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Q&A

How did you become involved in doing research?
After my freshman year of school as a Mechanical Engineering, I approached Erin Mannen, a Mechanical Engineering PhD student with a Biomechanical focus working in Professor Lisa Friis' lab. She talked to me about her project regarding a novel Spine Testing Machine and she suggested that proposing a project for an Undergraduate Research Award (UGRA) would be an excellent experience and enable me to work as part of her research over the summer. I got the UGRA and I became involved in her lab and her research.

How is the research process different from what you expected?
The research process is different than I expected because projects do not always go as planned. Sometimes it is necessary to find several different ways to approach a problem and if everything fails, you have to think of a new solution. This process can be tedious and very time-consuming, especially if you are talking of an experiment with a one month duration. If things did not work out at the beginning, the entire month's work can be wasted.

What is your favorite part of doing research?
My favorite part of doing research is the thinking process and trying to find new solutions, new approaches that usually build up from existing literature. Research can be a long process and sometimes it can take you in a different direction than originally intended, but having results that make sense and that take you a step closer to a solution is definitely a very rewarding experience.

Estimating Fluid Local Velocity within a Novel 3D Collagen Matrix Perfusion System

Ana Villanueva

ABSTRACT

Traditional cell culture, performed on flat surfaces under static conditions, does not accurately represent physiologic conditions. As an alternative, groups have applied interstitial fluid flow (0.1- 2.0 μm/s) through a 3D cell-embedded extracellular matrix (ECM). Cells sense the flow via mechanotransduction, a process by which cells sense mechanical forces and resultantly respond with biochemical signaling. Previous work demonstrates enhanced cell morphogenesis under interstitial flow conditions. However, fluid flow is poorly described within these systems, stressing the need for a well-characterized 3D interstitial flow system. Understanding fluid mechanics within a perfusion system will help elucidate cellular response to flow-induced mechanical forces.

The objective of this study was to quantify the fluid flow velocity through a controlled ECM. The changes in the collagen concentration are directly related to the fibril density of the collagen (stiffness).

A fluorescent Rhodamine solution was pumped at a constant flow rate through a collagen matrix-containing chamber. The resulting flow front was visualized at the center of the chamber using a fluorescent microscope. A Matlab program was developed to track the light intensity between time points to provide measures of flow velocity.

The results suggest that collagen concentration affects the estimated velocity measurements. As collagen fibril density increases, the resistance to flow increases, leading to a decrease in estimated velocity. These results validate the device's ability to consistently distribute flow over a range of ECM stiffness. Control and quantification of local fluid velocity is essential for future experiments in which cells will be embedded in the collagen matrix to observe cell response to fluid flow.
Keywords: Cell, matrix, interstitial fluid flow, perfusion chamber.

INTRODUCTION

Interstitial fluid surrounds and nurtures the cells in the tissues and supports nutrient and waste exchange. For more than half a century, the understanding of the functionality of interstitial fluid has increased and it has been established that it has a substantial role in cell signaling and cell morphology (1). It is also established that cells have an improved and physiologically relevant response in a 3D in-vitro system than in a two dimensional (2D) in-vitro system (2). About 20% of the human body is composed by interstitial fluid (3). However, groups working with in vitro systems had long been considering interstitial fluid a negligible variable due to its slow velocity in the human body (0.1-2.0 μm/s), ignoring the mechanical forces and pressures in the system. Recent works have investigated the effect of interstitial fluid flow in tissue engineering, such as inducing blood and lymphatic capillary morphogenesis in vitro (3), promoting morphogenesis in perfused organ cultures(4), inducing cell and matrix alignment (5), and inducing cell migration along streamlines (6).

Currently, perfusion systems for in-vitro cell culture purposes are used to study interactions between cells, their surrounding extracellular matrix (ECM) and the effect of interstitial fluid. Advances in microfluidic perfusion systems have filled several gaps in morphogenesis—the process that causes an organism to develop its shape— and angiogenesis—the process through which new blood vessels form from existing vasculature—assays, such as the control of the flow rate and complex networks. However, a deficiency in these microfluidic systems is that groups have developed working microchannel configurations involving Human Umbilical Vein Endothelial Cells (HUVEC) monolayers with cross sections that do not resemble the in vivo cylindrical lumens(7). Novel techniques that solve this challenge by creating a network that lines up with the HUVECs are difficult to reproduce and low throughput (8). Another disadvantage is the lack of control of the extracellular matrix (ECM), assuming that the mechanical forces are negligible. When flowing a controlled flow rate, the fluid path is observed on the top of the matrix, instead of visualizing the flow going through the ECM.

