Gas and Liquid Gradient Bioreactor to Mimic Tumor Microenvironment

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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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Gas and Liquid Gradient Bioreactor to Mimic Tumor Microenvironment

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Abstract:

A major barrier in cancer research is the inaccuracy of *in vitro* tumor models. *In vitro* research is critical to understand cancer biology and investigate anti-cancer therapeutics. Existing technologies either capture an incomplete picture of the tumor microenvironment or are expensive and require complicated instrumentation to operate. There is a need to develop a low-cost, easy-to-use tool that more accurately mimics the tumor microenvironment. The aim of this study was to design and validate a novel bioreactor that combines two distinct, gradient-generating technologies into a single device that simultaneously delivers gas and liquid gradients to cultured cancer cells. A bioreactor was designed with a baffle system to produce a liquid gradient and a chamber with a snaking chemical reaction channel to produce a gas gradient. The chamber was designed to allow for gas produced by the chemical reaction to enter into the cell culture area, resulting in a gas gradient. The device was produced using CAD and SLA 3D printing. A pump system was developed wherein the input solutes are delivered via peristaltic pumps controlled by an Arduino Uno. This pump system allows for real-time adjustment of solute flow rates. The gradient components of the device were validated by quantitatively measuring the liquid and gas gradients with benchtop testing. The device was further validated through delivery of a nutrient liquid gradient and an oxygen gas gradient to cultured U2OS cells. The cells were imaged, and their phenotypes and distribution were evaluated. The analysis of the cell culture platform showed that cells in the high oxygen, high nutrient regions of the platform were most proliferative. The fabrication and initial validation of this device serve as a vital first step in the creation of a novel bioreactor to study the dynamic tumor microenvironment and further advance anti-cancer therapeutic development.

Keywords: Tumor microenvironment, bioreactor, tissue engineering, microfluidic device, 3D printing

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Introduction

Cancer remains one of the leading causes of deaths across the globe, despite worldwide efforts to further our understanding of cancer biology and develop therapies.[1,2] An estimated 1.9 million people were diagnosed with cancer in the United States alone in 2021, and more than 600,000 cancer cases resulted in death.[2] One major barrier to cancer research is that many findings from preclinical studies do not translate to clinical results. Around 97% of cancer drugs fail clinical trial testing.[3] The two most common reasons cancer drugs fail clinical trials are lack of efficacy and problems with toxicity.[4] *In vitro* research is a low-risk tool to evaluate safety and efficacy of anti-cancer therapeutics before moving to testing in animal models and clinical trials.[5] There is a need to increase the accuracy of *in vitro* tumor microenvironment models in order to better estimate efficacy of therapeutics and better understand their mechanisms of action.

Currently, data on proliferation, differentiation, and migratory capacity of cells is obtained through the use of traditional tissue culture methods.[5] These methods subject cells to static conditions of fixed temperature, atmosphere, and media compositions, acquiring a molecular, biochemical, and histological “snapshot” of a dynamic cellular response.[6,7] These methods are extremely limited in their ability to replicate critical aspects of tumor microenvironments: the presence of multiple overlapping gas (NO, O_2, etc.) and solute (growth factors, cytokines, nutrients, etc.) gradients.[6,8] In order for tumors to grow, they depend on vascularization to supply oxygen, receive nutrients, and clear metabolic

waste products.[9] Tumors typically have a rich vascular network which primarily feeds the cells on the exterior of the cell cluster. These exterior cancer cells also release high levels of proangiogenic growth factors such as vascular endothelial growth factor (VEGF)[10]. This high vascularisation causes exterior cells to have unique cellular behavior, like rapid proliferation and increased likelihood of metastasis.[11] Many large tumors have impared vascular supply in their more central regions. The high vascularization in the exterior of tumors and limited vascularization in the interior of tumors leads to a gradient of oxygen and nutrients throughout the tumor body[12]. Cells located in the deeper layers of the tumor are quiescent.[11] As the tumor grows, the cells in the innermost portion become hypoxic and die, forming a necrotic core.[11] Cells in these hypoxic regions have also been shown to have increased metastatic potential[13]. These cells have additionally been shown to respond differently to a wide variety of anticancer therapeutics. Further understanding of how and why these cells behave differently in varied environments is critical for effective development of cancer treatments. There is a need to develop a laboratory tool that allows for the simulation of these natural oxygen and nutrient gradients.

