Using Neoblast Proliferation in Bioprinted Scaffolds as a Model for Metastasis

Grant Proposal

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Abstract

Cancer, characterized by the uncontrolled division of cells within the body, becomes particularly dangerous when it metastasizes, or spreads to other parts of the body. Among individuals with metastatic cancer, the median survival time is approximately 10 months, thus showing a critical need for improved research models. Current models for cancer metastasis, such as animal systems and 2D cell cultures, are both costly and challenging to reproduce, creating substantial barriers to cancer research and delaying the development of potentially life-saving therapies. As such, bioprinting technology offers a promising alternative by enabling the creation of cost-effective, reproducible models for studying cancer metastasis, provided that a functional microenvironment can be established within the bioprinted scaffold. The aim of this project is to develop a cost-effective, reproducible bioprinted model to mimic the proliferating aspect of cancer metastasis. Known for their abundant neoblasts with a highly proliferative nature, planaria serves as a biological analogue for metastatic cancer cells due to their ability to provide rapid cell proliferation and tissue regeneration. When the regeneration of planaria within the bioprinted model was compared to that in their natural environment, the results are expected to be consistent, demonstrating the feasibility of developing an initial model for cancer metastasis. Possible mechanisms to enhance the proliferation of planaria within the model to better replicate the behavior of aggressive cancers can be further explored by modifying parameters surrounding the bioprinting process.

Keywords: bioprinting, cancer, metastasis, planaria, proliferation

A Novel Approach to Cell Proliferation through 3D Bioprinted Scaffolds

When considering the future of regenerative biology, bioprinting stands at the forefront due to its ability to manufacture biocompatible scaffolds that are organized and controllable. This innovative technology promotes cell growth in models that are more effective and viable than those currently used for studying cell proliferation and reproduction (Cellink, 2019). To understand biology, cell proliferation and reproduction must be studied as they provide the foundation for various biological processes, which significantly impact health, disease, and medicine. One of the most significant areas is cancer, where uncontrolled cell proliferation leads to tumor growth; unchecked cell growth is a root cause of tumor development and metastasis (Puls et al., 2018). As a result, modern research surrounding cancer to develop

anticancer therapies requires a thorough comprehension of cell proliferation. Currently, models to study cell proliferation are produced in two ways: animal testing and 2D models. However, both methods have drawbacks which significantly impact their uses in a research environment. While models created through animal testing provide an accurate cell environment, it is expensive, time-consuming, and can potentially raise ethical concerns (Zhang et al., 2016). 2D models, such as traditional cell cultures grown in petri dishes, can cost significantly less but often has low reproducibility rates, suggesting that the alternative to animal testing is unable to properly showcase cell-to-cell and cell-to-environment complexities. Through bioprinting, these concerns can be mitigated while the benefits of each model are kept. Bioprinting allows for the facilitation of layer-by-layer scaffolds that loosely mimic native tissue architecture, offering a low cost, ethical, and highly reproducible approach by combining the precision of 3D printing with biological systems (Shukla et al., 2022). These scaffolds are a key to studying cancer cell proliferation as they facilitate an ideal environment for cellular behavior.

Bioprinting Methods 101

There are three common bioprinting methods: inkjet, stereolithography (SLA), and extrusion. Extrusion bioprinting is the most widely used method of the three types due to its lower cost, versatility, and ability to handle a larger range of biomaterials. Extrusion bioprinting most closely resembles fused deposition modelling (FDM) in traditional 3D printing, with a bioink being dispensed through a syringe to build a 3D structure layer-by-layer (Zhang et al., 2021). Resolution for this method is lower than inkjet and SLA printing, extrusion-based bioprinting allows for the creation of larger constructs with structural integrity, which is the best method to create scaffolds to support cell growth (*Extrusion vs. DLP 3D Bioprinting - Explanatory Comparison*, 2023). As such, this project utilizes extrusion based bioprinting in the form of a modified Prusa MK4S 3D printer.

