the two peristaltic pumps (Pump 1 – input, Pump 2 – output) need to be set on the left and right sides of the bioreactor respectively. Silicone laboratory tubing with an internal diameter (ID) of 3 mm and an external diameter (OD) of 5 mm was utilized and placed in the pumps surrounding the rotor piece and out the front of the pump. Two nylon plastic barbed connectors were placed onto the front of the bioreactor to connect the system and tubing. The tubing was then attached to the bioreactor by the connectors – one pump attached to each connector. Three pieces of Parafilm, sized 2 x 3 cm, were cut, and placed into the bioreactor scaffold holders.

These pieces were placeholders for the BAM scaffolds. Cosmetic wedge sponge applicators were cut in the shape of rectangular prisms by the dimensions of 2.54 x 3.81 x 5.08 cm, pre-dampened with water used to quantify the dye dispersion and placed in opposite corners (top left and bottom right) of the bioreactor. The sponges were positioned in the bioreactor such that they were held in place by semicircle cutouts in the bioreactor and positioned to not compress the sponges. Next, after the connectors were properly placed, the bioreactor was filled with 220 mL of water. To make the concentrated dye water used in place of "fresh media," we took 540 µL of blue food coloring ('Market Pantry' dye bought at Target) to serve as a proxy for fresh media into our bioreactor setup. and mixed it with 270 mL of water. This dyed water entered the bioreactor via the inlet connected to Pump 1. Therefore Pump 1 had the inlet tubing placed into the beaker containing the "fresh media." Pump 2 was connected to the bioreactor and the "waste" beaker. The FEBE motor was then started by plugging it into a power source and pressing "Go." The motor automatically runs 10 iterations of stretching the scaffolds before pausing for 60 min then restarting until unplugged from the power source. Once the motor was running and the scaffolds were visually moving, the Arduino code was uploaded and run. The code was updated based on the experiment trial and the independent variables. After the liquid in the beaker for Pump 1 was gone, both systems – Arduino code and FEBE motor – were stopped.

Once the power was turned off, the "waste" beaker was manually mixed by pipetting up and down to homogenize the contents. Three separate samples of 1 mL were taken from the "waste" beaker and placed in a microcentrifuge tube for storage. Next, each sample cuvette was labeled (1,2,3) along with the experiment number and stored with the other samples for later spectrophotometry testing. Next, the sponges were removed from the bioreactor and placed on a dry paper towel to dry completely for later testing. Finally, the bioreactor tubing, scaffold holders, and beakers were rinsed and the parafilm ("scaffolds") were discarded. The same steps were performed for each of our eight experimental groups with the respective independent variable values as featured in Table 1.

Group	Flow Rate (mL/min)	Volume pumped in/out (mL)	Pause time (s)
1	3	1	10
2	3	3	10
3	3	1	30
4	3	3	30
5	1.5	1	10
6	1.5	3	10
7	1.5	1	30
8	1.5	3	30

Table 1: Parameters for each experimental group.

Processing of Dye Samples

Spectrophotometer

Two spectrophotometers were used. The first one used was a Thermo Fisher Scientific NanoDrop One spectrophotometer to measure the absorbance spectra of the blue food coloring used in dye testing. 20 µL of dye was mixed in 4 mL of water and the optimal wavelength of 580 nm was estimated as the wavelength with the highest absorbance. This instrument is used for highly concentrated samples and only space for one cuvette sample at a time.[10] This spectrometer was used in this instance due to only using one sample of dye to find the wavelength needed.

Concentration Curve

Next, 2-fold and 10-fold serial dilutions of the dye were performed to visualize and create a concentration. The linear region of the 10-fold serial dilution was found after spectrophotometry and used to convert absorbance to concentration. The dilutions were completed before the plate was placed into the SpectraMax iD3 Spectrophotometer for the sample testing.

Sample Testing

Following storage over seven days while all eight experiments were being performed, the samples were not homogeneously mixed due to settling. Hence, samples were homogenized via agitating the cuvette by gently tapping the side before aliquoting 45 µL of each sample into a 96-well plate. The plate was analyzed using a second spectrophotometer called a SpectraMax iD3 Spectrophotometer at 580 nm. Absorbance values were normalized by

subtracting the average absorbance of blanks of water before being converted to concentration via the concentration curve acquired via serial dilution. This spectrophotometer is larger and was used in this case to read samples contained in a 96-well plate.

Imaging Sponges

To assess the dry sponge's appearance and potential dye concentration, the original aim was to perform the following protocol to analyze the diffusion of dye across the gradient of the side of the sponge. However, due to the inconsistent absorbance of the sponges, we did not end up using these *ImageJ* images for data analysis. Our process involved the following. The samples were placed in a "lightbox" provided by the lab, labeled with a name placard, and photographed using the lab *Nikon* camera. It was essential to ensure the camera settings were manual, with consistent aperture, ISO, and exposure settings for each sample. This process was repeated for all six sides of each sponge (sixteen sponges total since two sponges per experiment). If our sponges had consistently absorbed dye during the experiments, following image capture, *ImageJ* software would have been used to quantify the sponge darkness and analyze any variations based on height or location in the bioreactor. For future experiments, it may be beneficial to establish a concentration curve akin to the spectrophotometer's readings, to facilitate the conversion of color intensity to dye concentration for more precise analysis and comparison.

End Matter

Author Contributions and Notes

A.R.B, E.S.C, E.M.J, and M.A.L designed research. A.R.B and E.S.C built pumps, E.S.C wrote code, A.R.B and E.S.C performed pump calibration, A.R.B and E.M.J performed dye testing, E.S.C performed spectrophotometer testing and analyzed data. A.R.B, E.S.C, E.M.J, M.A.L, and G.J.C wrote the paper.
The authors declare no conflict of interest.

