MICROFLUIDIC GAS DIFFUSION PLATFORM

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Abstract

Myocardial infarctions, more commonly known as heart attacks, kill almost one in four Americans. Heart attacks lead to ischemia in the heart cells. Ischemia is the lack of proper oxygen and nutrients to these cells and can cause cell death. Because heart cells are terminally differentiated, the heart is weakened in mass apoptotic events. Current research is looking at grafting mesenchymal stem cells to ischemic heart cells to cause fusion and induce the production of new heart cells. The problem is that this is not something that can be easily studied in vitro. Current methods are not applicable for testing this particular question, time-consuming, and expensive. A solution to this problem is the fabrication of a microfluidics platform that can create an oxygen gradient mimicking ischemia in vitro. In order to design this platform, two design components must be considered: microfluidic channel design and oxygen sensing techniques. In creating this design, three channel designs were explored: the parallel flow channel, two-channel, and oxygenator designs. After considering each design, the two-channel design was selected for its simplicity. Additionally, three oxygen-sensing techniques were considered: thin film fluorescent measurements, fluorescent bead measurement, and oxygen probe measurement. Ultimately, the thin film sensor was selected as the most viable option because it has the least risk of interfering with the cell culture system and is expected to provide reliable sensing results. Moving forward with these design selections, both components will be fabricated and tested individually. Upon successful testing on the individual scale, the two components will be integrated and testing in order to test the success of the system.

Introduction

The United States Centers for Disease Control and Prevention (CDC) reported that 616,000 people died from coronary heart disease in the United States. This estimate means that almost one in four American deaths is caused by heart disease (CDC, 2012). Heart disease causes the build-up of plaques in the coronary blood vessels which can lead to myocardial infarction, commonly known as a heart attack. In a heart attack, blood flow to the heart is blocked and causes nutrient and oxygen depletion in the heart tissue which can lead to cell death (National Heart Lung and Blood Institute, 2012). In severe cases of nutrient and oxygen deprivation, heart cells (cardiomyocytes) will normal die within three to four hours (Freeman, unpublished). This is of particular importance in the heart because heart cells are terminally differentiated meaning that they cannot repopulate themselves when they die (Freeman, unpublished).

Current research in the area however has hypothesized that it might be possible to induce cardiomyocytes to repopulate after a large apoptotic event by grafting mesenchymal stem cells (MSCs) from bone marrow onto the injured cardiac area and inducing cell fusion through ischemic conditions (Freeman, unpublished). This work could ultimately lead to a clinical method by which the heart can be repaired after a heart attack. Unfortunately, there is currently not a good method for modeling ischemic conditions in vitro meaning that many of the details of this hypothesis cannot be effectively studied (Freeman, unpublished).

The goal of this design project is to fabricate a microfluidics platform that is replicable and capable of creating ischemic heart conditions in a way that is quantifiable. **This will require two major design components: the design of the microfluidics platform that mimics ischemic conditions, and the design of an oxygen sensing method that is both accurate and robust.**

Current Methods

The current methods for testing questions related to ischemic conditions are less than desirable at best. The most commonly used method is controlling oxygen levels in a hypoxic chamber. The problem with this method is that hypoxic chambers are expensive to operate and slow to equilibrate to the desired oxygen concentration. This makes it very difficult to study a question such as the one proposed above because it is currently unknown what level of hypoxia causes cell stress to a large enough degree to cause fusion between cardiomyocytes and MSCs. Therefore, in order to test this hypothesis using a hypoxia chamber would require a large amount of time to find the correct hypoxic conditions where cell fusion would be most optimal.

Microfluidics however has shown great promise in this field of research thus far. By creating small microenvironments for cells, it is easy to regulate multiple variables on one simple chip thus coining the phrase "lab on a chip."

Microfluidics is a relatively new field with great promise. The process starts by using photolithograpy to create a master template of the microfluidics channel design. A layer of SU-8, a very viscous polymer, is spun onto a silicon wafer to a precise height and then cross-linked in certain areas to the wafer via a photomask and UV light. The photomask is designed to cover the whole silicon wafer, being dark in parts where cross-linking is not desired and clear in areas where cross-linking is desired (figure 1). After a brief exposure to UV light, the silicon wafer can be washed removing any SU-8 that was not cross-linked. After this the master template with slightly raised SU-8 features stuck to the silicon wafer (figure 2).