FRESH Bioprinting

Freeform Reversible Embedding of Suspended Hydrogels Bioprinting (FRESH) bioprinting is a subset of extrusion bioprinting which specializes in bioprinting soft gelling biomaterials (*FRESH Bioprinting Enables More Complex Geometries*, n.d.). FRESH supports these softer biomaterials by printing in a support bath as a compatible bath supports the extruded bioink on all sides, allowing the ink to rapidly go through a gelation

process. This method enhances cell viability by providing a supportive, customizable aqueous environment that can include cell culture media and growth factors to promote cell vitality.

Hydrogels Used as a Support Bath in Bioprinting

The support bath used in bioprinting is most commonly a hydrogel, as it provides an environment that closely mimics the extracellular matrix (ECM) found in biological tissues. The extracellular matrix is a complex network that surrounds cells and supports cells within a tissue, providing a physical scaffold for cells within tissues. Thus, changes in the ECM's mechanical properties directly affect cells. One example is a stiffer ECM promoting excessive proliferation, while a softer ECM inhibits cell division (Wu et al., 2023). Hydrogels mimic the ECM through primarily being composed of water, supporting cell viability, and mimicking conditions found in human tissues. Hydrogels are also easily modifiable by adjusting a chemical property, crosslinking, or encapsulating another cell within its matrix, as well as structurally sound enough to provide a support scaffold for larger 3D tissue constructs. Due to these properties, they act as an ideal support for softer bioinks when extruded from the syringe, allowing them to stay in place until successfully cured.

Sodium Alginate Used as a Bioink in Bioprinting

Within bioprinting, the bioink acts as the "filament" in standard 3D printers of which the construct is printed out of. Bioink selection is crucial as it dictates the rheological and biocompatible properties of the final construct. In this case, sodium alginate was chosen as the bioink of choice in bioprinting. Previous studies have shown the success of sodium alginate for direct bioprinting as it supports cell growth and exhibits high biocompatibility, thereby presenting it as a viable option for printing tissues (Bociaga et al., 2019).

Section II: Specific Aims

This proposal's objective is to develop a 3D bioprinted microenvironment to facilitate planaria stem cell proliferation in a controlled and observable environment. As planaria stem cell division and differentiation during regeneration share similarities with cancer cells exhibiting rapid division, neoblast proliferation within a wounded planarian serves as a model for cancer cell metastasis. The long-term goal is

to develop a microenvironment for cell growth that is easily replicable, cost-effective, and provides a platform to study anticancer therapies through planaria biology. The central hypothesis of this proposal is that sodium alginate bioink printed in a calcium support bath will be able to foster cell division and proliferation of neoblasts within a cut planarian. The work we propose here will help create a more effective model compared to those currently used.

Specific Aim 1: Create a bioprinted model that supports planarian life.

Specific Aim 2: Use the model to study planarian neoblast proliferation.

Specific Aim 3: Determine most effective manufacturing parameters for the model (Tian et al., 2021). The expected outcome of this work is to create a bioprinted structure that supports planarian neoblasts and promotes cell growth in a way consistent with previously published research.

Section III: Project Goals and Methodology

Relevance/Significance

Studying cancer metastasis is challenging due to the complexities of the surrounding tumor-tissue environment and ECM, as well as the unpredictable timing surrounding cancer cell division and subsequent proliferation. *In vivo* metastasis models, such as mouse models, are particularly effective for studying how cells interact with surrounding tissue due to the multitude of methods available to introduce and monitor the cancer cells into the model itself (Hebert et al., 2023). However, any model involving studies with live animals pose extreme cost and ethical concerns. Alternately, 2D cell cultures are cost-effective and customizable due to their widespread use within labs and straight-forward approaches. Monolayer cell cultures have been invaluable when studying cancer proliferation and testing anti-cancer treatments. Despite this, 2D cell cultures are unable to capture all the complexities of the extracellular matrix and environment which supports cancer proliferation (Milagro et al., 2024). Additionally, results are difficult to replicate between studies due to the biological variability of cells. Therefore, there is a need

