OPTIMIZING A GAS AND LIQUID GRADIENT BIOREACTOR TO MIMIC TUMOR MICROENVIRONMENT

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By

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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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Abstract

A major barrier to cancer drug development is the inaccuracy of preclinical *in vitro* cancer models. *in vitro* preclinical research is a critical first step in evaluating potential effects of cancer therapeutics and understanding the mechanisms governing cancer. Current models are not adequate in simulating the dynamic tumor microenvironment or are too expensive and complicated to replicate and evaluate. There is a need to develop more accurate models of the tumor microenvironment that are more accessible to use and understand. The aim of this project was to design and validate a novel bioreactor capable of generating tunable and orthogonal gas and liquid concentration gradients to culture cancer cells. The bioreactor would rely on a baffle system to generate a liquid solute-specific concentration gradient and a network of parallel splitting channels to produce an oxygen concentration gradient. By layering both devices, adhered cancer cells would be cultured under a variety of liquid and oxygen concentrations to characterize the impact of hypoxia in the tumor microenvironment. Visualization of the effect of hypoxia would be through use of an inverted microscope setup to observe cell fluorescence of the adhered cancer cells on glass slide cell culture area. The device would be designed using CAD and printed with Stereolithography 3D printing. Individual verification of the gas and liquid components through benchtop testing demonstrated successful generation of respective linear concentration gradients. Full assembly of the device was only capable of generating a liquid gradient under a consistent low-oxygen environment. Regardless, the fabrication and initial validation of this device serves as a critical step for future design iterations of a novel bioreactor capable of simulating the dynamic tumor microenvironment and advancing the accuracy of preclinical *in vitro* tools for cancer drug development.

Keywords: Tumor microenvironment, microfluidic device, 3D printing

Introduction

Cancer is one of the leading causes of death globally. In the United States alone, 609,000 people are projected to die from cancer, along with 1.9 million new cancer cases projected to occur in 2023.[1] Although lifestyle changes can be made to reduce risk factors associated with cancer, it is impossible to prevent cancer entirely. This means that successful treatments and therapeutics are critical in lowering mortality rates. One major problem in cancer drug development is the poor success rate–3.4%–for novel therapeutics.[2] The ability to translate cancer research to clinical success is so low that clinical trials in oncology have the highest failure rate compared with other therapeutic areas.[3] Common problems with anti-cancer therapeutics that fail clinical trials include lack of efficacy and off-target toxicity.[4] The failure of anti-cancer therapeutics can be partially contributed to the inaccuracies of the *in vitro* studies with which the drugs are developed. Current *in vitro* tumor models are unable to capture all aspects of the tumor microenvironment (TME) and lack physiological relevance.[5] In addition to the faults of these in vitro studies, translation into clinical practice has been slow with an average of 4-8 years of research before clinical trials can even begin.[6] Quickening this transition could have enormous impacts as each year by which time

to drug approval is shortened has the potential of saving almost 80,000 lives worldwide.[7]

Traditional cell culturing or 2D cell culturing are widely used in *in vitro* preclinical research. The problem with 2D cell culture subjects cells to static conditions of fixed temperature, atmosphere, and media compositions. These conditions are unsuitable for simulating the progression of cancer cells *in vivo* as the dynamic TME and the tumor architecture can drastically change the availability of nutrients and gene expression of cancer cells.[8,9] In particular, the TME consists of multiple overlapping gas (NO, O2, etc.) and solute (growth factors, cytokines, nutrients, etc.) gradients.[9,10] The dynamic conditions of the TME also plays a key role in tumor metastasis as most cancer deaths are not caused by a primary tumor, but instead by a process called metastasis.[11]

Tumors develop vascular networks for interior and exterior cells. Exterior tumor cells will release high levels of proangiogenic growth factors leading to high vascularization and unique cell behavior which supports their rapid proliferation.[12] This rapid proliferation often leads to inefficient vascularization as nutrients and other molecules sourced from the blood vessels become restricted.[13] The result of this inefficient distribution leads to heterogeneity with spatially and behaviorally distinct cancer cells, as interior cancer cells will generally become quiescent and eventually necrotic.[12] Hypoxia, or low levels of oxygen, is a major driving force of this heterogeneity as low levels of oxygen stimulate the major hypoxia-inducible factor (HIF) pathway leading to downstream effects including increased blood vessel formation, increased aggressiveness of cancer cells, and development of treatment resistance.[14] Further understanding of the development of these mechanisms and differential behavior is critical for the development of effective cancer treatments. As such, there is a need to develop a laboratory tool that allows for the simulation of these oxygen and nutrient gradients.