After creating the master mold, the microfluidics platform can be fabricated by pouring liquid poly(dimethylsiloxane) (PDMS) or another gel forming polymer onto the master mold. After

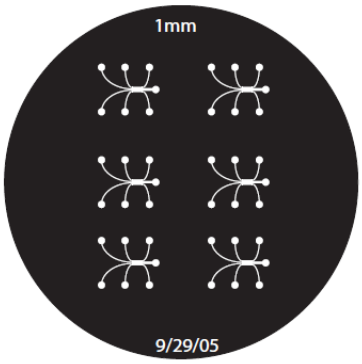

Figure 1: Example photomask design. Design is printed on clear slide. White areas are clear allowing UV light to pass through and cross-link SU-8 on master mold silicon wafer (image courtesy of

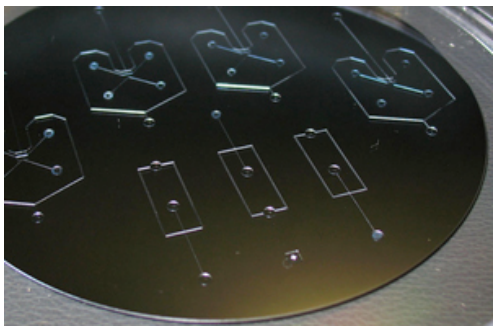

Figure 2: Example of silicon wafer after SU-8 has been cross-linked using photomask and excess SU-8 has been washed off. (image courtesy of www.blogs.rsc.org/chipsandtips/2008/04/22/integrated-reservoirs-for-pdms-microfluidic-chips/)

removing all oxygen and allowing the PDMS to solidify it can be removed from the master mold yielding a clear polymer with small wells where the raised SU8 features were on the master mold. The PDMS is then cross-linked onto a glass slide via exposure to a plasma torch. Once fused onto the glass slides, the channels formed in the PDMS are complete. In order to add liquid or gas to the channels, small holes can be punched through the top of the PDMS. This allows for the creation of small microenvironments (one microenvironment per channel) and the creation of multiple of these environments on one chip (figure 3).

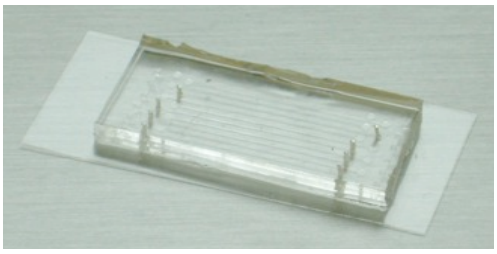

Figure 3: Example of PDMS polymer that has been cross-linked to a glass slide using a plasma torch (image courtesy of www.e27.ph.tum.de/index.php?id=26)

Microfluidics has however not yet been employed to study ischemia in the heart, but there have been numerous examples of microfluidics platforms being used to create varying oxygen concentrations on one chip (i.e. Li et. al 2011, Lo et. al 2010, and Lam et. al 2009). Based off of this previous research, the fabrication of a microfluidics platform to create an environment that mimics ischemic conditions in vitro is possible.

Problem Statement

Develop and validate a next-generation, microfluidic-based hypoxia chamber to facilitate studies involving oxidative stress, ischemia, and reactive oxygen species (ROS)-mediated cellular pathways.

Design Specifications

In order to create this in vitro microfluidic ischemia system, seven critical design specifications must be met in order to for the experiments to be conducted reproducibly and as close to in vivo conditions as possible. The first design specification is that the oxygen gradient produced by the platform should vary from 21% O2 (the concentration in ambient air) down to 1% oxygen gradient, much lower than the standard concentration under normal in vivo conditions. The next design criterion is that the conditions of the platform cannot interfere with cell culture. This means that all materials used in the construction of the platform must be biocompatible and non-cytotoxic. The third specification is that the channels should be made of poly(dimethylsiloxane) (PDMS). While there are other options available for construction of the channels, the use of PDMS in the design is critical for two reasons. The first being that it is oxygen permeable allowing gas to be passed into cell culture media at various oxygen levels thus establishing a gradient. The second is that it is the most commonly used material in cell-based microfluidic assays. This means that the body of literature surrounding PDMS is large (at least in comparison to other polymers) making its interactions with other components of the system more predictable. The fourth design specification is that the platform must be able to function inside of a cell culture incubator at 37^{o}C and 5% CO_2 concentration. This is the condition that normal cell culture occurs. By constraining this design to work under these conditions increases its desirability for use and study in other laboratories because the necessary equipment will likely be available. Finally, the channels in the platform need to be between 250μM to 500μM tall and 250μM to 750μM wide. The width is critical as the average cell is 10μM wide thus allowing for cells to easily enter the channel. The height is critical because the ratio of height to width in microfluidics is ideally a 1:1 ratio. By meeting these design criteria the platform design will be able to successfully mimic the in vivo system of ischemia to a level that is acceptable for this experiment and create a reproducible device that can be widely used.