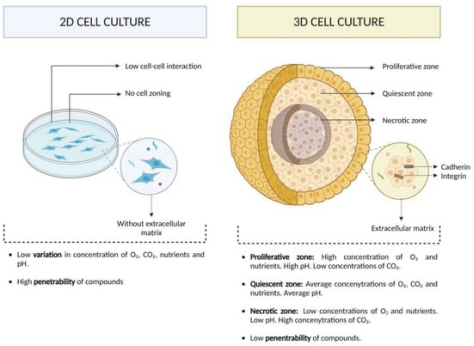

Figure 1: A diagram illustrating 2D cell culture and 3D cell culture. This figure shows the physiological structure of both models and provides basic information (Milagro et al., 2024).

to produce other models to study cell proliferation that is cost-effective, complex, and replicable. This model uses 3D bioprinting technology to improve pre-existing procedures to study cell growth.

Innovation

This study aims to utilize 3D bioprinting as a method to create a model which mimics a native tissue environment to promote cell proliferation in planarian neoblasts, representing cancer cells. A similar study for cancer research resulted in a tumor-tissue invasion model to recreate a tumor extracellular matrix, showing potential for similar models within high-throughput high-content drug screening (Puls et al., 2018). This study aims to modify past methods to generate a more robust, cost-effective, and replicable model to study cell proliferation to assist with future development of anticancer therapies.

Methodology

The following steps will be taken to complete the aims of this project:

1. Modification of a Prusa MK4S 3D printer to create a FRESH 3D bioprinter. The extruder of the printer will be removed and replaced with a syringe-based extruder (Kim, 2023). The extruder will be held together with 3D printed parts out of PLA or ABS filament. There will be a control board added to the printer as well to control the printer and calibrate it for a bioprint (Tashman et al., 2021).
2. Cut a planarian in half and submerge in water. Evaluate cell proliferation over a period by observing the spread of the cells while the planarian is regenerating.
3. CAD plate with measured distances marked. Create bioink by dissolving sodium alginate powder with deionized water. Prepare calcium chloride support bath.
4. Create print of sodium alginate. Vary density of the print and print conditions to create multiple tests to find the optimal environment.
5. Submerge planarian into print to test for organism viability. Expose planarian to stimuli (light, chemical desire) over three days to study long-term vitality within print (Inoue et al., 2015).
6. Cut an unmodified planarian in half and submerge it into the most successful print from Step #5. Angle cut side into the print and uncut side out.
7. Evaluate cell proliferation over period of time by observing spread of cells (Pijuan et al., 2019) using the predetermined distances marked on the plate.

8. The model will be validated through comparing cell proliferation within the print to that of the control group.

Specific Aim #1: Create a bioprinted model that supports planaria life.

A main objective of the 3D printed construct from sodium alginate is to successfully promote planarian neoblast vitality within the construct. The planarian itself should be able to survive within the print.

Justification and Feasibility. The construct must cure and maintain structural integrity after being removed from the support bath. It should support cell vitality by mimicking the native environment. The bioprinted scaffolds will be made of sodium alginate to ultimately host and support cut planaria. Establishing the construct's ability to sustain planarian life is a prerequisite for studying cell proliferation.

Summary of Preliminary Data. An initial print of the construct without added planaria should be able to keep a solid formation when removed from the support bath and then be able to support planarian life. The first metric of a solid formation will quickly be seen when the support bath is removed, as an improper print will be washed away with the support bath. In the initial phases of this project, planaria were assessed for viability. Figure 2.1 displays the mean length of the head fragment measured over time, while Figure 2.2 presents the mean length of the tail fragment. Statistically significant differences ($p < 0.01$) are indicated by asterisks. Both graphs illustrate regeneration from Day One to Day Three, demonstrating a correlation between time and growth. This measurement approach was also applied to a second, distinct planarian, which exhibited similar growth patterns, shown in Figures 3.1 and 3.2.