Critical factors affected in the usage of 2D cell culturing for tumor cell models include loss of the natural structure of tumors and their tissues, the loss in cancer cell morphology following transition from tumor to culture, and subjection of tumor cells to unlimited access to culture medium, oxygen, signaling molecules, and other metabolites. These factors are critical for cancer cell development and will result in changed cell behavior and gene expression. Thus there has been a growing interest in transitioning to 3D cell culturing methods.

There are a wide variety of 3D cell culturing methods, each with their distinct objectives and properties they utilize to model cancer cell behavior.[15] 3D cell culturing can generally be categorized into four groups: scaffold-based, non-scaffold-based and spheroids, specialized culture platform, and hybrid of spheroids and scaffold-based. Scaffold-based models rely on the addition of natural or synthetic extracellular matrix (ECM) like material, such as hydrogels, to mimic the mechanical, physical, and structural environment of cancer cells.[16] By enclosing a cancer cell within these scaffolds, the cell's behavior can reflect that of its natural environment and development. Spheroid models mimic the aggregation of cancer cells within tumors. Within these clumps the cell-cell and cell-matrix interactions can be accurately simulated to observe tumor microenvironment changes and interactions of nutrient and chemical gradients. Specialized culture platforms can either be microfluidics or organ-on-chip systems which are modified containers of the cell culture that allow for controlled delivery of liquid and other chemicals. Cancer cells within these platforms are continuously washed by various perfusions, such as liquid gradients, allowing for parallelization of cancer culture and automation.[17] The advantages of 3D cell culture is its ability to simulate the natural structure of tissues and tumors, controlled access to nutrients and other metabolites, preserves the normal in vivo morphology and gene expression. The limitations of 3D cell culture are its complexity, high-maintenance costs, worse performance and poor reproducibility, lack of commercial tests, and difficulty in data interpretation. For each of the various cell culturing methods, cancer cell models have been developed to examine different aspects of cancer cell behavior, as seen in Table 1.

To improve *in vitro* characterization of cancer cells for cancer drug development, a dual gas liquid gradient bioreactor will be created. The device will be a specialized microfluidics platform that will be used for studying cancer cell behavior under various conditions, particularly hypoxia. The concept of incorporating gas gradients to microfluidic culture platforms is not new, in fact there exist multiple methods to accomplish this.[18] Furthermore there exists multiple different dual gas and liquid gradient microfluidic platforms, though each platform utilizes different means of generating a gas gradient either through chemical reaction or by attachment to an outside gas supply.[19–22] This device builds upon existing designs and in its ability to generate smooth liquid gradients and specific, step-wise, gas gradients.

Current *In Vitro* Tumor Models		**Uses**	**Limitations**
2D	Traditional 2D Cell models	Modeling cancer cell proliferation, drug screening, characteristics, and molecular mechanisms	• Snapshot of cellular environment • Deprives cancer cell morphology and proper cell interaction • Uncontrolled access to nutrients and metabolites • Does not mimic natural structure of tissue or tumors
3D	Transwell-based models	Modeling cancer cell migration, invasion, and transendothelial migration	• Low physiological relevance • Models only single-cell motility • Migration and invasion studies can produce conflicting data
	Spheroid-based models	Modeling tumor proliferation, migration, invasion, drug screening, angiogenesis, and immune cell response	• Difficulty in controlling size of spheroid • Low throughput and difficult for long-term culturing • Difficult to collect cells for analysis
	Hybrid models	Modeling tumor proliferation, tumor remodeling, migration, invasion, drug screening, angiogenesis, cell gradient, metastatic extravasation	• Lack 3D environment • Lacks tumor complexity • Lacks flow through vasculature Disadvantages vary depending on the hybrid model.
	Tumor-microvessel models	Models tumor cells and the tumor vasculature interactions. Can study tumor proliferation, migration, invasion, drug screening, paracrine signaling, intravasation and extravasation, and dormancy	• Limited vessel diameter ranges • Unpredictable flow

Table 1. Table of Current *In Vitro* Tumor Models. General usage and disadvantage of each cell model is listed. Each model can utilize different 3D or 2D cell culturing methods to generate the cancer cells necessary to examine cancer behavior.

Results

Design Development

In order to simulate these oxygen and nutrient gradients the following specific aims were established based on the device requirements and specifications from by our advisor, Thomas Genetta, Ph.D: tunable generation of gas gradients with O_2 concentrations ranging from 0% to 19.8%, generation of solute-specific liquid gradients using fluid flow that is tunable in real time, biocompatible for cell culture, sterilizable for reusability, and suitable in size for an incubator and inverted microscope setup. The initial design of the bioreactor was based on those developed by the 2022 Capstone team of Evan Clark, Emma Lunn, and Elizabeth Wood (Fig. 1AB). Problems they had encountered with their iterations along with previous publications of devices that could mimic the tumor microenvironment were also used to help inform design changes. Design iterations following these criteria were developed in Autodesk

Fusion 360. The fundamental of the device would be splitting the liquid gradient and gas gradient into two microfluidic components. These two components would be layered on top of each other such that an orthogonal gradient of liquid and gas would be delivered to the cell culture area, consisting of cancer cells adhered to a cut glass slide. Microscopic imaging could then be performed using an inverted microscope setup to visualize cell fluorescence across the glass slide.