The standard *in vitro* models in cancer research and preclinical studies are two-dimensional (2D).[14] The advantages of 2D models include low cost, long cell culture times, high reproducibility, and ease of data interpratation.[15] However, in recent years, researchers have increasingly explored three-dimensional (3D) cell culture methods.[14] Spheroids, organoids, and tumors-on-chips are the major emerging 3D cell culture technologies. All three technologies have distinct advantages and disadvantages, but are all superior at mimicking *in vivo* tumors when compared to traditional 2D technologies. Spheroids are free-floating aggregates of cells with relatively low complexity.[16] Organoids are more complex structures with cells grown in 3D to resemble an organ.[16] Spheroids and organoids are costly, time-consuming, limited in analysis capacity, and are not viable for extended periods of time.[16] Tumors-on-chips (ToC) are microfluidic based systems. Due to the small dimensions of ToC, these systems can only model small tumors, allow for limited mixing of solutes, and analysis quality is reduced by surface effects.[17] One of the major derivations from *in vivo* tumors in traditional 2D cell cultures is uniform exposure to nutrients and growth factors in the medium.[15] A novel 2D cell culture technique that creates a gradient of nutrients to more closely mimic *in vivo* tumors, while maintaining advantages in terms of cost and high analytic capacity associated with 2D methods is needed.

The ultimate goal of this project is to design and validate a novel bioreactor that will allow for more accurate 2D, *in vitro* simulation of the dynamic tumor microenvironment. We proposed to combine two distinct, gradient-generating technologies into a single device that enables the delivery of simultaneous gas and liquid gradients to cultured cells, with real time adjustment capacity.

Our major aims were to 1) design and fabricate a bioreactor capable of subjecting cancer cell cultures to both liquid and gas gradients simultaneously, 2) quantitatively measure gas and liquid concentrations within the cell culture platform, and 3) perform proof-of-concept testing using cultured cells and evaluate phenotypic changes. In order to accomplish these aims, we set forth five major design constraints for our bioreactor: i) sterilizable ii) biocompatible cell culture platform, iii) tunability of liquid and gas gradients, iv) suitable for incubator in size and material, and v) ability to image the cell culture platform.

Results

Design Development

The initial design for the bioreactor was developed based on a cell culture device previously proposed by our advisor, Thomas Genetta, Ph.D. Previous publications of devices that similarly mimic the tumor microenvironment were used to help inform initial designs and functionality requirements. Both the initial proposal and previous art lead to the following design criteria: a modular design to allow for switching of parts and easy assembly, low material cost, fabrication precision, small overall design, and serializability. Autodesk Fusion 360 was used in order to model each bioreactor iteration.

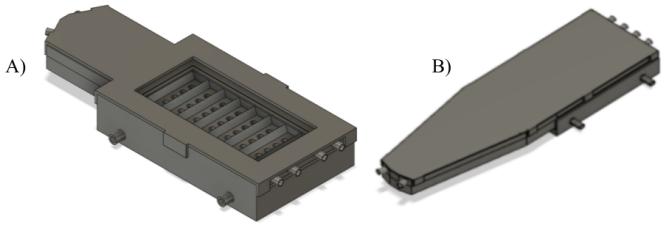

Fig. 1 Bioreactor designs A and B: Iteration A was the initial design for the bioreactor. This iteration failed due to an inadequate baffle system for the production of the liquid gradient. Iteration B was the secondary design for the bioreactor. This iteration failed due to media leakage within the cell culture area.

Three iterations of the bioreactor were produced. The initial iteration was unable to generate the desired

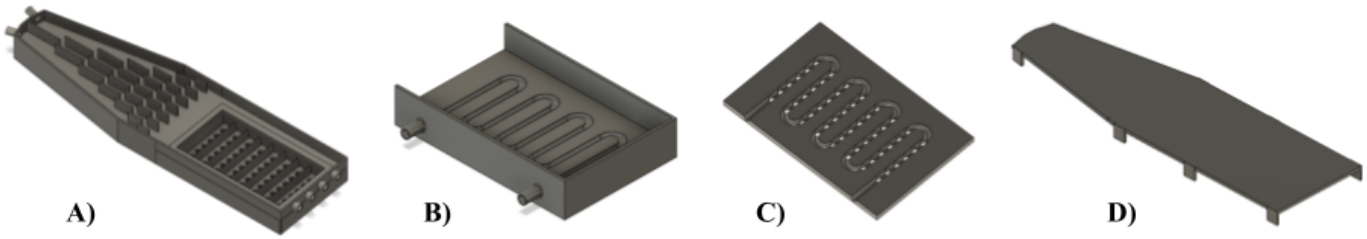

Fig. 2. Bioreactor CAD Design. **A)** Main bioreactor body, containing a baffle system for liquid gradient generation, cell culture area that allows for gas movement, inlet and outlet ports. **B)** Chemical reaction chamber bottom, contains a channel for the chemical reactor to take place underneath the cell culture platform. **C)** Gas gradient generator top, fits on top of the chemical reaction chamber bottom, contains holes for gas movement. **D)** Bioreactor top, contains the fluid within the bioreactor and prevent contamination.

liquid gradient and warped during printing (Fig. 1A). The second iteration of the bioreactor had points of leakage (Fig 1B). The final iteration alleviated these previous issues while satisfying our original design criteria (Fig. 2).