Design Options

Platform Design

Parallel Flow

The parallel flow design, illustrated by the block diagram in figure 4, relies on regulated injection of oxygen and nitrogen into the system. Nitrogen is used as a complimentary gas, because it does not adversely affect the cells. Both gasses flow into the solenoid manifolds at a constant rate, with oxygen being further regulated by pulsating solenoids, switching between open and closed at a designated frequency. In order to accomplish this, four solenoids are required, along with a mass flow regulator in the oxygen input line. After passing through the solenoids, the gases enter channels within the PDMS platform, from which the gases diffuse into the cell cultures, represented by the darker grey circles in figure 4. Diffusion into the cell cultures establishes a gradient from high to low based on the frequency of the solenoid pulses, as well as oxygen diffusion through the PDMS, allowing for tightly controlled gradients.

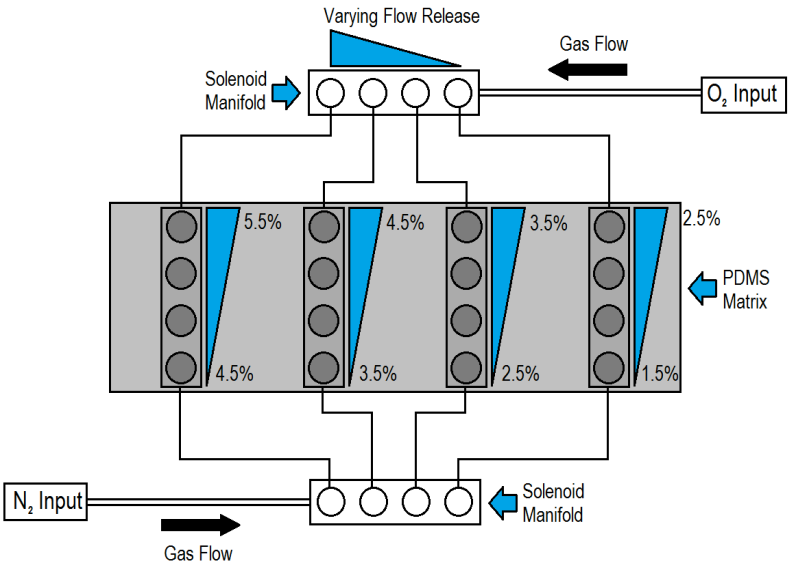

Figure 4: Block diagram of the Parallel Flow platform.

Two-channel

Relying similar principles to the Parallel Flow design is the Two-channel design. figure 5 is a depiction of the channel layout, including gas inputs and the anticipated gradient that will be formed. The Two-channel

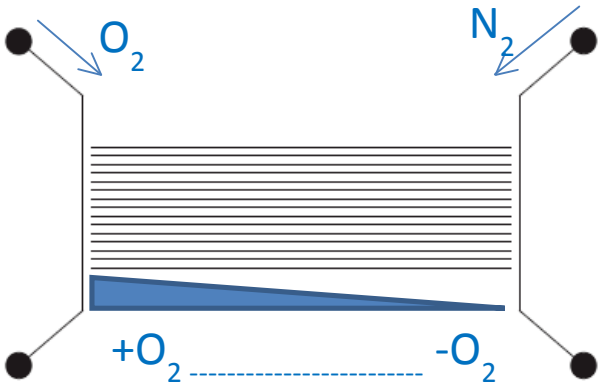

Figure 5: Two-channel platform design schematic.

design works fundamentally the same as the Parallel Flow design, but does so without the use of costly mechatronic components. The cells, which are housed in the longitudinal channels, receive the diffusing oxygen, with the highest concentration of oxygen being nearest to the injection channel. As the oxygen in the culture channels diffuses towards the opposite end, a gradient is formed, allowing for the observation of cell life at different levels of oxygen.