Figures 2.1 and 2.2

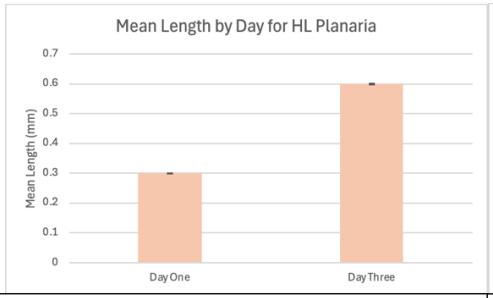

Figure 2.1: Mean length by day for the head of the "lighter" planaria. Asterisks denote statistical significance ($p<0.01$)

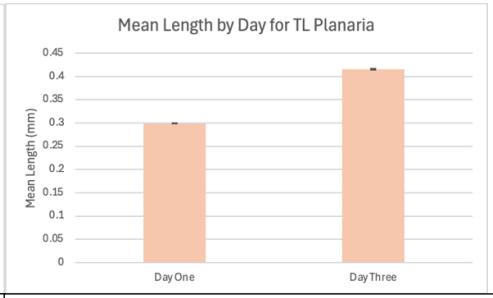

Figure 2.2: Mean length by day for the tail of the "lighter" planaria. Asterisks denote statistical significance ($p<0.01$)

Figures 3.1 and 3.2

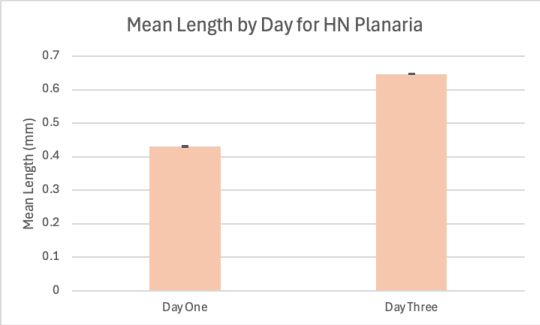

Figure 3.1: Mean length by day for the head of the "newer" planaria. Asterisks denote statistical significance ($p<0.01$)

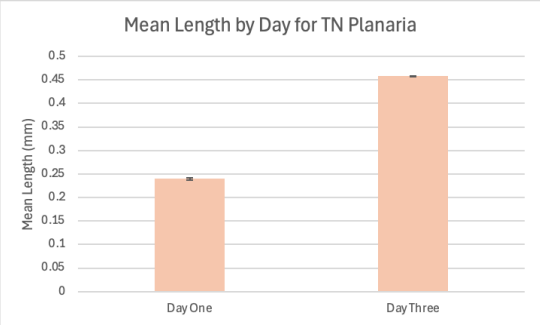

Figure 3.2: Mean length by day for the tail of the "newer" planaria. Asterisks denote statistical significance ($p<0.01$)

A comparison of the two planarians revealed that both exhibited growth as expected; however, the extent of growth was not identical. This finding is significant for future experiments involving planaria embedded in the bioprinted construct, as uniform growth should not be assumed in that context either.

Expected Outcomes. Creating a print that supports planarian life is the first step to building a model to study planarian cell proliferation. This aim sets the stage for the rest of the procedure, as organism life is essential to cell life. The prints that are able to stabilize planarian life will be used in the study.

Potential Pitfalls and Alternative Strategies. As with any 3D print, there is potential for failure. Within bioprinting, this potential is exacerbated by the specific print requirements of the bioink and support bath on the XYZ axes. The bioink needs to be carefully extruded into the support bath for accurate printing and for the support bath to hold the print while it is being cured. The print will be observed closely to determine required calibrations to the printer to mitigate this pitfall (Bessler et al., 2019). The resultant print can be cross validated with previous 3D printed models as well as the original CAD design to ensure there is design consistency.

Specific Aim #2: Use the model to study planarian neoblast proliferation.

Planaria can be used as a model for cancer cell metastasis due to their unique property of being made primarily of neoblasts, which are stem cells with the ability to proliferate extensively and differentiate into different cell types. The migratory behavior of neoblasts during regeneration is similar to the movement of metastatic cancer cells as they invade an organism (Pearson & Alvarado, 2010).

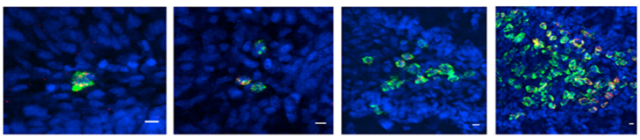

Figure 4: NB2 (neoblasts) growing back 4, 7, 9, and 14 days after irradiation (Zeng et al., 2018).