The final bioreactor design consisted of four components (Fig. 2). The main body of the bioreactor consists of a baffle system with inlet ports on either side and a cell culture area (Fig 2A). The baffles increase from one baffle to seven baffles, with a one baffle increase per level. Each intersection of baffles allows for mixing of fluid that ultimately produces a linear gradient of the two fluids being pumped into the system. Polydimethylsiloxane (PDMS) was used as the cell culture platform and was glued into place using biocompatible superglue. PDMS was selected due to its biocompatibility and use in similar applications in literature.[18] It is gas permeable, liquid impermeable, and can be treated to allow for cellular adhesion. A network of holes that allow for diffusion of gas from the chemical reaction chamber are located below the cell culture platform. The chemical reaction chamber consists of a top and bottom component (Fig 2B, 2C). A snaking channel was extruded into the chemical reaction chamber to allow the reagents to flow through it. The chemical reaction chamber top contains holes which align with the snaking channel as well as the holes in the main body, allowing gas to pass from the chemical reaction chamber into the main body. A second piece of PDMS was adhered to the top of the chemical reaction chamber to prevent the movement of fluid between the two chambers. A top cover was developed to fit over the top of the main body. It was adhered to the body using biocompatible superglue during cell culturing in order to prevent fluid spills, minimize contamination, and create an airtight container (Fig 2D).

Pump System and Circuitry

In order to produce the gradients and provide oxygen and nutrients to the cells, a pump system was developed (Fig. 3). Several criteria influenced the design and implementation of the pump system: slow flow rates to minimize reagent usage and more accurately mimic the flow rates *in vivo*, ability to operate for extended periods of time, utilization of inexpensive components, real time adjustments of flow rates, and ease of sterilization. Peristaltic motors were selected as our primary pump system to allow for easy cleaning and sterilization of the tubing used in these pumps. A system of four peristaltic pumps was used to deliver the various reagents. Initial iterations of the pump system implemented 6V DC motors; however, these motors were unable to run at the required low flow rates and overheated with extended periods of usage. Stepper motors were ultimately utilized in the final design. These motors provided low flow rates while being far less expensive than the alternative, research-grade syringe pumps. In order to power and control the pumps, an Arduino Uno and a stepper motor driver were used. The Arduino Uno was programmed to allow for easy adjustment of the pumps with user inputs on a computer. The pumps were capable of being adjusted during operation. Two identical pump systems were fabricated each containing an Arduino Uno, a stepper motor driver, two stepper motor peristaltic pumps, and a 24V external power supply (Fig 3B). The pump system was allowed to run continuously for a 48 hour period to ensure the system would not overheat or shut off with use. The system was able to operate consistently over this period.

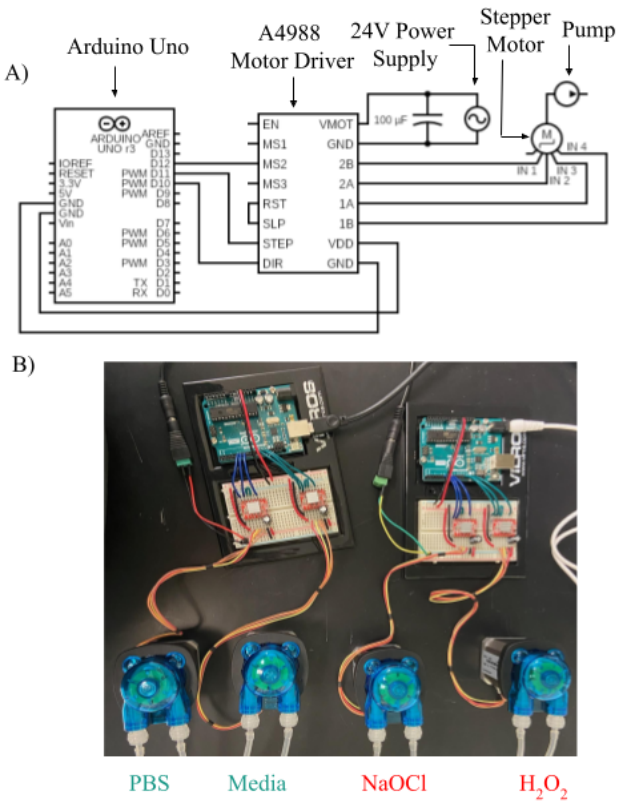

Fig. 3 Pump System: A pump system was assembled to control the flow of solutes through the bioreactor. **A)** Wiring schematic for stepper motors controlled by a motor driver with an Arduino Uno used to control the system. **B)** Full motor setup.

Liquid Gradient Quantification

In order to quantify the liquid gradient, colorimetric analysis was employed. Two tests were used to generate coloretric data: 1) water dyed with red and blue food coloring and 2) Dulbecco's Modified Eagle's Media (DMEM) and water. In our first validation experiment, three drops of blue food coloring were added to 200 mL of water and three drops of red food coloring were added to 200 mL of water. The blue solution was fed through the right inlet to the baffle system and the red solution was fed through the left inlet. Testing was performed at various flow rates, ranging from 0.5 mL/min - 1.5 mL/min. This range was selected to reduce reagent usage, while still being fast enough to produce the desired gradient. After allowing the bioreactor to run for 10 minutes, pictures were taken (Fig. 4A).