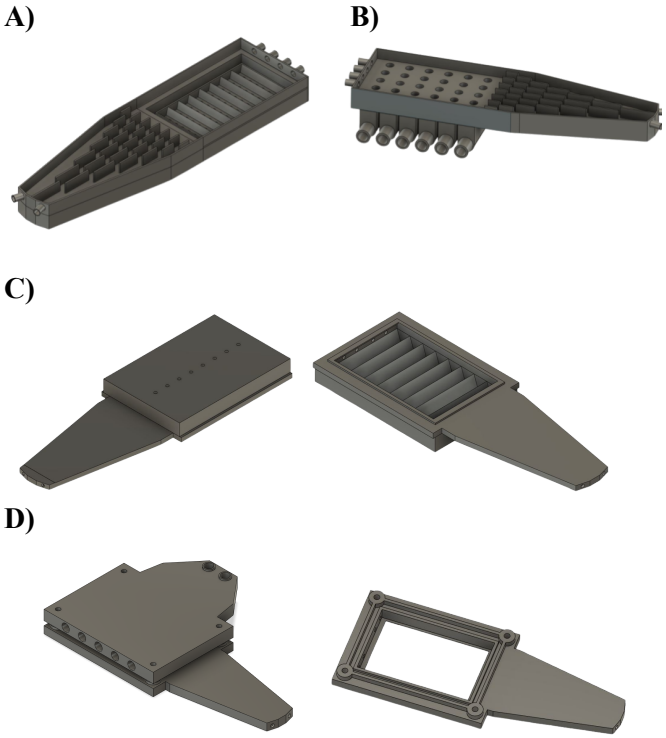


Fig. 1. Initial Bioreactor Designs. A) Main bioreactor body of the old design which was reliant on baffle and chemical components for gradient generation. **B)** Bioreactor design which relies on several mixtures of O_2 input for gradient generation. **C)** First iteration of bioreactor design which was reliant on several mixtures of O_2 input for gradient generation and designed for imaging under an inverted microscope setup. **D)** Second iteration of bioreactor design when fully assembled and its liquid baffle component

Three iterations following the design guidelines were developed with various changes between design iterations due to limitations in materials, difficulties in assembly, and leakages. The initial iteration was unusable as it relied on numerous tanks of O_2 mixtures which would be too expensive to implement (Fig. 1C). The second iteration was unusable due to difficulties encountered during full assembly of the device as the PDMS membrane between the liquid and gas components would not set evenly and created points of leakage (Fig. 1D). The final iteration resolved the assembly issues we had previously encountered with PDMS and was found to be fully sealed (Fig. 2A).

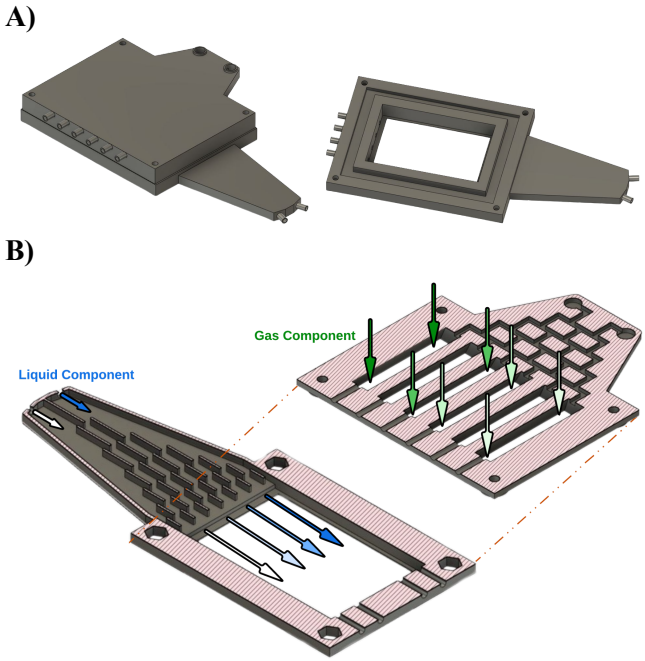


Fig. 2. Final Bioreactor CAD Design. A) Full assembly and liquid baffle component of final iteration. **B)** Functional diagram of gradient generation and creation of orthogonal liquid and gas gradient for cell culturing

