BMEG 101 - Cellular and Molecular Bioengineering
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Final Project Report: Engineering a signalling gradient by guided self-organized cell populations

INTRODUCTION

The topic of tissue self-organization is one which is currently being heavily researched in the field of cellular bioengineering due to its tremendous potential clinical and research applications. As a biotech team based in Vancouver, BC, we leveraged various cellular signalling processes to develop a reproducible and robust protocol for the self-organization of functional tissue made of two cell types, cell A and cell B. Following our design, one should be able to set up several outlined initial conditions and allow tissue to self-organize and grow to a circular geometry with Cell A in the center and Cell B in a ring around A. Our protocol is specific to an initial circular shape of 1mm diameter which grows to 10mm, but in theory these results can be scaled by altering initial conditions and modifying our provided calculations. We also discuss potential cell types for cell A and B which could be used in this protocol, as well a few potential applications of this project in therapeutics.

METHODOLOGY

With the goal of self-organizing tissue design in mind, we first split the problem into two parts: cell migration and cell proliferation. These were treated separately. We also realized that for the system to be completely self-sufficient, there needed to be cell signalling processes integrated into both the migration and the proliferation steps. The signalling involved in the migration step would need to indicate to Cell B to move to the outer edge of the 1mm conformation, and the signalling in the proliferation step would first need to trigger the release of growth factor from Cell A which would be received by Cell B, and then signal to B to stop growing once the geometry reaches a 10mm diameter circular configuration. Finally, we searched for cell types which could potentially meet all the requirements for our protocol.

TOOLS AND PROTOCOL

Deliverable 1:

Materials:

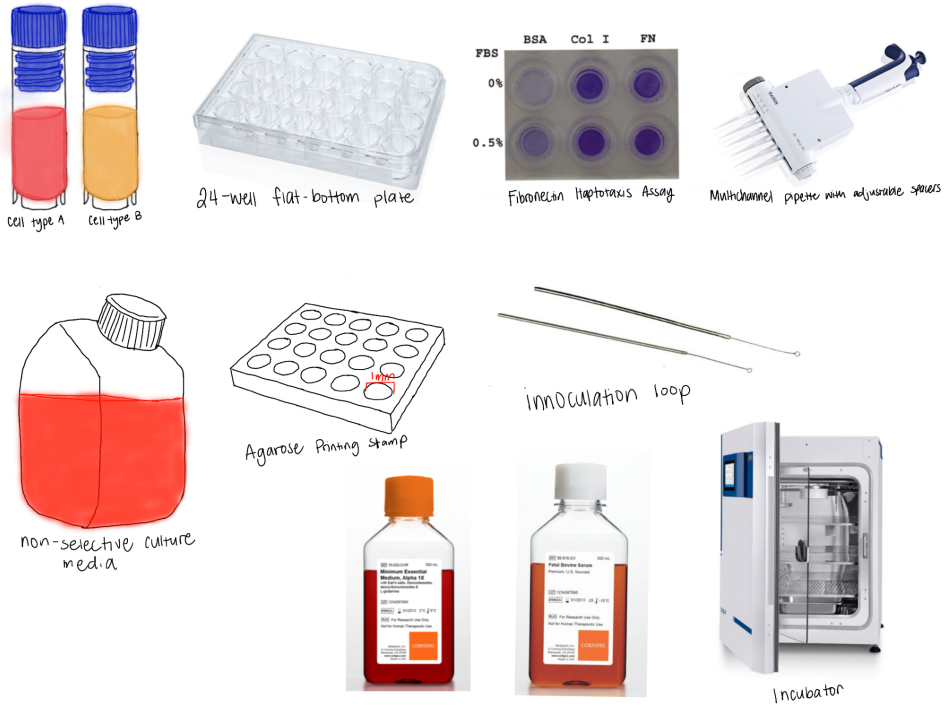
a. Stock reserves of Cell type A and Cell type B in cryopreserved vials
    i. Assume these cells have already gone through the seeding, passaging, culturing processes and are ready to be thawed and plated.
b. Tissue cultured 24-well flat-bottom plate (10mm working area per well)
    i. This can be found at https://assaycell.com/
    ii. The plate must be tissue cultured to provide ECM properties for cell adhesion and migration and to be a favorable environment for cell proliferation (Primary Cell Culture Basics, n.d.)
    NOTE: ensure the tissue culture is uniformly distributed over the surfaces of all the

plates or else there will be unwanted migration of the cells towards any gradients (haptotaxis).