Images were then processed using ImageJ. The images were converted to gray scaled 8-bit images. A line was drawn vertically through the cell culture platform, and gray scale quantification was performed. The gray scale values were generated at various points throughout the cell culture platform over three trials (Fig. 4B). The same procedure was performed for quantification of trials using cell culture media and PBS. Flow rates, trial duration, and analysis procedures were kept constant. Results from trials using media and PBS mimicked those seen using the dye solution. Overall results from these trials indicate that a satisfactorily linear gradient was formed due to the baffle system (Fig. 4B).

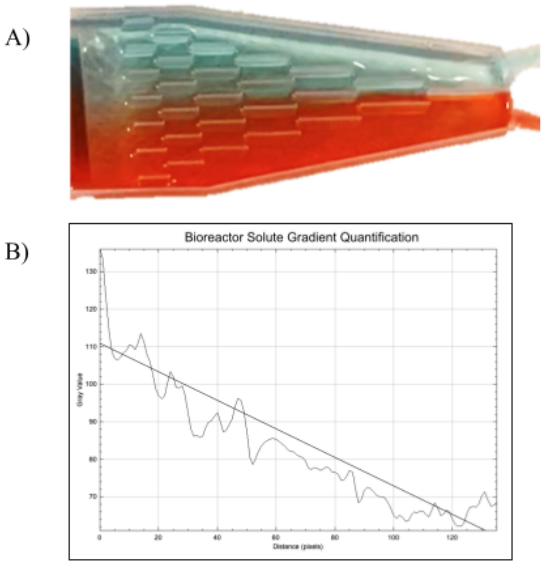

Fig. 4 Liquid Gradient Validation: Red and blue food coloring were individually mixed with water to generate two dyed solutions. The blue solution was fed through the upper inlet of the liquid gradient generator and the red solution was fed through the lower inlet. ImageJ was using to convert figure 3A into 8-bit grayscale. Gradient values were measured throughout different points on the cell culture platform for quantification. **A)** Mixing of red and blue water pumped through the liquid gradient after ten minutes **B)** Example grayscale quantification from the cell culture platform

Gas Gradient Quantification

Pyrogallol, an oxygen sensitive dye, was utilized to quantify the gas gradient. When exposed to oxygen, pyrogallol turns from a nearly colorless solution to a dark brown solution. Pyrogallol was prepared at 200 mg/mL in 1.0 N NaOH. The color of the pyrogallol was then determined at various oxygen concentrations. Three solutions of pyrogallol were prepared using a hypoxic chamber glove box with an oxygen concentration of 1.0%. The first vial was left in an environment of 1.0% oxygen, the second vial was exposed to 10.4% oxygen inside the hypoxic chamber, and the third vial was exposed to the atmosphere, 20.9% oxygen (Fig. 5A).

Images of the three vials were then taken and used to generate a calibration curve to model the relationship between oxygen concentration and pyrogallol color (Fig. 5B). In order to test the ability of the bioreactor to create an oxygen gradient, a testing apparatus was developed (Fig. 6A). First, identical copies of the gas gradient

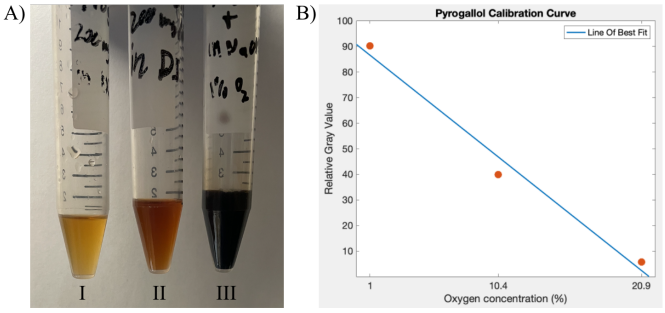

Fig. 5. Pyrogallol Calibration Curve: A pyrogallol solution was prepared in a 1.0% oxygen environment. Alloquates of the solution were then exposed to varying oxygen concentrations **A) I.** 1.0% oxygen, **II.** 10.4% oxygen, **III.** 20.9% oxygen. **B)** These three solutions were then analysed using ImageJ to produce a calibration curve correlating pyrogallol color to relative oxygen concentration

generator bottom and top were 3D printed. These pieces were secured together with biocompatible superglue and a sheet of PDMS was adhered on top. The 3D printed testing apparatus was then secured to the PDMS using glue. Under hypoxic conditions, each square of the grid was filled with 0.15 mL of pyrogallol solution. In order to produce oxygen, solutions of sodium hypochlorite, and 3.0% hydrogen peroxide were then pumped through the inlet port of the gas gradient generator (Equation 1).

Equation 1: $NaOCl + H_2O_2 \rightarrow NaCl + H_2O + O_2$

As the chemicals progressed through the reaction chamber, the reagents were consumed and the production of oxygen decreased, producing an oxygen gradient. A chemical flow rate of 0.95 mL/min was selected to allow the reaction to be completed approximately three-fourths of the way through the chemical reaction path. After 10 minutes, an image was taken of the pyrogallol solutions. After converting the image to grayscale using ImageJ, the oxygen content at each point in the grid was determined using the previously generated calibration curve (Figure 6B).