The final design of the bioreactor relies on two microfluidic components. The liquid component was based on the works of Irimia et al[23] for creating stable chemical concentration gradients from two inputs. The liquid component utilizes a series of baffles, with the number of baffles beginning with one and increasing by one per level to a total of seven (Fig. 2B). At each level intersection, fluid from the previous level splits into two with mixing occurring across each baffle to ultimately generate a smooth linear concentration gradient. The gas component was based on the works of Szmelter et al[24] for delivering a linear range of oxygen concentrations to a 96-well plate. The gas component utilizes a network of splitting parallel channels that split and mix an input of

two gas concentrations into a linear gradient of concentrations ranging between the concentrations of the input to the downstream gas compartments (Fig. 2B).

Liquid Gradient Verification

To verify the generation of a linear concentration gradient of liquid, colorimetric analysis was performed. Red and blue food dye solutions were created using three drops of respective food dye into 200 mL of water. The red dye solution was fed through the left inlet and the blue dye solution was fed through the right inlet. Testing was performed by first filling the liquid component half full of water before switching to the food dye solutions. Each solution was pumped into the devices at the same flow rates ranging from 1 to 2 ml/min until a gradient was established across the cell culture area. After allowing for the pump system to run for 5 minutes, pictures of the cell culture stage were taken (Fig. 3).

ImageJ was then used to process the images of the cell culture stage. Each image was converted into an RGB stack, which converted each image into an associated gray-scale image depending on the red, green, or blue pixel intensities. The generated red and blue stacks were analyzed by drawing vertical lines across various points of the cell culture stage parallel to the direction of the linear liquid concentration gradient and gray scale quantification was performed. The gray scale values along the vertical lines drawn were then graphed to verify the generation of a linear concentration gradient (Fig. 3AB). Overall results from these trials indicate that a stable linear gradient over time was formed due to the baffle system. The stability of the gradient across the cell culture stage was not consistent as the linear regressions of Line A and Line B resulted in different lines of best fit (Fig. 3AB). Future modifications to flow rates to ensure stable gradient generation across the cell culture area will be required.