Perfusion systems at a macro and micro scale involving the cell, matrix and fluid dynamics are challenged by the compaction of the ECM and the fluid channeling around the matrix (5). The fluid mechanics of these macro scale systems are solved using Brinkman's equation to estimate velocity (9-11) and 3D modeling (computational fluid dynamics) of the perfusion chamber (12). While a controlled fluid flows through the collagen matrix, the effect of the resistance in the system is not taken into consideration (13). A disadvantage to Brinkman's equation is the assumption of continuum fluid and uniform velocity, as they do not mimic physiological conditions, instead of local values for the velocity profile.

While few groups have been able to recreate these complex cell-matrix-fluid dynamics within in-vitro assays, their systems have been limited on both a matrix and fluid flow standpoint. When developing a 3D perfusion culture system, it is essential to define matrix structure-mechanical properties as well as quantify and validate the flow and velocity profiles through the fibrous system.

This study addresses the two main questions regarding interstitial fluid flow through tunable collagen matrices: the effect of the matrix structure on the velocity profile and the flow rate measurement.

METHODS

A. Device specifications

A compliant red silicon was acquired (McMasterr, Elmhurst, IL) with a 1.58mm thickness. The device is a square cut from the rubber with 18mm on each side. The inside chamber is a square with 8mm on each side. Glass coverslips seal the chamber using glue (Gorilla Epoxy 25ml Syringe). Also, a pair of 23G needles were placed on opposite sides of the perfusion chamber (Figure 1). A subsequent surface

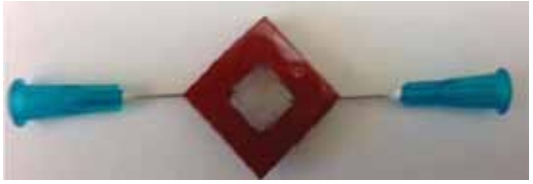


Figure 1. Perfusion chamber with 18mm on each side. The inside square has 8mm on each side. Glass coverslips sealed the chamber using epoxy glue. 23G needles.

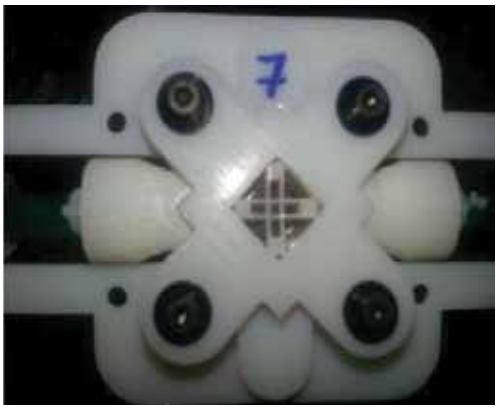


Figure 2. 3D printed structure modeled in Solidworks to ensure stability and to center it with the microscope.

treatment injecting the Poly-L-Lysine to the chamber and rinsing it with Milli-Q ensures the adhesion of the collagen matrix to the chamber walls. The perfusion chamber was placed into a 3D printed structure to ensure stability (Figure 2).

B. Matrix Formulations

Type I collagen oligomers were isolated from porcine dermis and standardized as described previously (Kreger et al., 2010). Collagen-fibril matrices were prepared by neutralizing the oligomer solution according to established methods (Kreger et al., 2010). The oligomer concentration of the polymerization reaction was modulated to obtain different collagen matrix stiffness values, which is directly related to the fibril density of the collagen (Kreger et al., 2010). Collagen-fibril matrices were prepared at 0.74mg/mL, 1.45mg/mL,1.94mg/mL and 2.56mg/mL to achieve matrix stiffness values of 50Pa, 200Pa, 350Pa, and 600Pa, respectively. The neutralized collagen solution was injected into the perfusion chamber and subsequently inserted into an incubator to polymerize.

C. Interstitial flow in 3D system

To analyze the velocity of the interstitial flow mimic, fluorescent dye (Rhodamine 123, *50μM*) was pumped through the perfusion chamber at a constant flow rate of *10μL/sec* using a peristaltic pump (FCS2 & FCS3 Micro-Perfusion Pump (Low-Flow), Bioptechs, Butler, PA) connected to the device (Figure 3). The flow rate was measured at the inlet and outlet of the device. The inlet value was set by the controls of the peristaltic pump and the outlet value was measured by the distance traveled by the fluid on the outlet tubing at a measured time (Figure 4).