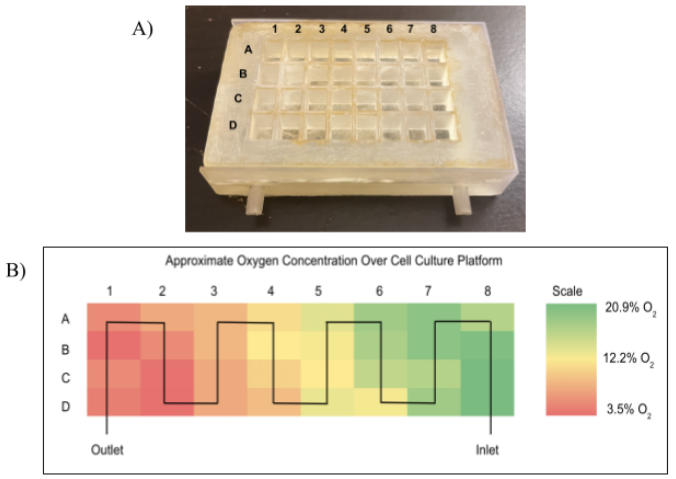

Fig. 6 Gas Gradient Validation: A grid was 3D printed and adhered to the top of the reaction chamber. A hypoxic chamber was used to provide a deoxygenated environment. Pyrogallol was placed in each well of the grid. The chemical reaction was run through the gas chamber and the grid was imaged. The images were analyzed using imageJ to determine oxygen levels. **A)** Gas gradient testing grid. **B)** Oxygen concentration grid over gas gradient chamber.

Initial Proof of Concept

Proof of concept testing was performed to ensure that cells were able to adhere and grow in the bioreactor. First the viability of PDMS as a cell culture platform was tested. Under a tissue culture hood, sections of PDMS were cut and placed in a 10 cm petri dish. Next, 15.0 mL of a 0.1 mg/ml solution of poly-L-lysine was added. Poly-L-lysine enhances electrostatic interactions between the cells and the PDMS, promoting cellular adhesion. The solution was allowed to coat the PDMS for two hours. The solution was then aspirated and the PDMS was washed three times using sterile water. After allowing the PDMS and petri dish to dry for an additional two hours, cells were introduced to the PDMS. Approximately 4.4E6 Human bone osteosarcoma epithelial cells (U2OS cells) were added to 12.0 mL of complete media. Complete media consists of DMEM, 10% fetal bovine serum (FBS), L-glutamine, and an antibiotic solution. The U2OS cells were then thoroughly suspended in the media and added to the petri dish. Cellular adhesion was allowed to occur for 24 hours prior to qualitative analysis of cellular adhesion.

The cells showed a primarily spread out morphology, as opposed to a more circular morphology. This spread, rectangular morphology is indicative of cellular adhesion in U2OS cells. Additionally, gentle rocking of the petri dish did not dislodge the cells from

the PDMS, further confirming cellular adhesion to the PDMS. During testing, cells were plated at a relatively high initial confluency of 50% to allow for the maximum cellular adhesion. Given the approximate 24 hour doubling time of U2OS cells, a confluency of nearly 100% was expected. The PDMS showed a final confluency of approximately 55%. This finding indicates that nearly half of the initial cells did not adhere to the PDMS or had difficulty with cellular replication. We believe a potential cause for the loss of cellular adhesion was due to decreased poly-L-lysine coverage, as a result of the relative hydrophobicity of the PDMS. This issue was compensated for in future trials by seeding cells at a higher density and increasing the poly-L-lysine coating time. Based on these trials we determined that PDMS treated with poly-L-Lysine was a suitable candidate for use in the bioreactor.

Next the ability of cells to remain adherent to the PDMS under the shear stress of fluid flow was tested. PDMS was similarly treated with poly-L-lysine solution in a petri dish. Coating of the PDMS was performed outside of the bioreactor to prevent poly-L-lysine coating of the inside of the bioreactor, limiting cellular attachment to just the PDMS. The bioreactor was sterilized using both 70% ethanol and UV light exposure for a duration of 30 minutes. Although gamma irradiation would be more ideal for sterilization, this method was sufficient, given the short duration of the trial. Following treatment of poly-L-lysine, the PDMS was glued inside of the bioreactor with biocompatible superglue. The inlet and outlet ports of the bioreactor were then closed off. Next, approximately 5E6 U2OS cells were added to 10 mL of media and introduced into the bioreactor. Cells were allowed to adhere to the PDMS for 24 hours in an incubator. Following cell adhesion, complete media was introduced at both inlet ports at a flow rate of 0.7 ml/min. This flow rate was selected in order to minimize reagents usage while still being fast enough to produce the respective gradients that will be required in future gradient trials. No liquid or gas gradients were used in this trial to isolate the effect of the shear stress on the cells. The bioreactor was then run for 24 hours. After 24 hours, the PDMS was carefully cut away from the bioreactor, placed on a glass slide, and analyzed under a microscope. Results showed cells were able to adhere and proliferate in this environment.

