Enhancing Spheroid Development with Microfluidic Pulsatile Pressure

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Abstract:

Spheroids are an example of three-dimensional cell culture and are commonly used to model a cell's complex *in vivo* environment. However, the viability of spheroids is hindered due to a lack of understanding of spheroid growth and development. Since spheroids lack vascularization, nutrients, such as oxygen and glucose, have a difficult time reaching the center of a spheroid. Due to the lack of nutrients in the center of a spheroid, a necrotic core begins to develop. This study aims to introduce nutrients deeper into a spheroid as a result of changing the environment the spheroid lives in by subjecting it to external mechanical forces, such as pulsatile pressure. Microfluidic devices will be designed and fabricated for the spheroids to grow in. The spheroid will then face various mechanical stimuli while in the microfluidic device. An improved understanding of how these mechanical forces affect spheroid growth and development will be instrumental in optimizing the viability of spheroids, creating a further step towards personalized medicine, disease mapping, and predicting patient drug response.

Background:

Three-dimensional cell culture is an *in vitro* method of culturing cells that most accurately models their complex environment *in vivo*. Spheroids, the model used in this study, are a type of three-dimensional cell culture formed by growing a differentiated cell type (HEK293 cells, e.g.) in an environment that encourages self-assembly into a spherical tissue structure. There are many applications of three-dimensional cell culture found in tissue engineering, drug testing, and biocompatible microfluidic devices [1]. Not only do cells experience a more realistic *in vivo* environment in three-dimensional cell culture, but cells are also able to maintain their extracellular matrix, have improved cell physiology and viability, are able to proliferate and differentiate, and are able to express more physiologically relevant genes compared to two-dimensional cell culture [2]. Despite the many benefits of three-dimensional cell culture, there are drawbacks as well. The most notable drawbacks of three-dimensional cell culture include the lack of homogeneity between samples, difficulty with microscopic imaging and analysis, hindered nutrient delivery to the center of the three-dimensional structure, increased cost, and greater difficulty to use. This study was tasked with addressing the issue of hindered nutrient to core delivery present in spheroids as well as microscopic imaging and analysis of spheroids. A phenomenon known as a necrotic core develops in spheroids as a result of the lack of nutrients, such as oxygen and glucose, reaching the center of the spheroid due to their absence of vascularization [3]. As the outer cells of the spheroid proliferate, nutrients have an increased difficulty reaching the spheroid's core [4]. Microfluidic devices were used in this study to grow spheroids and introduce mechanical forces, such as cyclic pressure patterns, to encourage deeper penetration of nutrients into the spheroid core. Microfluidic devices were used not only because they are relatively inexpensive and easy to reproduce, but they are also optimal for spheroid formation, culture, handling, and manipulation. HEK 293 spheroids, genetically modified to express three fluorescent proteins when exposed to the drug doxycycline, were used in this experiment. When a high enough drug dosage exposure is present, the genes encoding for red, green, or blue fluorescence will be expressed. It is important that the spheroids fluoresce intensely and that they express triple positivity of all three colors, as this is indicative of successful gene expression and sufficient drug exposure to induce this expression.

Objectives:

- Design and fabricate microfluidic devices that will optimize nutrient to core delivery.

- Introduce external mechanical forces through various flowrates to reduce the size of the necrotic core long term.

Methods and Materials:

The spheroids were cultured using a HEK 293 cell line. A 1% agarose gel was created by mixing cell culture-grade agarose in deionized (DI) water and heated until boiling to dissolve the agarose. 75 µL of liquid agarose was placed in the inner wells of a 96-wellplate and allowed to cool under UV light for 10 min to sterilize and allow the agarose to solidify. A 200 µL aliquot of genetically modified HEK 293 cells, lipofected with the 3-plasmid gene construct tagged with three fluorescent protein markers, were loaded into the inner wells of the 96-well plate on top of the solidified agarose. The outer wells of the well plate were filled with DI water. Once the spheroids were grown and ready to be tested on (2-3 days), they were loaded into a microfluidic device.

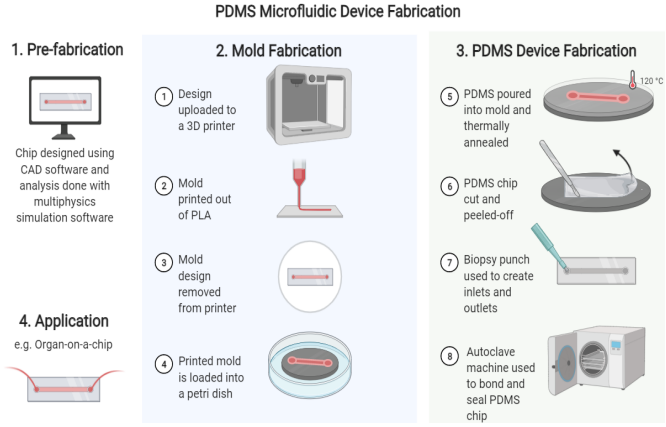

Figure 1. The PDMS microfluidic protocol used to fabricate our devices.