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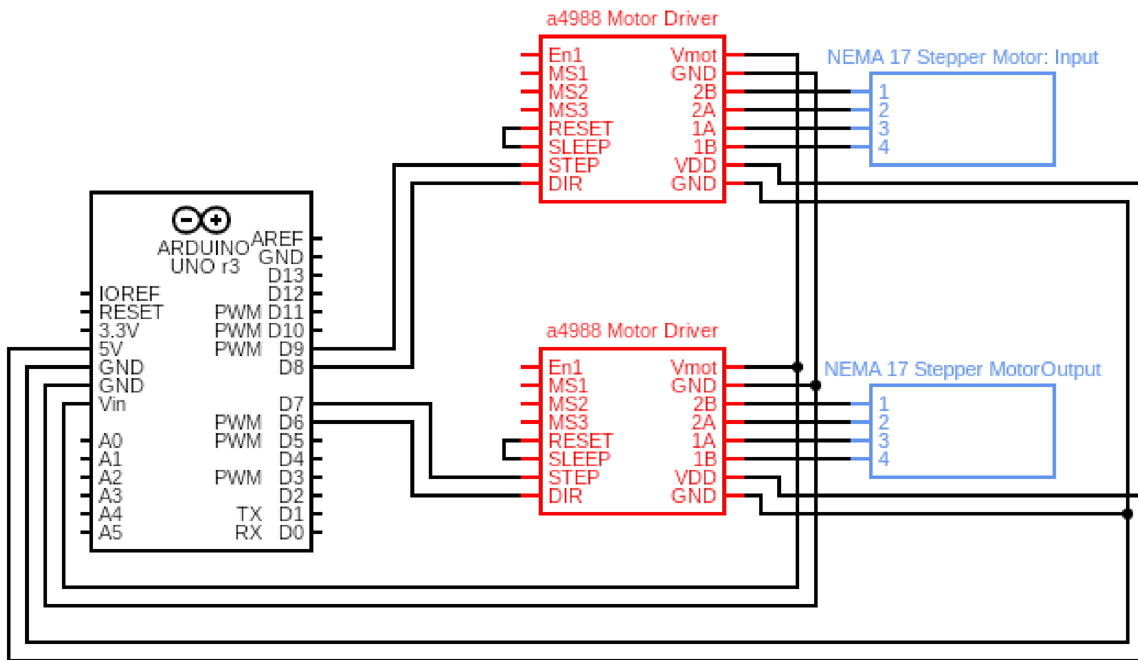
Supplementary Figures

Supplementary Figure 1: Arduino Code used to run the perfusion system.

```
/*
  Example sketch to control a stepper motor with A4988 stepper motor driver,
  AccelStepper library and Arduino: continuous rotation.
  More info: https://www.makerguides.com
*/

// Include the AccelStepper library:
#include "AccelStepper.h"

// Define stepper motor connections and motor interface type.
// Motor interface type must be set to 1 when using a driver
#define dirPin1 6
#define stepPin1 7
#define dirPin2 8
#define stepPin2 9
#define motorInterfaceType 1
//set parameters for pump function
#define pause 10 //time pump off between cycles in seconds
#define flowRate 3 //desired flow rate (mL/min)
#define volume 3 // volume of media to be pumped in and out (mL)
#define timeBwInOut 10 //time in s, should equal pause
#define pump1correlation 182.5548737 // sps/(mL/min) flow rate =
#define pump2correlation 310.7945151// sps/(mL/min)
#define pump1Offset 34.40839898 //pump speed = pump1Correlation*flowRate + pump1Offset
#define pump2Offset 77.40648193

//calculate speed in sps for pumps
float pump1Speed = flowRate*pump1correlation + pump1Offset; //mL/min * sps/(mL/min) = sps
float pump2Speed = flowRate*pump2correlation + pump2Offset;
float timePump = 60000*volume/flowRate; //volume/flowRate gives time in minutes. convert to milliseconds


// Create a new instance of the AccelStepper class:
AccelStepper stepper1 = AccelStepper(motorInterfaceType, stepPin1, dirPin1);
AccelStepper stepper2 = AccelStepper(motorInterfaceType, stepPin2, dirPin2);
int numLoops = int (125/volume);
int i = 0;
void setup() {

  // Set the maximum speed in steps per second:
  stepper1.setMaxSpeed(1000);
  stepper2.setMaxSpeed(1000);
  // Set the speed in steps per second:
  stepper1.setSpeed(pump1Speed);
  stepper2.setSpeed(pump2Speed);
```

```
}

void loop() {
  if (i < numLoops){
    //run pump in for timePump
    long starttime = millis();
    long endtime = starttime;
    while ((endtime - starttime) <=timePump){
      stepper1.runSpeed();
      endtime = millis();
    }

    //wait for the time between in and out
    delay(timeBwInOut*1000);

    //run pump out for timePump
    starttime = millis();
    endtime = starttime;
    while ((endtime - starttime) <=timePump){
      stepper2.runSpeed();
      endtime = millis();
    }

    //wait for pause time
    delay(pause*1000);
    i++;
  }
}
```

Supplementary Figure 2: Circuit Diagram


a4988 Motor Driver
En1
MS1
MS2
MS3
RESET
SLEEP
STEP
DIR
Vmot
GND
2B
2A
1A
1B
VDD
GND
NEMA 17 Stepper Motor: Input
1
2
3
4
ARDUINO
UNO r3
AREF
GND
D13
D12
PWM D11
PWM D10
PWM D9
D8
D7
PWM D6
PWM D5
D4
PWM D3
D2
TX D1
RX D0
IOREF
RESET
3.3V
5V
GND
GND
Vin
A0
A1
A2
A3
A4
A5
a4988 Motor Driver
NEMA 17 Stepper MotorOutput