Figure 3. A peristaltic pump was connected to the perfusion chamber at a flow rate of *10μL/sec* to flow fluid through the chamber.

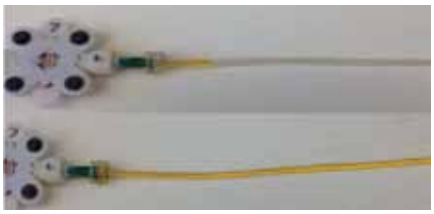


Figure 4. The outlet flow rate was measured by the distance traveled by the fluid on the outlet tubing at a measured time.

D. Microscope imaging

Upon initiation of flow, time-lapse images within the center of the tissue construct were collected using a 4X objective on a fluorescence microscope (Nikon Eclipse TE2000-S), with a numerical aperture (N.A.) of 0.2 and a B-2A filter.

E. Matlab Program

A Matlab (MathWorks, Torrance, CA) program was developed to process the time-lapse fluorescent images and determine temporal changes in light intensity across an image (a single time point) (Figure 5). It then plotted the light intensity curve for all time points. Half maximum intensity of the fitted data (light intensity for all time points) was plotted to find the location of the time points over the time per pixels. The results were plotted in a position over time graph and its slope is the velocity.

RESULTS

The images of collagen microstructure indicate that the collagen-fibril matrices have sufficient porosity and mechanical integrity to support interstitial fluid flow as the ECM remains intact throughout the duration of perfusion experiments (Figure 6). Future studies would involve taking 3D stacks of the collagen microstructure before and after flow conditions, and using correlation techniques to determine the stress and strains on the ECM. Understanding fluid-induced ECM deformation will provide insight on its stability over time.

Results indicate that the flow rate measurement at the inlet and outlet of the device was not statistically different (Figure 7) over the range of collagen-fibril matrices tested.

The measured average velocities were statistically different between 50 Pa and 200 Pa matrices, with 200 Pa measuring a slower velocity than 50 Pa (Figure 8). Interestingly, however, the velocity increases and is statistically significant when comparing 200 Pa to 350 Pa. However, velocities for 50 Pa and 350 Pa were not statistically different.

DISCUSSION

The flow rate measurement denotes that a range of matrix stiffness does not have an effect on the flow rate. However, it was observed that an increase in fibril density imposes larger resistances that would affect flow rate. Using a 600

Pa stiffness ECM (n=2), the flow was significantly impeded (less than 10% of input flow rate), with no leaks found in the system (data not shown). This suggests that the system has an upper-limit on the pressure that can be applied to the inlet; however, further work must be done to form a more conclusive statement. Furthermore, the method for measuring flow rate should be improved, as error could have been introduced through a couple of ways. The tubing was very compliant, making it difficult to keep straight when taking measurements. Additionally, slight human error could be introduced, as marking the start and end position by eye has its obvious limitations. A more accurate method would be to use a scale to weigh the volume of output fluid. This would eliminate all previously mentioned issues.

The velocity decreases as the fibril density (stiffness) increases, due to the change in resistance of the collagen matrix when the collagen concentration is changed. The inconsistencies found for 350 Pa could occur due to many factors that come into play. First, the average max intensity of 350 Pa ECMs (n=4) was 141.6 units, while 50 Pa was 83.42 units, and 200 Pa was 93.45 units. The light intensity of the 350 Pa matrix was 51.5% higher than the 200 Pa matrix and 69.7% higher than the 50 Pa matrix. Since light intensity is a correlated measure of Rhodamine concentration, these results suggest an increased Rhodamine concentration within the 350 Pa matrix. One explanation of these results is that the increased fibril density decreases the diffusivity of the system, therefore leading to increased concentration. The concentration build-up occurs when Rhodamine enters the system at a faster rate than it is able to travel through the ECM. Additionally, preliminary experiments were performed to optimize fluorescent