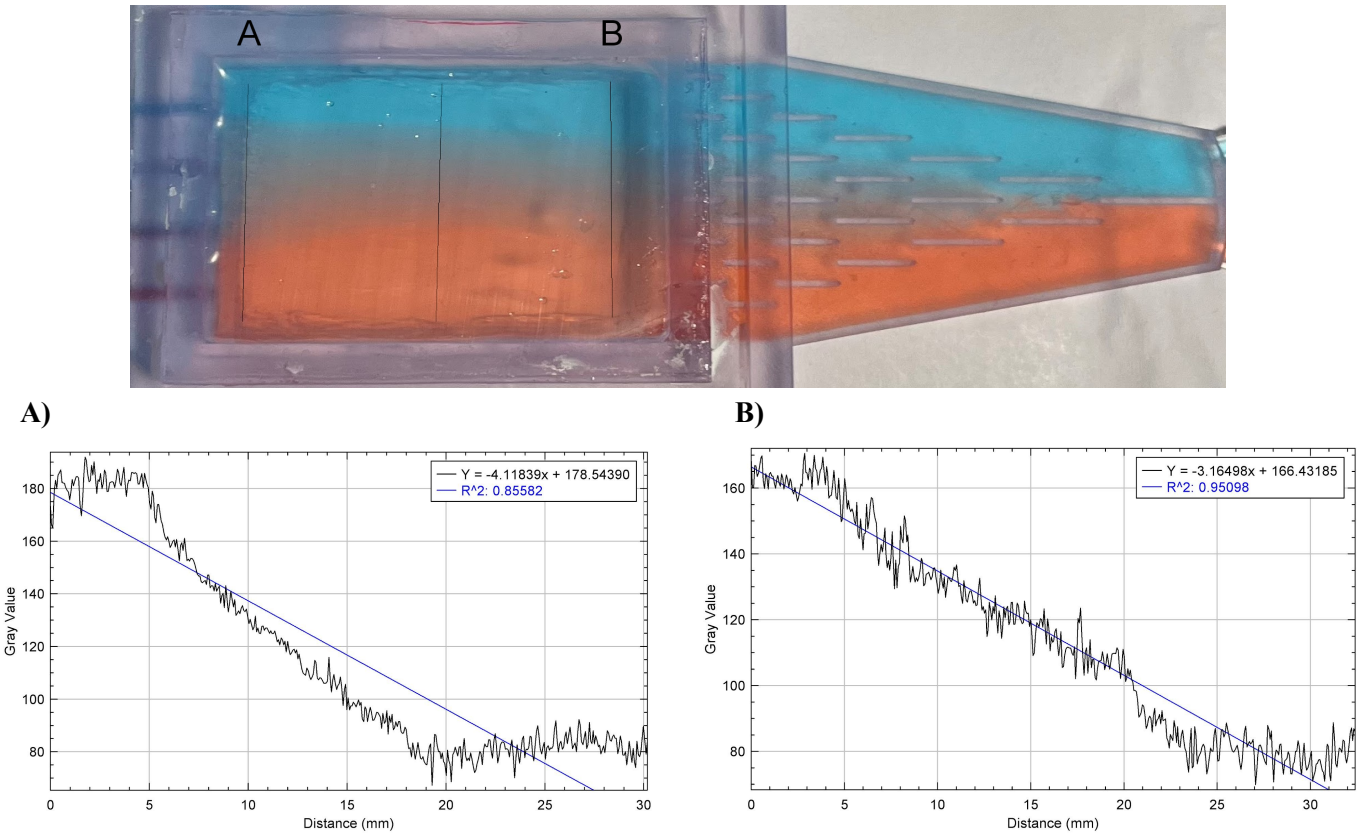
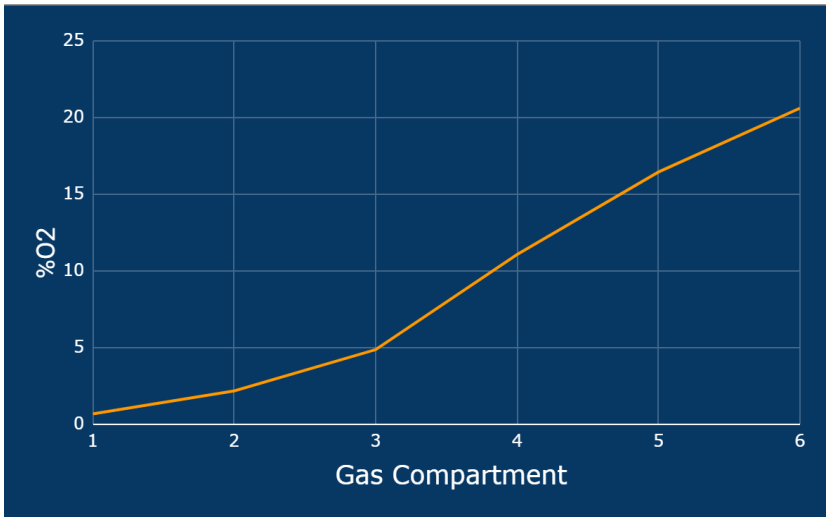


Fig. 3. Liquid Gradient Verification. Overview of the liquid gradient generated with red and blue dye solution. Linear gradient verification occurred at the above three lines. **A)** Gray scale quantification of Blue image stacks with linear regression of pixel intensity values across Line A. **B)** Gray scale quantification of Blue image stacks with linear regression of pixel intensity values across Line B.

Gas Gradient Verification

To verify the generation of a linear concentration gradient of O_2 from the gas component, optical oxygen dot sensor patches were attached to the walls of the gas component. Optical oxygen sensors utilize fluorescent quenching of luminophores to detect the amount of oxygen present. When exposed to a specific wavelength of fluorescent light, the luminophores become excited and emit specific wavelengths of fluorescence. Oxygen molecules will interact with the excited luminophores and decrease the emittance of specific wavelengths of fluorescence relative to the concentration of oxygen present. The ratio of emission intensities from the luminophores can therefore determine the concentration of oxygen present.[25]

Gas flow of both inputs were adjusted using stop valves and set such that relatively low flow rates and pressure were inputted into the component inlets. Sampling of oxygen concentrations at each downstream gas compartment was performed using the optical oxygen dot sensor probe to verify the generation of a linear concentration gradient of gas across the gas compartments from 0% to ~21% O_2 (Fig. 4). Overall the results of this test indicate that a stable linear gradient of O_2 gas ranging from 0% to 21% will be generated from the gas component.



Gas Compartment #	% O_2
1	0.68%
2	2.17%
3	4.88%
4	11.08%
5	16.46%
6	20.62%

Fig. 4. Gas Gradient Verification. Graph and values of the % O_2 concentrations sampled across the six gas compartments

Full Assembly Verification

To fully assemble the bioreactor, hex nuts were glued onto the liquid component allowing for liquid and gas components to be screwed together (Fig. 2A). An addition of a film of PDMS would also be sandwiched between the liquid and gas component to also test the full assembly. Verification that the full assembly of components would generate an orthogonal gradient of liquid and gas to the cell culture area would utilize the previous verification methods for liquid and gas components. Optical oxygen dot sensor patches would be attached to the cell culture area and red and blue dye solutions would be pumped through the liquid component. Following the successful verification of the orthogonal gradient, verification and validation would then be performed using cancer cell lines stably transformed with reporter cDNAs that would express fluorescent proteins at subthreshold levels of hypoxia. Microscopic imaging would then be performed to analyze the change in cell fluorescence across the cell culture area.