Full Bioreactor Testing

Final proof of concept testing was performed with both gradient systems implemented. A similar procedure to the previous tests were performed by treating the PDMS, adding the PDMS to the bioreactor, adding the cells, and allowing them to adhere for 24 hours. The liquid gradient was generated by administering complete media into the left fluid gradient port and phosphate buffered saline (PBS) into the right fluid gradient port. Both pumps were set to a flow rate of 0.7 mL/min. The gradient from complete media to PBS simulates the nutrient gradient present within a tumor. PBS was selected as the secondary fluid in order to maintain an isotonic solution throughout the bioreactor. Sodium hypochlorite (bleach) and 3.0% hydrogen peroxide were pumped through the oxygen gradient generator at a rate of 0.95 ml/min to produce the oxygen gradient. The bioreactor was allowed to run for 24 hours prior to analysis. The PDMS was carefully excised from the bioreactor and cut into sections. Each section of the grid was imaged and cellular density and phenotype were analyzed (Fig. 7). Results from these trials showed slightly increased cellular density in high oxygen and high nutrients environments. low oxygen and low nutrient environments had far reduced cellular density. Through this trial we were able to show that the presence of the oxygen and nutrient gradient had effects on overall cellular density.

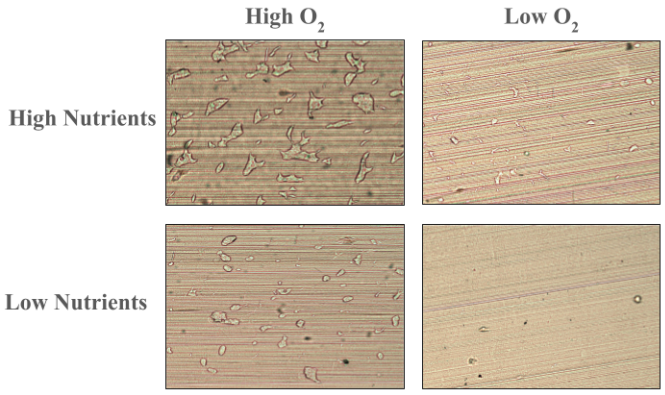

Fig. 7. Cell Culture Images: Cells were initially cultured and allowed to adhere within the bioreactor prior to gradient introduction. The gradients were then introduced for 24 hours before cellular imaging and analysis was performed. Different cellular morphology and density was seen based on the location within the bioreactor.

Discussion

The results of this project provide the basis for a novel 2D *in vitro* technology to model the tumor microenvironment. This bioreactor allows for simultaneous delivery of both liquid and gas gradients to

cultured cells, with flow rates that can be adjusted in real time. The liquid baffle system produced a linear nutrient gradient, and the chemical reaction within the gas chamber produced an oxygen gradient ranging from hypoxia to normoxia. Cancer cells were successfully cultured in the device, and the expected changes in cellular behavior were observed after the application of the gradients.

Limitations

Budget constraints and quantity of time were the two largest limiting factors. One major goal of this project was to keep the cost of the bioreactor lower than alternative technologies to make the device more easily accessible for any lab or classroom settings.

Stepper motor pumps were used instead of syringe pumps due to their cost. Syringe pumps can pump at much lower flow rates and have much higher levels of accuracy. A lab with access to syringe pumps could benefit from using the more advanced pumps to administer fluids to the liquid gradient system instead of the stepper motor pumps used for this project. The SLA printers used have a theoretical accuracy of 0.05mm.[19] Multiple printing attempts demonstrated that bioreactors smaller than the current design experienced detrimental warping and part merging. Access to micro-SLA printers would allow the bioreactor to be printed with much better accuracy, allowing for smaller designs. Clear resin was used to print the final design, but the resin accessible through the School of Architecture did not provide an optically clear body. This hindered the ability to image cells in real time. The PDMS which served as the cell culture platform had to be cut out of the bioreactor for cell imagining. This eliminated the possibility of allowing the culture to continue incubation after imagining. Optically clear resin is available, though it is more expensive, and could be used to remedy this problem as needed.[20] When printing the device a lab can pick their resin type based on the specific needs for any given experiment. An incubator capable of simulating a hypoxic environment would be necessary for the most accurate data collection and ease of use. Such an incubator was not accessible during the duration of this project. Since the bioreactor was not run inside a hypoxic environment, any section of the bioreactor not perfectly sealed could lead to extraneous introduction of oxygen, reducing the range of hypoxia in the cell culture.