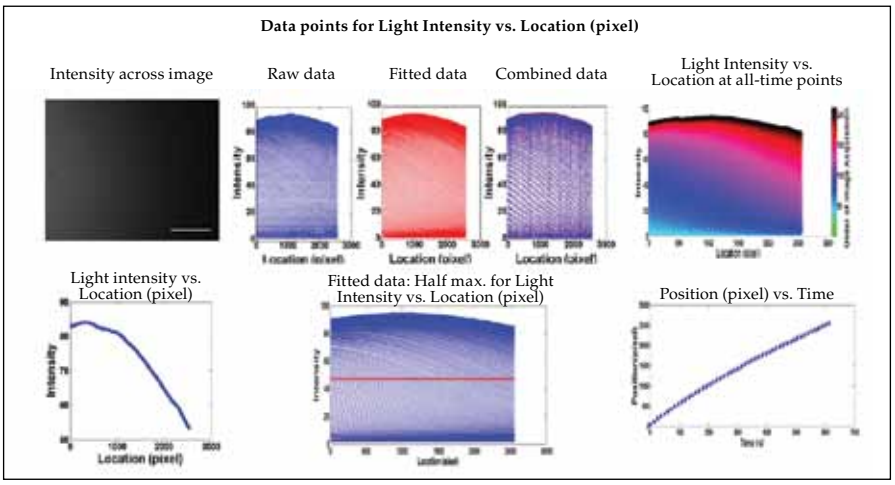


Figure 5. A Matlab program calculates the velocity profile. Using the fluorescence microscope, images are taken during the flow front experiment. The program processes the images and plots a light intensity vs. location (pixel) curve for all images. Half the maximum intensity from the fitted curves for the light intensity vs. location (pixel) finds the location. Then, it plots the light intensity vs. location at all-time points of the images to obtain the curve for the position (pixels) vs. time. The slope of the position vs. time is the velocity profile. Scale bar = 500 µm.

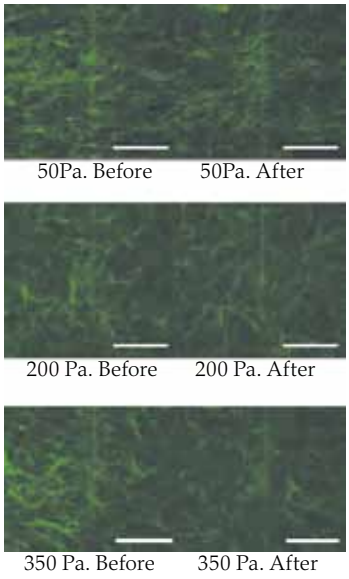
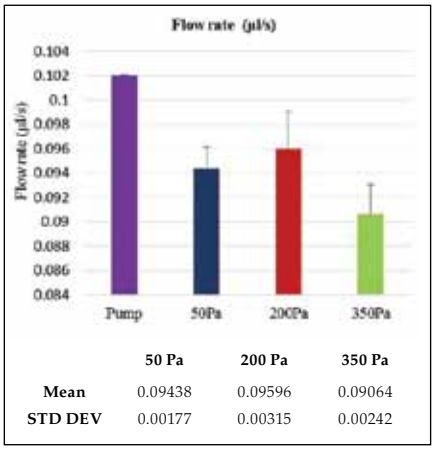
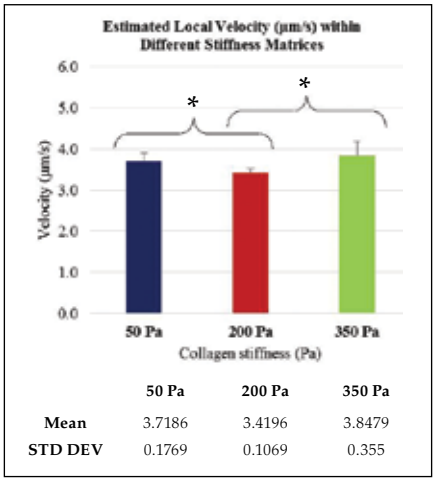


Figure 6. A confocal microscope collected images of the microstructure before and after the presence of the fluid flow. The images of collagen microstructure indicate that the collagen-fibril matrices have sufficient porosity and mechanical integrity to support interstitial fluid flow as the ECM remains intact throughout the duration of perfusion experiments. Scale bar = 100 µm



	50 Pa	200 Pa	350 Pa
Mean	0.09438	0.09596	0.09064
STD DEV	0.00177	0.00315	0.00242

Figure 7: Flow rate measurement (µL/s) for a flow front at the outlet of the perfusion chamber (error bars represent STD DEV of calculations).