iii. The high quantity of wells will allow for high throughput; multiple circular tissues can be formed simultaneously.

c. Fibronectin Haptotaxis Assay
   i. This is used to create the haptotactic gradient for Cell B (as will be seen in Deliverables 2 and 3)
   ii. Can be found at https://www.cellbiolabs.com/
d. Micropipette or multichannel pipette with adjustable spacers if greater efficiency is required.
   i. Adjustable multichannel pipette can be found at https://www.mt.com
e. Non selective culture media
   i. This is used to create Cell A and B mixture and must be non-selective because it must be able to support both cell types at the same time.
f. 1mm diameter circular microcontact printing agarose stamp
   i. This stamp must be formed in a specific way so that when stamped, it creates a radial gradient.
   ii. This will be used to create a haptotactic gradient for cell migration.
g. Inoculation loop
h. Incubator
i. Minimum Essential Media (MEM α) and Fetal Bovine Serum (FBS)
   i. Needed to create favorable condition for cell growth

Figure 1: Materials needed for the experimental protocol of self-organizing tissue growth.



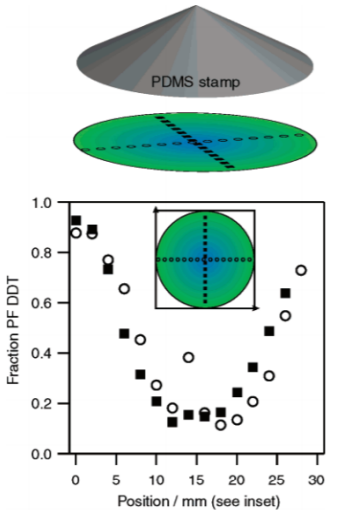
Deliverable 2:

NOTE: For the experimental protocol outlined below, we assume we are only making one circular tissue. However, this number can be scaled up to as many as we want, as long as there are enough starter cells, plates, and assays.

Experimental Protocol:

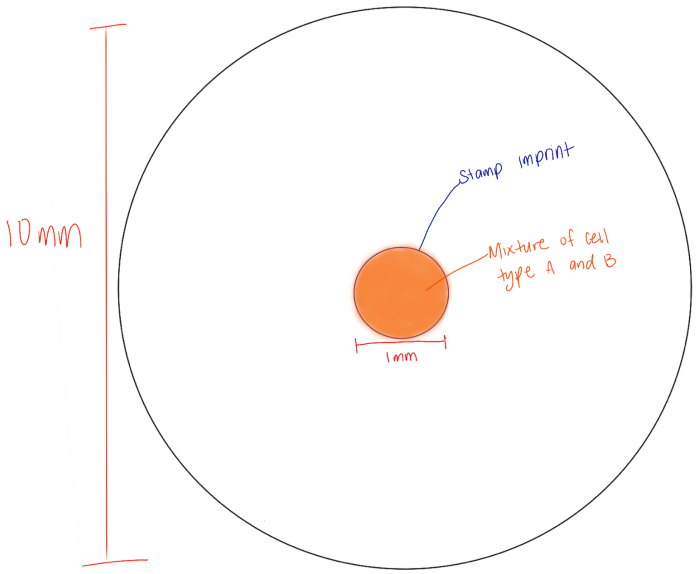
1. Create a 1mm diameter radial substrate adhesion gradient in the center of the plate. To do this, soak the specially contoured 1mm agarose stamp into the fibronectin haptotaxis assay then stamp it onto the center of a plate (Kraus et al., n.d). The gradient will look similar to Figure 1 with higher concentration of fibronectin towards the outer edge.

Figure 2: Agarose stamp with radial gradient used to create a haptotactic gradient of fibronectin assay. Image borrowed from https://pubmed.ncbi.nlm.nih.gov/16089385/.