Unfortunately, verification testing of the bioreactor using the previous verification methods demonstrated that the generation of the orthogonal gradient was not successful (Fig. 5). The liquid concentration gradient was consistent with its individual verification testing. The oxygen concentration gradient was not consistent with its individual verification testing. The oxygen sensors on the cell culture stage found a consistent concentration of O_2 present on the cell culture stage ranging from 9.57% to 9.9%, suggesting potential gas mixing occurring across the different gas compartments. Further verification testing with and without the film of PDMS between the liquid and gas gradient demonstrated similar results albeit a much lower presence of oxygen ranging from 0.3% to 1%. Further design changes will be needed to resolve this issue. Regardless of these results, the current iteration of the bioreactor is capable of generating a low oxygen concentration environment suitable for cell culturing purposes.

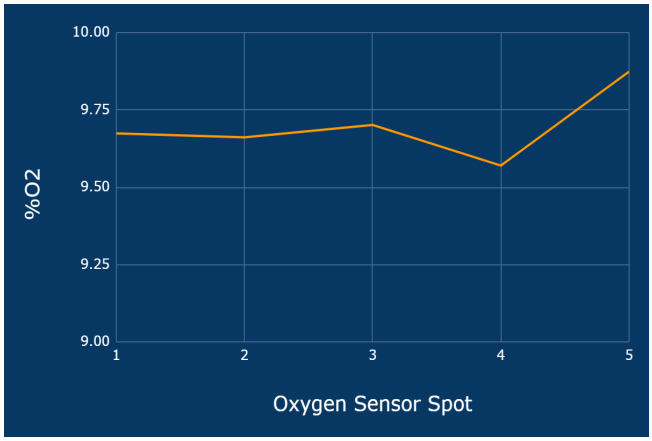


Fig. 5. Full Assembly Verification. Readings from the oxygen dot sensors placed on the cell culturing area.

Discussion

The results of this project provide a novel *in vitro* method for studying the effects of developing anti-cancer therapeutics on cancer cells. The simultaneous, orthogonal gas and liquid gradients act to model the tumor microenvironment and deliver a variety of conditions to cultured cells. The liquid baffle system generates a solute-specific linear nutrient gradient, while the gas parallel splitting channel network produces a linear oxygen gradient ranging from hypoxia to normoxia. Both gradients are tunable with flow rates that can be adjusted in real time.

Limitations

The largest limiting factor for this project was the time restraints. There were several obstacles and missteps that required a significant amount of troubleshooting. With many of these impediments happening during validation testing, little time was left for adjustments to be made to the final assembly of the bioreactor. Though we were able to solve problems that came up while testing the individual components, full assembly testing was constrained to one day, disallowing design alterations. With more time, we are confident that a solution for generating the gradients simultaneously would have been found.

In early stages of the design process, major delays were placed on beginning to print, as we realized that the entire existing concept would need to be flipped. With the goal of creating an easily accessible device that can be used in most lab or classroom settings, the bioreactor needed to be compatible with inverted microscopes. Live cell imaging is most commonly done with these specific microscopes, so we needed to change our upright design to somehow allow for a transparent cell culture platform at the base of the device. These changes not only took time, but also created unforeseen issues down the road.

The process of validating the liquid gradient took almost triple the expected time. Arduino Unos, though simplistic and affordable, tend to be unreliable and temperamental. Whether it be slight movements of jumper wires or switching around pumps, the liquid pumping system was overall inconsistent and prone to malfunction. A lot of maintenance was required to ensure the stepper motors were hooked up correctly and to make the pumps run as desired. Using more accurate pumps, such as syringe pumps, or an alternative circuit may save time and effort moving forward. Another problem we found with the liquid component was that due to small diameters and low placement, the fluid would not flow through the liquid outlets. This created

air bubbles that were nearly impossible to remove and was the ultimate reason that a third pump was added to the circuit to draw liquid out of the bioreactor. Adding the outlet pump came with a number of additional issues, as it created a vacuum inside the cell culture chamber.