	50 Pa	200 Pa	350 Pa
Mean	3.7186	3.4196	3.8479
STD DEV	0.1769	0.1069	0.355

Figure 8: Velocity calculations (µm/s) for a flow front through different collagen matrix stiffness (error bars represent STD DEV of calculations). Means with different letters are significantly relevant (*, p<0.05).

dye concentration (data not shown). These experiments determined the working range of the Rhodamine solution, and additional observations of higher velocity measurements when more concentrated Rhodamine solutions. An important consideration on the optical setup is the area of excitation. This area significantly affects overall light intensity readings, as an excessively large illumination area results in background intensity contributed by surrounding Rhodamine beyond the field of view. It is therefore important to choose the minimally acceptable illumination area, large enough to encompass the camera's field of view while small enough to reduce background noise. Secondly, the diffusivity of a species depends on its concentration, with higher concentration gradients increasing the rate of diffusion. Therefore, employing a higher concentration would in effect increase the measured velocity, as the diffusive component of the flow will have increased. Therefore, the increased velocity in the 350 Pa could be attributed to background illumination and altered diffusion. Further experimentation with different microscope settings and concentrations is required to confidently determine the cause for the increased velocity in 350 Pa.

Data suggests that flow remained constant between the different ECM formulations at the inlet and outlet of the tubing (Figure 7); fluid velocity decreased as the fibril density and matrix stiffness increased (Figure 8). An explanation, supported by the law of conservation of mass, would be that the velocity streamlines were narrower (and therefore faster due to a nozzle effect) in the 50 Pa matrix, and streamlines became increasingly wider (and slower) as the matrix stiffness increased since more fibrils obstruct the path. Probing this would require a closer study of the obtained concentration profiles. While there was no geometric quantification on the concentration profiles, there are notable differences in the concentration gradients and shape that could inform streamlines. For example, increasing fibril density decreased the observed velocity from 50 to 200 Pa, as it was measured over the center of the construct. A possible explanation for this would be that the streamline within the 50 Pa is narrower, and therefore should move through the ECM faster; however, the 200 Pa matrix has a wider streamline, therefore perceived velocity would be lower. Further development of Matlab code to quantify concentration profiles, along with COMSOL model development, will allow accurate predictions of streamlines within a device.

CONCLUSION

The flow rate measurement denotes that a range of matrix stiffness does not have an effect on the flow rate. However, previous experiments suggest that an increase in the fibril density imposes larger resistances that would affect flow rate. A more accurate method for the flow rate measurement would be to use a scale to weigh the volume of output fluid.

The velocity decreases as the fibril density (stiffness) increases, due to the change in resistance of the collagen matrix when the collagen concentration is changed. However, further experimentation with different microscope settings and concentrations is required to confidently determine the cause for the increased velocity in 350 Pa.

Images collected by the confocal indicate that the collagen-fibril matrices have sufficient porosity and mechanical integrity to support interstitial fluid flow as the ECM remains intact throughout the duration of perfusion experiments.

The next step is the Fluorescence Recovery after Photobleaching (FRAP) technique to obtain diffusion coefficients to enter into the COMSOL model to estimate the local fluid velocities for more accurate results (Figure 9). The control over local fluid velocity is essential for future experiments in which cells will be embedded in the collagen matrix to observe cell response to fluid flow.

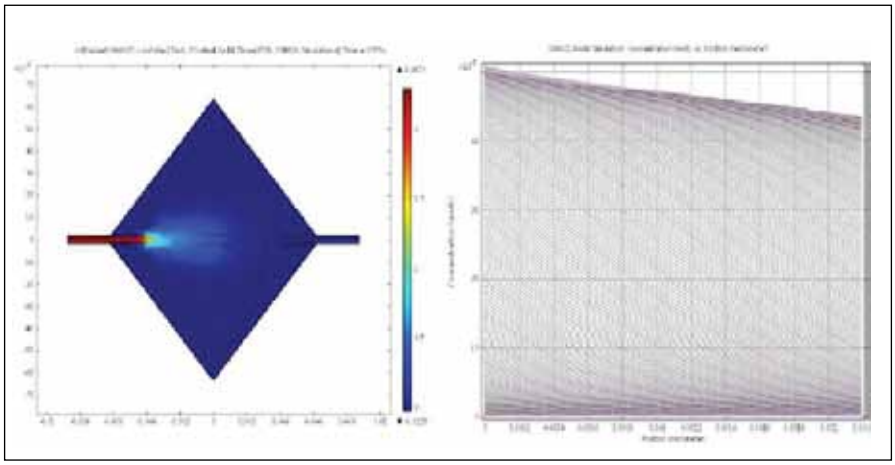


Figure 9 . COMSOL program models the inside of the perfusion chamber and the trajectory of the fluorescent particles flowing through the device to estimate the local fluid velocities.

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