2. Thaw cell types A and B. Use a sterile inoculation loop, scrape equal parts of Cell type A and type B from the starter tubes and transfer them into a Falcon tube filled with enough culture media to cover all the cells. Thoroughly mix the resulting solution with a micropipette and then pipette just enough of the solution onto the center of the plate to cover the stamp's imprint (~1mm diameter drop) as seen in Figure 2 below.
    a. At this point in time, there are no visible domains of cells A and B. They are completely mixed in a 1mm diameter hemispherical drop on the plate. Since the height of the drop will be <0.5mm, this configuration can be thought of as a monolayer of cells.

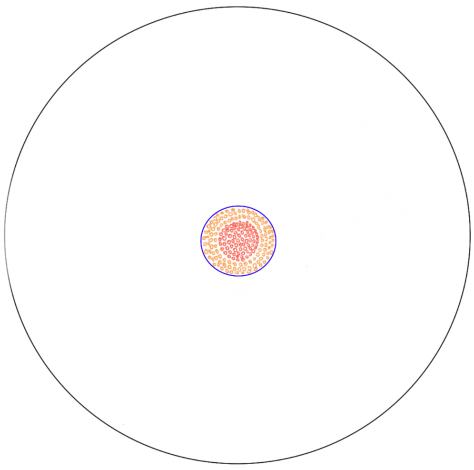
Figure 3: Plate with 1mm diameter circular homogeneous monolayer of cells containing cells A and B. The previously stamped fibronectin gradient is found underneath this monolayer.Note: only one plate from the 24-well plate is shown.



3. Wait for approximately 8-9 hours for the type B cells to migrate and form an outer ring on the 1mm circular conformation, as seen in Figure 3 below. This time is based on the average speed of fibroblasts migration for *in vitro* experiments, which is ~1μm/min (Trepat et al., 2012). If one Cell B begins at the center of the circular 1mm geometry, then it must move 0.5mm (500μm) to reach the outer diameter. It then follows:

$$Time\ for\ B\ cells\ to\ reach\ outer\ ring\ = \frac{500\mu m}{1\mu m/min} = 500\ mins = 8.33\ hours$$

Figure 4: Plate with 1mm diameter circular monolayer with visible cell A (shown in red) and B (shown in orange) ring pattern.



4. Add Minimum Essential Media (MEM α) and Fetal Bovine Serum (FBS) to the plate, ensuring not to damage the cell conformation, and incubate at 37°C with 5% CO_2. It will take approximately 3-4 hours (BMEG 101 Lecture 4, 2021) for Cell A to begin proliferating and secreting growth factors.
5. Wait for 1-1.5 days until the resulting self-growing tissue reaches a 10mm circular geometry. In other words, it will at this point have reached the outer edge of the plate. Type A cells are not expected to not grow much during this subprocess so their domain will remain a circular shape of approximately 1mm diameter in the center, perhaps slightly larger due to any diffusion effects. Cell B is the cell type which is mainly proliferating during this subprocess so it will occupy the remaining space in the plate that is not taken up by type A. This 1.5 day time frame comes from the time it takes for type B cells to receive and interpret the growth factors,which is 3-4 hours (BMEG 101 Lecture 4, 2021), and also the time it takes for type B cells to proliferate from a 1mm diameter to a 10mm diameter with its doubling time of 13h. This doubling time is an approximate of the doubling time of mammalian cells (Sitton & Srienc, n.d.). All the calculations for Step 5 are as follows:

   $\frac{dP}{dt} = kP(t)$ where P is the population of B cells and k is some constant.

   Solving for k using a doubling time of 13h, we get:

   $k = \frac{1n(2)}{13}$

   And the final expression for the current population of B cells is:

   $P = P_0(2)^{t/13}$

   Solving for t using and initial population of around 130 and a final population of around 1300 (see Deliverable 4 for this calculation), we get:

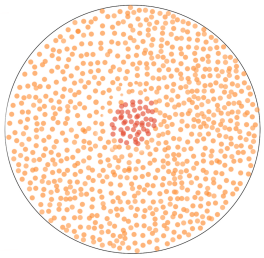
   $t = 13\frac{ln(1300/130)}{ln(2)} = 27h$

Therefore:

$Total\ time\ =\ 4\ hours\ +\ 27\ hours\ =\ 31\ hours\ =\ 1\ -\ 1.5\ days$

6. Type B cells will begin to feel mechanical stress from the edge of the plate and it will take this as a cue to begin apoptosis at the same rate as proliferation, effectively stopping the growth of B. The final 10mm circular confirmation is now satisfied, as seen in Figure 4 below.