Oxygenator

The third design, named the oxygenator, is shown in figure 6. This design, like the other two, uses nitrogen as a complimentary gas to oxygen. Like the Two-channel design, the oxygenator relies on diffusion to establish a gradient, not mechatronic control.

As the gases travel through the channels, gas pressures are halved at each node, yielding a gradient that ranges from 0 – 100% oxygen upon exiting. This platform device is intended to be multi-layered, with the cells situated above C_{out} to allow for diffusion of the gases into the cell culture.

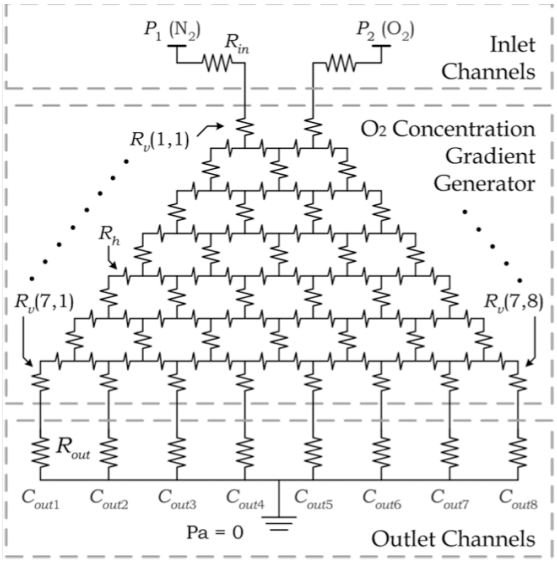

Figure 6: Oxygenator platform design.

Platform Design Matrix

Based on our design constraints, three different platform designs were proposed, and evaluated using the weighted factors shown in table 1.

Platform Design				
Factors	**Weight**	**Rating (1-10)**		
		Parallel Flow	**Two-channel**	**Oxygenator**
Ease of production	**0.25**	4	9	2
Span of gradient range	**0.20**	4	7	9
Cell-culture isolation	**0.15**	8	5	6
Gradient Control	**0.25**	8	4.5	2
Cost	**0.15**	1	6	7
TOTAL	**1**	**5.15**	**6.425**	**4.75**

Table 1: Platform design matrix.

The five factors that were considered with each design were, ease of production, span of gradient range, cell-culture isolation, gradient control, and cost.

Cost was weighted lowest at 0.15, because the materials (PDMS, SU-8, etc.) used to make the platforms are standard across all three designs, aside from the Parallel Flow design, which requires costly mechatronics to control gas flow. For this reason, the Parallel flow design received a 1 for cost, while the Two-channel and Oxygenator received comparable scores of 6 and 7. These two designs do not require solenoid manifolds, nor do they require as precise of flow regulators, lowering their overall price. Also weighing in at 0.15 was cell-culture isolation.

Cell culture isolation refers to the design's ability to isolate the cells not only from external influence, but also from each other. The Parallel flow design earned an 8 because it utilizes cell cultures plated in micro-wells, which gives users several, isolated cultures to base results off of. The Two-channel design places cells in long channels between the gas input channels, lacking definitive separation within the gradient. The Oxygenator, similar to the

Parallel Flow design, uses micro-wells to plate the cells, but only allows for one population of cells receiving gas output from each channel in the tree.

The feasible gradient range that each of these designs can produce was weighted at 0.20. This factor strictly refers to the difference between the highest and lowest concentration values produced in the gradient, which is required to encompass 1 – 21%. This is especially important in first trials to narrow down the concentration at which cells begin to die. The Oxygenator was rated highest, due to its ability to generate a full span gradient from 0 – 100%. Behind the Oxygenator was the Two-channel design, which doesn't produce the full-scale gradient, but still generates a gradient that is expected to include a concentration range spanning from 1 – 21%. Lastly, the Parallel flow design has the potential to create a tight, linear gradient, depending on the programming of the solenoids. What the Parallel Flow design lacks in gradient span, it makes up in gradient control, which was weighted at 0.25.