Thus, a model that studies cell proliferation within planaria neoblasts can be applied to study cancer metastasis as well.

Summary of Preliminary Data. Preliminary data for this step will be gathered from the models to identify parameters that support planarian life. A planaria will be cut and then inserted into the edge print with the cut side facing into the bioprinted plate. By measuring cell proliferation as neoblasts attempt to regenerate the cut planaria, a quantitative value for cell growth can be achieved.

Expected Outcomes. The expected outcome of this aim is to advance the model from merely supporting cell viability to replicating a natural environment that actively promotes and sustains cell growth. This knowledge will be used to quantify success of the overall model in a future step (Augustine et al., 2021).

Potential Pitfalls and Alternative Strategies. Unlike the pitfall in Aim #1, this aim will be challenging due to potential cell viability issues. Planarian neoblasts may not survive successful division due to insufficient nutrient/waste exchange once inserted into the scaffold. The potential of this occurance happening can be lessened through optimization of the bioink properties, such as viscosity.

Specific Aim #3: Determine most effective manufacturing parameters for the model (Fu et al., 2021).

Justification and Feasibility. After the model has been created, it must go through an iterative process to truly determine the most effective parameters of manufacturing. As previous studies have shown, development of models is a linear and iterative process (Puls et al., 2018), especially when creating a microenvironment for a cell to study invasion.

Summary of Preliminary Data. This aim extrapolates from Aim #2: Use the model to study planarian neoblast proliferation. After a model that passes Aim #1 and Aim #2 is created, cell proliferation from the inserted planarian will be measured using the predetermined marks for distance on the bioprinted plate. A model that fosters cell proliferation with ≥85% similarity with the control group will be considered a success. Unsuccessful models will require a modification of printing parameters.

Expected Outcomes. The overall outcome of this aim is to improve the ability of the plate to promote cell proliferation for regeneration from the cut planaria. This knowledge will be used to finalize print manufacturing parameters and has applications in regenerative biology research as a whole.

Potential Pitfalls and Alternative Strategies. This aim is projected to be the most challenging if a model that passes both Aim #1 and Aim #2 does not return as successful, as it is difficult to determine the correct parameters for a print while facilitating life and proliferation. Creating these parameters will require the most iteration and time. However, this pitfall can be mitigated through thorough research, testing, and adjustments.

Section III: Resources/Equipment

Manufacturing

The bioprinter itself will be a Prusa MK4S modified to be a FRESH extrusion bioprinter. Prints will be made from an off-the-shelf filament ABS. A needle will be required, as well as a control board.

Print

The print itself will be made of sodium alginate printed into a support bath of calcium. A dish will be required to hold the calcium bath.

Organism

The model organism in this study will be planaria. Planaria will be required for this project. Distilled water will also be required to facilitate planaria health in the control group and when the flatworms are not being tested.

Other

Due to the organisms' miniscule size, a microscope will be required to analyze cell growth throughout the experiment. Other equipment required includes culture dishes, a box to hold the planaria for storage, a camera, and food for the planaria.

Section IV: Ethical Considerations

This model uses planaria cells as a model for human cancer cells. This model cannot be applied in a clinical setting due to the differences between fostering planarian cell life and human cell life and requires further research to be implemented to study metastasis of specific cancer cells.

Section V: Timeline

Link to Gantt chart with updated steps:
https://prod.teamgantt.com/gantt/export/pdf/?baselines=&color=default&color_filter=&company_resources=&date_filter=&date_format=&display_dependencies=1&display_name_in_bars=0&display_resources=1&hide_completed=0&include_blank_dates=0&orientation=p&page_size=Letter&pdf_font_face=dejavusans&pdf_font_size=7&pdf_task_date_format=&project_resources=&projects=4147733&public_keys=&rand=224159&show_estimated_hours_column=0&show_name_next_to_bar=1&show_percent_column=0&show_project_name_on_bar=0&task_list=default&user_date=2024-12-01&user_resources=

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Mindmap