Future testing could be performed to find the best type of PDMS to use for the cell culture platform. PDMS was used due to its biocompatible properties and its permeability of gas and not of liquid. Though PDMS is often used in cell culture models, it is slightly hydrophobic which results in issues with cell adhesion. Different types of PDMS have different properties which could affect cellular behavior in different microenvironments. Different types of tissue culture plate treatments could be tested to determine their effects on cell adhesion as well. Further testing needs to be performed in order to determine the best combination of PDMS and tissue culture plate treatment.

Future Work & Alternatives

This technology can be used to study a wide variety of cancers. Human bone osteosarcoma epithelial cells were used for the validation testing of the device due to their robust nature and short doubling time. However, researchers can use other cancer cell lines or any other adherent cell type. Multiple cell types can be easily cultured together to study cell-to-cell interactions while in these varied environments. This bioreactor can also be used for personalized medicine applications. Cells from a patient's tumor can be isolated and analyzed to determine the optimal therapies for that patient in particular. Depending on the cell type and the method of analysis, an alternate membrane and attachment factor may be desired. Tests can be performed to determine the most effective combination of membrane and attachment factor for the specific cell type. The design of the device allows for these changes to be made without disrupting the function of the device as a whole.

The bioreactor design allows for a variety of gas and liquid gradients to be delivered to the cells. Chemokines, cytokines, growth factors, and gasotransmitters are all important components of the tumor microenvironment and regulate a key number of biological functions.[21,22] Therefore, these solutes are important gradients to study in future experiments. In addition to the type of solutes, the flow rates of the solutes can be altered. These flow rates can additionally be adjusted in real-time. The number, size, and spacing of the baffles within the liquid gradient generator can also be modified to create various gradient types such as exponential or reflective gradient.

Cellular responses to the tumor microenvironment can be measured by fluorescently tagging proteins of interest and imaging the cell culture using fluorescent microscopy. For example, two proteins of interest are hypoxia inducible factors HIF-1α and HIF-2α. These factors are known to play a key role in tumor development.[23] HIF-1/2α are thought to induce gene expression that ultimately results in increased angiogenesis and metastasis.[23] However, their exact

signaling pathways and effects on cellular response are unknown[23]. Studying the expression of HIF-1/2α in the varied environment and their effect on cellular behavior could advance cancer treatments immensely.

This bioreactor can also be utilized to test the efficacy of anti-cancer therapeutics in varied microenvironments. Current 2D in vitro testing does not accurately capture the range of microenvironment, and thus cellular phenotypes, within an *in vivo* tumor. This bioreactor more accurately simulates the range in oxygen concentration and nutrients within a tumor, and thus may be more effective in testing therapies. Anti-cancer therapeutics can be administered to the entire cell culture platform after delivery of the gradients for a set time. Then, various metrics can be measured within the cell culture to evaluate efficacy of the therapeutic, such as through metabolic activity, protein expression, and tumor cell death.[24]

In addition to modeling alternate cancer types, this device can be used to model complex cellular microenvironments associated with other conditions. In ischemic stroke, the brain microenvironment becomes hypoxic and neuroinflammation occurs.[25] Modeling the stroke microenvironment with this device could help researchers learn more about cellular behavior and help develop therapeutics. Following myocardial infarction, the cardiac microenvironment becomes similarly hypoxic and inflamed.[26] This bioreactor could help provide insights into the tissue remodeling process that occurs post infarct. The easily tunable design of the bioreactor paired with the importance of the microenvironment in many disease states creates endless possibilities for alternate applications of this technology.

Materials

Bioreactor Fabrication

Autodesk Fusion 360 was used to generate a 3-dimensional rendering of the device. Formlab 2 Stereolithography (SLA) 3D printers were used to print the components. Clear V4 photopolymer resin was utilized for printing. A Formlabs wash station and Formlabs cure station were used to clean and harden the resin. Pieces were secured together using the Loctite 681925 superglue plastic bonding system. 0.01” thick Polydimethylsiloxane (PDMS) was utilized in the bioreactor and was acquired from Interstate Specialty Products.

Gradient Production

The liquid gradient was produced using phosphate buffered saline (PBS) and complete cell culture media. Complete media consists of Dulbecco's Modified Eagle's Media (Sigma Aldridge, D5030), a 1:10 dilution of fetal bovine serum Sigma Aldridge, F0804-500mL), a 1:100 dilution of L-glutamine (Thermo Fisher Scientific, 25030081), and an antibiotic solution. The gas gradient was produced using household bleach and 3.0% hydrogen peroxide. Pyrogallol (Sigma Aldrich, P0381) was used as an oxygen-sensitive dye. Pyrogallol was prepared at 200 mg/mL in 1.0 M NaOH.

Pump Circuit

Two identical pump systems were constructed. Each system consisted of two Kamoer KPAS-100 24 V stepper motors with peristaltic pump attachments, two A4988 stepper motor drivers, an Arduino Uno, two 100 μF capacitors, a 24 V external power supply for the motors, a 9 V power supply for the Arduino Uno, jumper wires, and a breadboard. Code developed through the Arduino’s computer software interface was used to operate the pump system and change the flow rate of each pump in real time.