Gradient control is the ability of the device to maintain a tight, linear range between concentrations. Figure 4 of the Parallel Flow platform shows that a gradient of 1.5 – 5.5% can be formed, earning this design an 8 for gradient control, as it allows the user to really hone in on precise percentages of oxygen concentrations. The Two-channel design received a 4.5, because it relies purely on diffusion to establish a gradient, unlike the Parallel Flow, which can be further regulated by changing the frequency in solenoid pulses. The Oxygenator is able to generate the 0 – 100% gradient, but, according to its original designers, the injected gases have a tendency to flow to the outer most channels of the tree (figure 6), decreasing the gradient linearity. In order for all of these designs to function, precise microfluidic work is required to ensure all channels form properly.

Ease of construction was the final factor considered prior to deciding on a preliminary design, and was weighted at 0.25. As one could infer, the Oxygenator requires extreme precision to develop the tree of channels, which is why it was given a score of 2. This device is multi-layered adding another degree of difficulty to the process that isn't required by the Parallel Flow or Two-channel design. The Two-channel design was given a score of 9, because it is a single layer device, and does not demand as extreme precision due to its linear channel qualities. Finally, the Parallel flow device was given a 4, because it involves the setup of multiple mechatronic components including feedback between computer and microcontroller, as well as an intricate array of microwells and adjacent channels, all connecting to micro capillary tubes that interface between the solenoid manifold and PDMS platform.

Gas Detection

Gas Detection Methods

The second design component for the microfluidic gas diffusion platform involves implementing a way of validating the oxygen gradients created by the PDMS platform. Three methods have been proposed for measuring the oxygen concentrations present in the cell cultures. The first two methods involve detection through continuous epifluorescence microscopy (EFM). In these methods, a fluorescent molecule is excited with a specific wavelength of light to produce microscopic points of light in microscope's field of view. The molecule continues to emit energy in the form of photons until it is quenched by a bound oxygen molecule. Thus, by comparing to a standard intensity curve, the amount of fluorescence directly corresponds to the amount of oxygen within the system (figure 7). The third option presents an external sensor apparatus for taking point measurements of oxygen concentration at different locations on the platform.

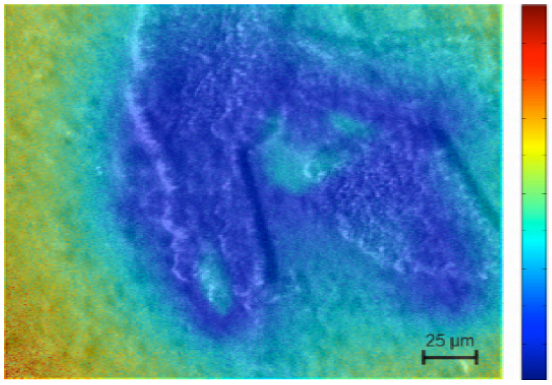

Figure 7: Fluorescent image overlay taken by EFM for detection of dissolved oxygen gas. (Ungerbock, et. Al 2010)

Thin-Sensor Film

The first fluorescence method involves creating a thin film composed of a fluorophore that will be quenched in the presence of oxygen (figure 8). The fluorophore would be dissolved in a semi-porous silicone matrix and spun out on a plate to achieve desired thickness. The silicone matrix is required to prevent the comparatively larger cardiac cells from coming into contact with the fluorophore while still being permeable to dissolved oxygen. Once fabricated, this film can be placed above or below the cell culture media depending on the final design. Selection of the appropriate fluorescence molecule for this application requires consideration of possible cytotoxic effects to the cell cultures as well as the wavelength of light required to excite fluorescence. Several fluorescent, ruthenium-based compounds have

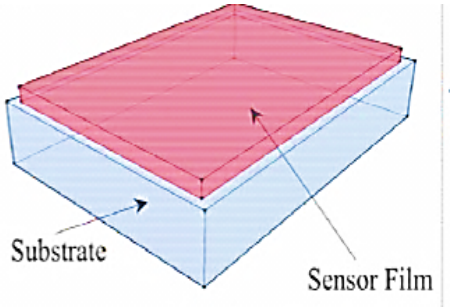

Figure 8: Thin film sensor composed of fluorophore embedded within a semi-porous silicone matrix. Oxygen diffuses through matrix to quench the fluorophore (Grist, et. Al 2010).

been applied to optical oxygen sensing. Compounds of reuthenium-tris-4,7-diphenyl-1,10-phenanthroline ($[Ru(dpp)_3]^{2+}$) and ruthenium(II)-tris(1,10-phenanthroline) ($[Ru(phen)_3]^{2+}$) are commonly-used examples, and they have been modified to be soluble in silicone films for oxygen sensing (Grist, et. Al 2010). Grist *et. Al*. investigated indicator leaching into various simulated fluid compartments and found that the leaching of $[Ru(dpp)_3]^{2+}$ from the polymer matrix into most aqueous solutions could not be detected (Grist, et. Al. 2010).