Figure 5: Plate with fully formed 10mm diameter circular tissue growth reaching the edges of the plate. The majority of the configuration consists of type B cells (shown in orange) with a minimal amount of type A cells (shown in red) at the center.



Since type A cells will only slightly proliferate in this entire protocol due to the minimal amount of FBS and MEM α, their domain remains in approximately a 1mm diameter circle at the center of the plate throughout the entire process. The final configuration of B cells as a ring around A cells will have an inner radius of approximately 0.5mm (half of A's final diameter) and an outer radius of 5mm. Adding the time of all the subprocesses, we find that the total time for this experiment would be 8.33h + 4h + 31h = 43.33h = ~2 days.

RESULTS

Deliverable 3: The experimental protocol described above was designed for a specific pair of cells which meets the following conditions (assumptions):

- Cell type A has the natural ability to produce and secrete growth hormones in a favorable environment.
    - The only requirement for the growth factor production and secretion is the cell growth and proliferation of cell type A, which is driven by the media added (MEM α & FBS) to supply nutrients and the simulation of a natural environment for mammalian cells (37°C & 5% CO_2).
    - To ensure cell type A will not expand beyond a 1mm diameter, we only add a minimal amount of MEM α and FBS.
- Only Cell type B is sensitive to the stamped haptotactic gradient.
    - Only cell type B will be growing outwards from the initial 1mm conformation. This will ensure that cell type B will always be the outer ring in the conformation.
- The association rate of the ligand (growth factor) /receptor (found on the surface of cell type B) complex is relatively higher than its dissociation rate.
    - In order for cell type B to grow and proliferate, it needs to receive the growth factor from cell type A. This will occur through paracrine signalling, with the growth factor binding to the receptors found on the surface of cell type B.

- Since there would be less amount of cell type A than B, the association rate of the receptor-ligand must be high to ensure that cell type B is receiving enough for its growth to continue expansion despite degradation.
- The binding of the growth factor to cell type B can be represented by the binding kinetics of receptor-ligand complex:

$$Ka = \frac{k_{on}}{k_{off}} = \frac{[RL]}{[R][L]}$$

- After the final conformation is reached, equilibrium will be reached, where there will be no more changes in the amount of activated cell type B (represented by the complex)

$$k_{on}[R][L] = k_{off}[RL]$$

- Cell type B would be following the Hippo Pathway during its proliferation.
  - Once cell type B reaches the desired size, the walls of the plate will be restricting the expansion of cell B beyond the 10 mm diameter. This mechanical stress from the walls would also cause signals through the hippo pathway (Boopathy & Hong, 2019) to make cell B start degradation at the same rate it is proliferating, therefore making the change in the amount of cell B equal to 0 (Chang et al., 2019).

Deliverable 4: With the assumption that cell type A has the natural ability to produce and secrete a growth factor, simulating a natural environment should trigger the production and secretion of the growth factor. To do this, we propose to add a MEM α and FBS (Fetal Bovine Serum) to the plates to promote the cell growth of cell type A. The plates would then be placed into an incubator at 37℃ and 5% CO_2 for a few days. We also assume that cell type A is a continuous cell line, as they can be grown and expanded over long periods of time, allowing for a self-organized system in which it can sustain itself. The rate of production and secretion of the growth rate should be dependent on the concentration of cell type A. Another trigger to release the growth factor, which is the ligand in this experiment, is the presence of receptors on the surface of cell type B. Ideally, cell type A would expand until it covers an area within 1mm diameter until the rate of change of its concentration becomes zero, then its concentration would stay constant and the rate of production the growth factor would also be constant.

The rate of the production of the growth factor can be represented by an expression for the absolute synthesis rate of a protein *i*, k*i* in the units of molecules per generation.

$$k_i = \frac{\phi_i P}{MW_i}$$

Where ϕ_i, MW_i, P, are the mass fraction of protein *i* relative to all other proteins produced, molecular weight, and weight of protein per cell, respectively (Li et. al., 2014). In this case, the growth factor is represented by protein *i*.