Cell Culturing and Imaging

Polydimethylsiloxane (PDMS) with a 0.01” thickness was acquired from Interstate Specialty Products. PDMS was treated with Cells were cultured on PDMS treated with poly-L-lysine (ThermoFisher Scientific, A-005-M). A 10 cm Polystyrene tissue culture treated dish was used to hold the PDMS and poly-L-lysine during treatment. Tissue culture grade sterile water was used to wash the PDMS following poly-L-lysine addition. Human bone osteosarcoma epithelial cells (U2OS) cells were used for cell culturing in complete media. Cells were analyzed using an OM900-T 100X- 640X inverted Trinocular Biological microscope. Cell images were taken using a Leica DM6 microscope.

Methods

Bioreactor Assembly

Following SLA printing, each component was washed in isopropyl alcohol for 15 minutes and cured for an additional 15 minutes at 60˚C. Pieces were then sanded using a dremel to allow for a tight fit. The chemical reaction chamber top was secured to the chemical reaction chamber bottom using superglue. Care was taken to ensure there was no leakage of glue into the reaction channels and that the glue did not adversely affect the resin. A section of PDMS was then cut and secured on top of the chemical reaction chamber top.

The PDMS prevented spillage of the chemicals in the reaction chamber while allowing for the passage of gas. The bioreactor main body was then glued into the main chemical reaction chamber body, on top of the PDMS. PDMS treated with poly-L-lysine was then glued into the main bioreactor body over the cell culture area. Following plating of cells, the bioreactor top was placed on the top of the main body and secured with superglue around the entire piece to ensure a watertight seal.

Pump System Development

Two Arduino Unos were used to control the four pump system responsible for the administration of fluids to the bioreactor. An Aurdino Uno was selected as our microcontroller due to its versatility, simplicity, and low cost. Each Arduino Uno was used to control two motors. Six volt DC motors with peristaltic attachments were initially used due to ease of access and low cost. The DC motors could not pump at low enough flow rates necessary for a microfluidic device. Stepper motors were ultimately used in order to achieve the desired flow rates while maintaining a low overall cost for the device. H-bridge motor drivers and a 24 V external power supply were utilized to power and operate the stepper motors to ensure proper function of the motors. 100 µF capacitors were implemented to prevent back surges from the stepper motors, protecting the motor controllers and arduino.

Prototype Validation: Liquid Gradient

In order to verify the generation of the desired liquid gradient, water containing blue food coloring was pumped through one port of the fluid gradient and water containing red food coloring was pumped through the second port of the fluid gradient. Both pumps were set to the desired flow rate for cell culturing, 0.7 mL/min. This flow rate was chosen to minimize reagent usage and to produce the desired solute gradient. The fluid gradient was quantified using ImageJ. The CAD design was altered until the desired fluid gradient was seen during testing.

Prototype Validation: Gas Gradient

In order to verify the generation of the desired gas gradient, an additional 24 grid piece was designed using Autodesk Fusion 360 and similarly printed out of clear resin using an SLA printer (Figure 4B). The grid piece was glued into the chemical reaction chamber assembly that consisted of the bottom piece, top piece, and PDMS. The assembled piece was placed into a hypoxic chamber with an oxygen level of 1%. Pyrogallol solution was placed in each well of the verification grid. Sodium hypochlorite and hydrogen peroxide were both pumped into the reaction chamber. Trials were conducted for 10 minutes. Images of the pyrogallol were taken and ImageJ was used for quantification.

Prototype Validation: Cell Culture

Cell culturing was first tested on PDMS treated with poly-L-lysine solution in a petri dish. Sections of PDMS were cut and placed in a 10 cm petri dish. 15.0 mL of 0.01 g/mL poly-L-lysine solution was added and allowed to sit for 2 hours. The remaining poly-l-lysine solution was aspirated, the PDMS was washed three times using 15 mL of sterile water, and allowed to dry for an additional 2 hours. U2OS cells were then introduced in 12.0 mL of complete media. Adhesion was determined based on cellular morphology and by rocking the plate back and forth. Cells with a spread morphology and that remained in place after rocking were considered adherent. To test the bioreactor, PDMS receiving the same coating procedure was glued into the cell culture platform and allowed to cure for 12 hours. U2OS cells were then introduced and allowed to adhere for 24 hours. Media and PBS were used in the liquid gradient generator, and NaOCl and H_2O_2 were used in the gas gradient generator. The bioreactor was run for 24 hours in an incubator. Media and PBS bottlers were also placed in the incubator. Following the trial, the remaining media and PBS was aspirated and the PDMS was cut from the bioreactor, placed on a slide, and analyzed under a microscope.

End Matter

Author Contributions and Notes

E. Clark, E. Lunn, and E. Wood wrote the paper, designed and conducted research, created CAD models, fabricated the pump system, and performed both liquid and gas gradient validation testing. E. Clark cultured cells. T. Gennetta Ph.D. provided initial research and bioreactor designs from a previous grant proposal. T. Gennetta also provided necessary materials for bioreactor development and cell culturing.

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