The process of fabricating microfluidic devices is displayed in Figure 1. The microfluidic devices were created by designing a mold using SolidWorks. The mold design was then uploaded and printed on a PLA 3D printer (Ultimaker 3). The PLA mold was then filled with liquid polydimethylsiloxane (PDMS) and allowed to thermally anneal in an oven heated to 50°C. The PMDS was then cut and removed from the mold. The inlet and outlet channels were formed with a biopsy punch, and the PDMS mold was bonded and sealed to a glass slide or sheet of PDMS using oxygen plasma. Once the spheroids were loaded into their microfluidic devices, a syringe pump was connected and used to introduce the external mechanical forces.

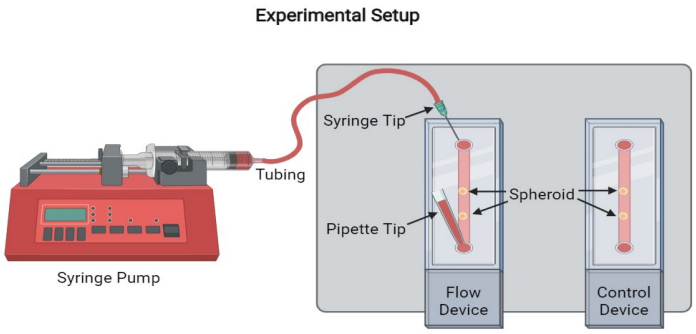

Figure 2. The experimental setup used to subject the spheroids to external stimuli.

The setup used to run experiments is displayed in Figure 2. In this experimental setup, there is a control device that is used to observe the development of the spheroids in just the device. Whereas the experimental device has the syringe pump connected to a syringe tip in the inlet punch with a pipet tip inserted in the outlet punch to prevent media from spilling all over the device. The syringe pump is preset to a designated flowrate, usually 350 µL a minute for a target volume of 7 µL. The experiment ran for a preset amount of time, usually 30-90 minutes. After the spheroids were unloaded from their microfluidic device, they were analyzed using confocal microscopy. It was hypothesized that the spheroids were too dense for the confocal microscope to pierce through [5]. Therefore, the spheroids were fixed in PFA and cleared using the CUBIC clearing method in order to ensure all the fluorescent cells in the spheroid were visible [6].

Result Discussion:

We discerned that we were seeing most, if not all, of the fluorescence in the fixed and cleared spheroids. It was decided that the tissue in the normal spheroids were too dense for the laser of the confocal microscope to pierce through. Moving forward, tissue fixing and clearing was a necessary step for imaging spheroids exposed to doxycycline. Once we were confident in our ability to see inside of the spheroid, we began quantifying the spheroid fluorescence. Fluorescent images of the spheroids were captured using a Zeiss 700 laser scanning microscope. Z stack images were taken in ~15 µm intervals to capture cell fluorescent at various depths into the spheroid tissue. The z stack layers of each spheroid were uploaded into ImageJ. Each z stack layer was then separated into their respective red, green, and blue, fluorescent channels. It was then observed that a bright, outer band of fluorescence appeared on each fluorescent color. This bright band gave us the penetration depth of the doxycycline into the spheroid. Therefore, we decided to quantify this bright, outer band in ImageJ.

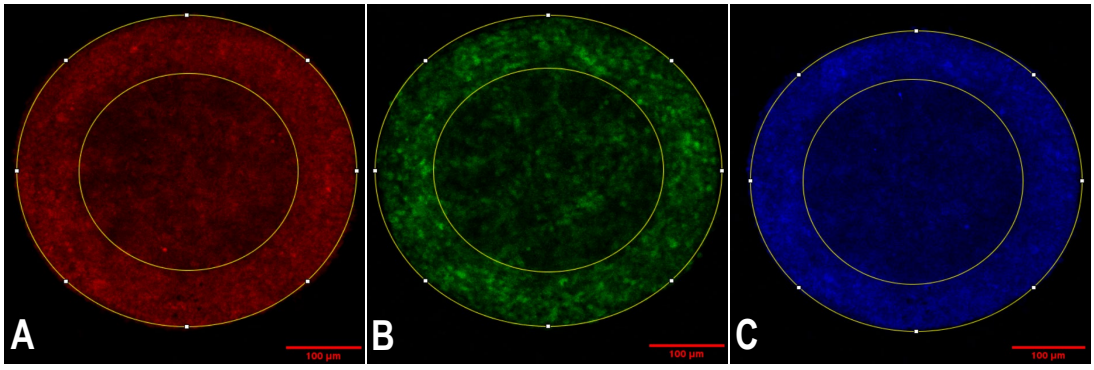

Figure 3. ImageJ analysis used to quantify red (A), green (B), and blue (C) fluorescence.

The method of analyzing the spheroid's fluorescent channels is displayed in Figure 3. We drew an ellipse that encompassed the entire spheroid and an ellipse that encompassed the darker, inner area of the spheroid. We then recorded the area, diameter, and angles from each ellipse. The inner ellipse area was then subtracted from the outer ellipse area to give us the entire penetration area of the doxycycline. This process was repeated for each fluorescent channel on every available z stack layer. A MATLAB program was also written that quantified the intensity and prevalence of each fluorescent marker on every z stack layer of the spheroids. For each fluorescent color, the penetration area of each z stack was displayed on a line graph that compared the control device and the experimental device.