Deliverable 5:

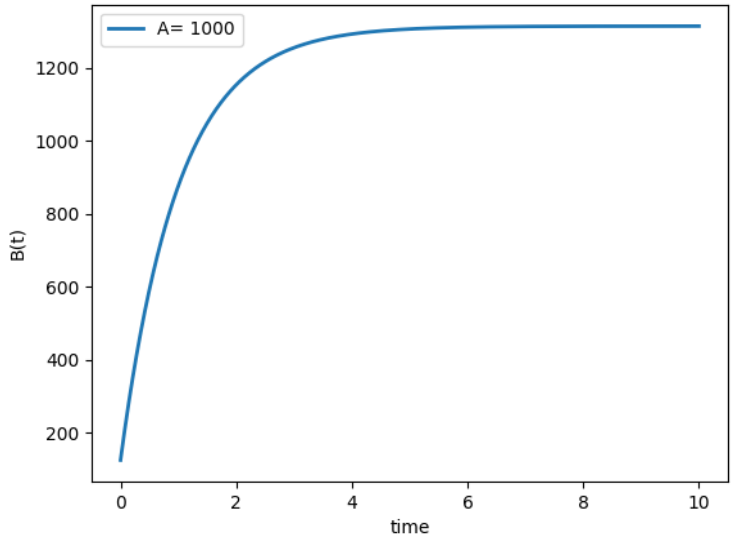
As cell type A activates cell type B, the change in concentration of cell type B can be modeled with the gene expression model:

$$\frac{d[B](t)}{dt} = r\beta \frac{[A]^n}{1+[A]} - \gamma\beta\,[B]$$

Where rβ represents cell type B's production or growth factor and ϒβ [B] represents cell types B's degradation.

As the initial diameter of the tissue is 1 mm, the initial circumference would be π mm. We can assume the diameter of cell type B to be around 25 μm (Göktepe et al., 2010). Thus, we can find that the amount of cells of type B would be π divided by 0.025 mm (~126 cells). As cell A is contained in a ring of cell B, we set the initial diameter of the circle cell type A is contained in as 1 mm, making the area 0.25π mm^2. The amount of cells could be found by dividing π mm^2 by the area of one cell A ($(0.025/2)^2 \cdot \pi$ mm^2) and factoring in spacing of the cells. Thus, we approximated the number of cell type As to be around 1000 cells. With the final diameter of the tissue being 10 mm, the circumference would have to be 10π, leading the number of cells to be around 1257 cells. As cell A is dependent on MEM α and FBS, the hill coefficient (n) would have to be more than 1, thus we set the value to be 2. We assumed there was minimal cell death of cell type B, setting ϒβ to be 1. We can assume rβ to be around 1315, and use the equation above to make the graph shown in *Figure 6*. The graph implies that cell type B evens out to be around 1260 cells as the rate of cell growth and rate of cell death cancel each other to have a steady amount of cells.

Figure 6. Graph modeling the growth of cell type B (image made using https://trinket.io/features/python3)



DISCUSSION

Deliverable 6:

"Cardiomyocytes and endothelial cells in the heart are in close proximity and in constant dialogue" (Giacomelli et al., 2017, pg. 1008). From extensive research, it was found that two promising real cell types that could be used in this procedure are endothelial cells (cell type A), and cardiomyocytes (cell type B).

In the case of cell type A, endothelial cells (abbreviated to “ECs" in many articles) are versatile in their capacity to arrange themselves and populate to suit their local environment. In addition, they “create an adaptable life-support system, extending by cell migration into almost every region of the body” (Alberts, 1970). This indicates the important characteristic of endothelial cells being able to migrate in the arrangement of the tissue design that is of concern in this project. Another specification of cell type A is that when prompted, it releases a growth factor (typically a protein or hormone) to promote the proliferation of cell type B. Endothelial cells fit this criterion; in response to stimuli, they “secrete a set of proteins and other factors, angiocrines, which act on the neighbouring cell types” (Talman & Kivelä, 2018, pg. 3). A benefit of ECs being able to release different growth factors is that we are able to choose and experiment with those that best fits the experimental procedure. One such example is neuregulin-1 (NRG-1), which binds to and activates receptors in cardiomyocytes, ultimately promoting their proliferation and growth (Talman & Kivelä, 2018, pg. 3-4). The manner by which ECs release this growth factor (and others) would be by paracrine signalling. As Kivelä et al. (2019) reports, “present findings shed light on [this] mechanism of how angiogenesis induces physiological [cardiomyocyte] growth and highlight the importance of [endothelial-cardiomyocyte] cross talk in cardiac homeostasis” (pg. 2579). What this essentially means is that paracrine signalling facilitates the interactions between these two cell types to promote the maintenance of heart tissue.