In testing the gas component, a layer of PDMS was glued to the top of the liquid component so that each of the six gas compartments would be sealed and isolated. This allowed for the generation and maintenance of six unique oxygen concentrations. When the full assembly test was initially performed, the PDMS was incorporated to maintain the integrity of the oxygen gradient, and chosen due to its biocompatibility and permeability of gas and not liquid. Different types of PDMS have different properties, including oxygen diffusion rate. We found that the gas from the compartments was not diffusing through the PDMS. This may have been due to the thickness of the PDMS or the pressure of gas in the compartments. With no time to perform the necessary calculations to solve this diffusion problem, the PDMS was removed altogether. This, as seen by the full assembly test results, was not a proper solution either. Future tests could be performed to find the ideal PDMS type or another, better material with similar properties.

Future Work

After necessary design changes are made to allow for the successful combination of the solute and oxygen gradients, cell studies in the device can begin. Some tests will need to be done, first, to determine how well cancer cells can be cultured in the bioreactor. Depending on the intended use of the bioreactor, some considerations should be made regarding whether the glass slide can or should be placed in the device prior to adhering cells. Once there is a clear protocol for this process, the applications of the bioreactor are endless.

The first recommended utilization of our dual gradient bioreactor is to experiment with fluorescence. Inside the device, researchers can culture cancer cell lines, such as MDA-MB-231, MDA-MB-468, LnCAP, PC3, HT-29, HCT116, and A549, that have been stably transformed with reporter cDNAs to express fluorescent proteins at subthreshold levels of hypoxia. This will not only provide further verification of the oxygen gradient, but allow for live visualization of the conditions experienced by the cells. Cell characterization tests can also be performed by switching out what kind of solutes are administered to the cells. With standard media flowing into one inlet, scientists can pump glucose-free media, media with added growth factors, or even experimental anti-cancer drugs into the other inlet to induce a new environment. This device even allows for multiple cell types to be cultured together for cell-to-cell interaction observation.

The use of the bioreactor is not limited to cancer research, but can be expanded to model the complex microenvironments of other conditions and disease states. Ultimately, the ease of tunability in real-time, flexibility of use, and importance of the microenvironment in many disease states means for innumerable uses of this technology.

Materials

Bioreactor Fabrication

Three-dimensional renderings of the liquid and gas components of the device were created in Autodesk Fusion 360. Both components were printed with a Formlabs Form 3 Stereolithography (SLA) 3D printer. Printing was done using Formlabs Biomed Clear Resin. Prints were post-processed using a wash procedure with 99% (v/v) isopropanol (IPA). After air-drying the prints for 30 minutes, prints were then cured using Formlabs FormCure for 1 hour at 60 °C. Liquid components printed for cell culture prototypes were coated with parylene to prevent indirect cell exposure to resin toxicity and extend cell viability. Medium-strength, class eight, M3 x 0.5 mm thread steel hex nuts and M3 x 0.5 mm thread, 15 mm long zinc-plated alloy steel socket head screws from McMaster-Carr were used to combine the two components into the full bioreactor. Plastic quick-turn tube coupling sockets for 5/32” barbed tube inner diameter were used for attaching tubing to the gas inlets of the device. Early iterations utilized plastic barbed tube fitting connectors for 1/16” tube inner diameter (for air and water) at the liquid inlets and

outlets and the gas outlets. All tubing connectors were also ordered from McMaster-Carr. The glass slides used in the base of the device were cut from precleaned, 1.0 mm thick glass microscope slides. Some iterations of the bioreactor utilized 0.01" thick polydimethylsiloxane (PDMS) obtained from Interstate Specialty Products. McMaster-Carr hardware, glass slides, and PDMS were incorporated and fixed into the device using Dermabond Advanced Topical Skin Adhesive (Ethicon, Inc.), a medical-grade superglue. A waterproof and biocompatible silicone grease sealant was used on the bottom of the liquid component to ensure a water-tight base for the bioreactor.

Liquid Pumping System

A singular pump circuit was assembled to power three Kamoer KPAS-100 24V stepper motors with peristaltic pump attachments. The circuit consisted of a 24V external power supply for the motors, an Arduino Uno with a 9V power supply, a breadboard, three A4988 stepper motor drivers, three 100μF capacitors, and jumper wires. Arduino Integrated Development Environment (IDE) 2, Arduino's computer software, was utilized in the development of code for operation of the circuit and pumping system and allowed for real time change of flow rates. Fisherbrand manifold pump tubing with an inner diameter of 2.29 mm was used for the entirety of the system. Separate lines were connected with plastic barbed wye connectors for 3/32" tube inner diameter from McMaster-Carr as needed. Two media bottles with GL-45 thread caps were used to hold two different solutes. Each bottle had a specialized cap with three holes, one for pump tubing, one for a sparger, and one outlet hole. The bottle cap assembly was used to degas solutes prior to entry into the bioreactor.