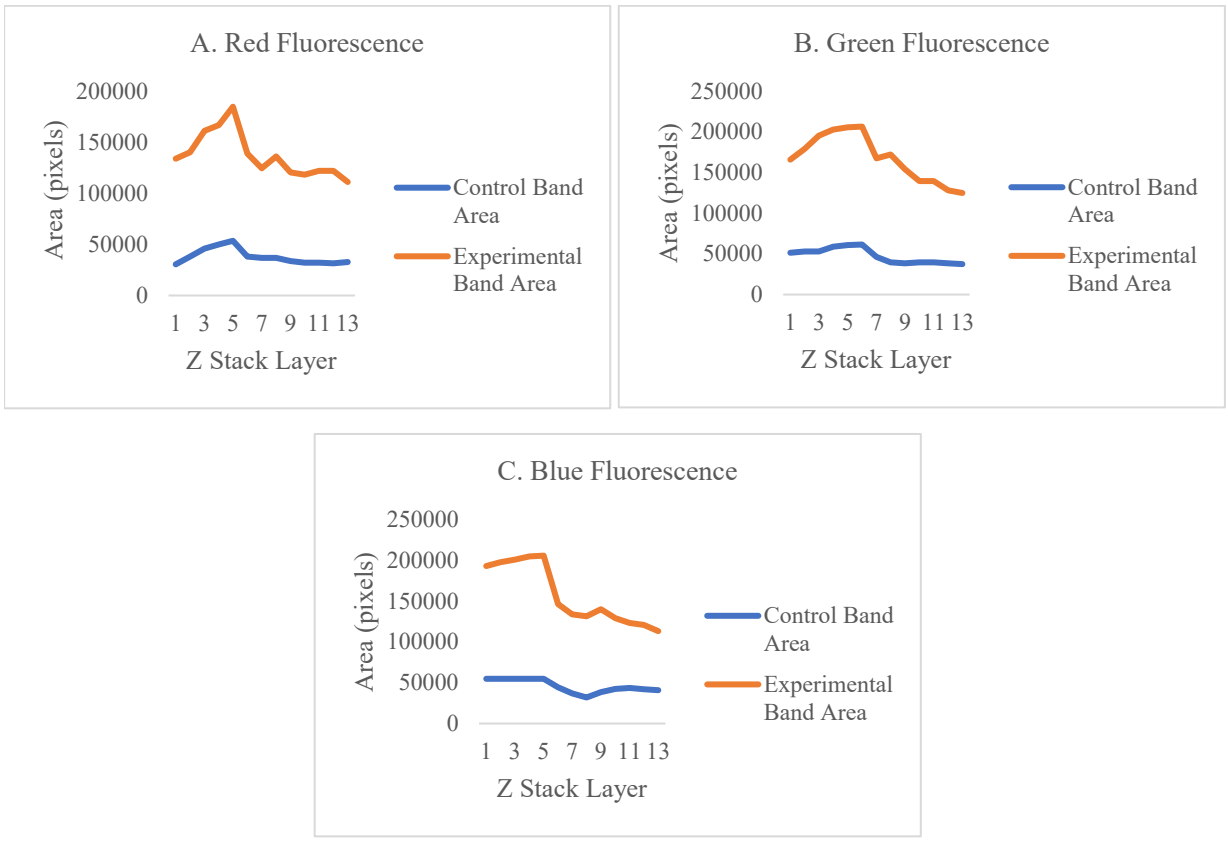

Figure 4. Red (A), green (B), and blue (C) fluorescent analysis comparing control and experimental penetration area from drug exposure to doxycycline.

Based on the graphs of each fluorescent color in Figure 4, the experimental device had the largest doxycycline penetration area compared to the control device. It is important to note that only one experimental and one control spheroid were compared to each other. Therefore, more data is needed to confirm statistical significance. However, we discerned from the graphs that spheroids exposed to external mechanical forces experienced a deeper penetration of doxycycline.

Conclusions:

In conclusion, we successfully created a microfluidic device that could subject the spheroids in the device to external mechanical stimuli. We were able to successfully load the spheroids into the device without any bubbles inside of the channels and without any media leaking out the plasma bonded seal. We were also able to address the issue of microscopic spheroid imaging and analyzing because we successfully rendered

our spheroids transparent by utilizing the CUBIC clearing method. Finally, we were able to quantify the penetration area of the doxycycline drug into the spheroids. We were able to run a successful experiment that resulted in the ability to compare the experimental device penetration area results to the control device penetration area results.

Future Works:

- Experiment with organoids in these devices to determine their optimal flowrate and necessary mechanical forces.
- Improve the parameters of the syringe pump flowrate to represent *in vivo* blood circulation.
- Optimize the microfluidic environment for multiple types of organoids.

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