Meanwhile, cardiomyocytes (abbreviated to “CMs”), which we have classified to be of cell type B, are abundant cells that enable the heart to contract, as stated by Woodcock & Matkovich (2005). A crucial attribute of cardiomyocytes is that they are able to “undergo enlargement (hypertrophy) in response to chronic demand for increased contractile force” (Woodcock & Matkovich, 2005). However, for this project, it is a necessary requirement that cardiomyocyte hypertrophy can be limited to fit the desired parameters. In an article by Frey et al. (2004), two different types of hypertrophy occurred for cardiomyocytes: “(1) concentric due to pressure overload… and (2) eccentric hypertrophy due to volume overload” (pg. 1580). Though further testing is required for this specific project, it was found that for the first time, “hypertrophic growth could be abolished while the inciting stimulus, pressure stress, was maintained” (Frey et al., 2004, pg. 1581). This finding opens up inquiry for how pressure can be a manipulated variable to limit the hypertrophy of cardiomyocytes, and ultimately the size to which these cells may grow. Following the size manipulation of cardiomyocytes, its ability to migrate into positions for the tissue is also important for stable tissue formation. In particular, a research article by Itou et al. (2012) studied the role of cardiomyocyte migration in the hearts of zebrafish. An important conclusion from this article was found: “the abnormal localization of proliferating CMs suggests that the migration of proliferating CMs, rather than local activations of CMs in the injury site, is necessary for heart regeneration” (pg. 4137). Though the activation of CMs are incredibly important in their own right, their migration will be a significant player into producing the tissue in conjunction with endothelial cells.

Ultimately, as previously mentioned above, cardiomyocytes are in constant communication with endothelial cells for heart tissue regeneration. By extensive research, these two indeed make a sufficient pair when it comes to engineering potential tissue for drug development.

Deliverable 7:

Therapeutic agents are considered more effective when they can reach their target faster and more easily. These agents not only need to reach their tissue and cells but also to their relevant subcellular structures (Fu et al., 2020). The size of the therapeutic agent affects whether it is able to effectively enter

smaller structures, thus affecting the drug's efficacy. With a size pattern similar to the one created by cell type A and B, the drug is able to remain small when entering the body, and expand to its actual shape when it reaches its final destination. This, in turn, allows the drug to efficiently reach the target without getting blocked by blood vessels. For example, if the drug needs to activate in the stomach, the final tissue conformation (to release growth factors from cell type A to cell type B) could require an acidic environment to be fulfilled.

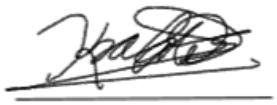
The cellular surface area of the drug can affect its efficacy, as it can affect how quickly the active agent can react with its target. The higher the surface area, the more of the agent there is to react with the target, allowing for a faster therapeutic effect. A study done by Xie et al. (2017) found that a triangle (in terms of several non-spherical shapes) was the most effective shape for cellular uptake. A circular shape (the 2D shape of our tissue) also has a relatively large surface area, allowing for a good cellular uptake and a relatively fast therapeutic effect. The shape pattern of our tissue remains circular, however, it would be possible, through cell migration, to vary the shapes to benefit different targets and different areas of the body.

AUTHOR CONTRIBUTION AND INTEGRITY PLEDGE

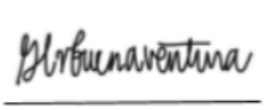
All work done in this project was distributed equally among all Group 12 team members.

I affirm that I will not give or receive any unauthorized help on this assignment, that all work will be my own, and that I will abide by any special rules for conduct set out by the examiner.

Latif Dhalla



Kyle Palacios



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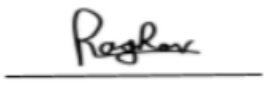

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