Gas Flow System

Two gas tanks, each with pressure reduction valves, were used as inputs to the gas component. One gas tank contained a gas mixture of 95% air and 5% CO_2. The second tank contained a gas mixture of 95% N_2, 5% CO_2, and therefore 0% O_2. Multiple sizes of tubing were used to step down gas flow. Tubes with an inner diameter of 5/32" were connected directly to the device with plastic quick-turn tube coupling plugs.

Methods

Bioreactor Fabrication and Assembly

Following SLA printing, each component was washed in 99% IPA for 5 minutes, with a syringe used to flush the internal channels, followed by a 5 minute soak also in 99% IPA. After air-drying each component for 30 minutes, prints were cured for an hour at 60℃. Pieces were then sanded using sandpaper and a dremel to allow for a tight fit. Tube coupling sockets were cut with a heated scalpel, sanded to the desired length, and superglued into the gas inlets. Tube fitting connectors were super glued into the liquid inlets, outlets, and gas outlets for prototypes without printed connectors. Precleaned, 3 x 5" glass microscope slides were cut to fit the dimensions of the device with a waterjet and sanded for smooth insertion. For each prototype, the prepared glass slide was attached to the base of the liquid component with superglue before sealant was applied to the other side. For prototypes with a PDMS membrane separating the two components, a piece was cut and super glued on top of the liquid component. Finally, the gas component was screwed to the top of the liquid component.

Liquid Pumping System Development

The liquid pumping system was designed for use of three pumps, two for the administration of fluids to the bioreactor and one for pulling liquid out. Tubes 16" in length were attached to each of the four liquid outlets and connected with wye connectors until merged into one tube to connect to the pump. One 16" tube per liquid inlet was used to attach the to the appropriate solute pump An arduino Uno was used to control the system in a straight-forward and cost-effective way. The utilization of stepper motors was a low-cost way to attain slower flow rates. A 24V external power supply, H-bridge motor drivers, and 100μF capacitors were implemented to ensure proper operation and function of the stepper motors while protecting the system by preventing back surges. Each solute used in the device was placed in a media bottle with a degassing bottle cap. These bottles

were placed in a bead bath to maintain a physiological temperature of 37℃ for optimal cell viability. Gas from the tank containing 0% oxygen was pumped through the sparger into the solutes to remove oxygen and prevent contamination to the gas gradient.

Prototype Validation: Liquid Gradient

The successful generation of a solute-specific linear concentration gradient was verified with the employment of colorimetric analysis. Two solutions were made with 200 ml of DI water and 3 drops of food coloring each. A solution with blue food dye was pumped through one inlet and a solution with red food dye was pumped through the other. Both pumps were set to flow rates ranging from 1-2 ml/min. The fluid gradient was then quantified with ImageJ.

Prototype Validation: Gas Gradient

The successful generation of a linear concentration gradient from ~0 to 21% O_2 was verified utilizing Presens Precision Sensing technology. Oxygen Sensor Spot SP-PSt6-NAU sensors with a 5 mm diameter were placed into the gas component of the device. One sensor was secured into each of the six gas compartments. A layer of PDMS was super glued to the top of the liquid component prior to screwing the device together. The two gas tanks were then attached to the device and turned on with matching flow rates. After allowing the gas to fill the compartments, a polymer optical fiber (POF) probe was used to transfer excitation light to the sensors and the sensors responses back to an OXY-1 SMA oxygen meter (POF, PreSens GmbH, Regensburg, Germany). The meter, connected to a computer, provided oxygen percentage readings for the sensors in PreSens Measurement Studio 2, a compatible software. Five readings were taken from each sensor (compartment).

Prototype Validation: Full Assembly

A final verification test was performed for the full assembly in order to determine whether the two gradients could combine successfully. Five Oxygen Sensor Spot SP-PSt6-NAU sensors with a 2 mm diameter were secured onto the center of the glass slide in an evenly spaced, straight line. A layer of PDMS was then super glued to the top of the liquid component prior to screwing the device together. The two gas tanks were attached to the device and turned on with matching flow rates. DI water was used to fill both solute bottles and was degassed prior to entering the bioreactor. The POF probe was then used to collect oxygen percentage readings from the sensors across the slide. Every sensor read under 0.5% O_2 indicating a lack of gas diffusion through the PDMS. The device was disassembled and the PDMS layer was cut out before reassembling and repeating the test.

End Matter

Author Contributions and Notes

V. Jian and S. Pugh wrote the paper, designed and conducted research, fabricated the liquid pumping and gas flow systems, and performed validation testing. V. Jian created CAD models. T. Gennetta Ph.D. provided initial research and bioreactor designs. T. Gennetta also provided necessary materials for bioreactor development and cell culturing.

The authors declare no conflict of interest.

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