Polymer-Coated Nanoparticles

The desire to create a versatile sensor with both the advantages of indicator encapsulation and the possibility of intracellular measurements has led to research on the development of micro/nanoparticle sensors (figure 9) for microfluidic applications (Grist, et. Al. 2010). The principle of fluorescence quenching for oxygen quantification explained in the previous section is also applicable for these sensors. Sensor beads are created by doping a polymer such as polystyrene with the selected fluorophore in solution. The nanoparticles are suspended within the cell culture media and offer a very direct measure of the extracellular oxygen concentration. These particles can also be designed to incorporate chemical ligands that induce endocytosis of the sensor. This process would be used when wishing to study the metabolic activity within the cell. Thus, in high concentrations both inside and outside of a cell, these particles can become cytotoxic or interfere with the experimental procedure.

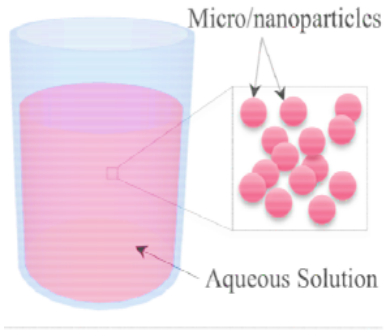

Figure 9: Representation of PDMS coated microparticles in solution (Grist, et. Al 2010).

M*icroelectronic Probe*

The use of a microelectrode array (figure 10) for measuring dissolved oxygen concentrations has been proposed by Lim et. Al (Lim, et. Al. 2009). Microelectrodes are among the most accurate and reliable monitoring devices for measuring the dynamics of biological processes, and have been widely used in the past. The system operates upon the principle of oxygen reduction potential (ORP) at the gold (Au) cathode of the electrode. The oxygen reduction produces a voltage relative to

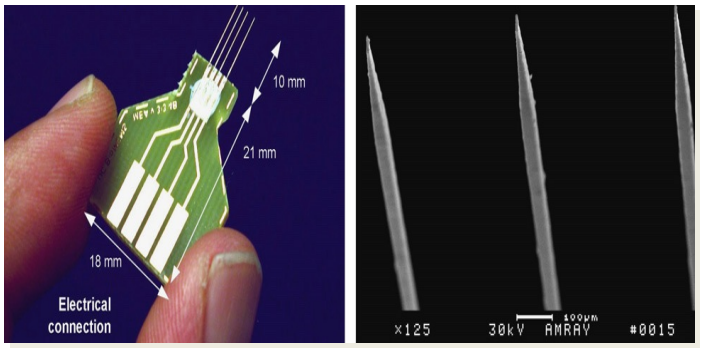

Figure 10: Dissolved oxygen microelectrode (Left) and dissolved oxygen sensing tips (Right) (Lim, et. Al 2009).

some reference electrode in the absence of oxygen. This voltage is amplified and oxygen concentration at the electrode is extrapolated using a standard calibration curve. However, because these probes rely the on reduction of oxygen at the electrode, they actually consume oxygen. Especially for a microfluidic device, this oxygen consumption could disturb the actual dissolved oxygen level in the system (Sin et. Al. 2004)

Gas Detection Design Matrix

Gas Detection Matrix				
Factors	**Weight**	**Rating (1-10)**		
		Thin Sensor Film	**Fluorescent Particles**	**O_2 Probe**
Accuracy	**0.30**	7	8	2
Cost	**0.15**	4	5	3
Ease of Use	**0.25**	7	4	7
Biocompatibility	**0.30**	8	6	8
TOTAL	**1.00**	**6.85**	**5.95**	**5.2**

Table 2: Design matrix comparing methods for detecting and measuring oxygen concentrations present within the PDMS microfluidic platform.

With assistance from the client, each of the aforementioned oxygen detection methods were compared numerically with each other to deduce which will best fulfill the design specifications listed on page 8. Cost and ease of use attributes are fairly self-explanatory with low-cost, easily fabricated methods scoring the highest. Accuracy and biocompatibility are of paramount importance and are weighted as such. Accuracy refers to each method's ability to correctly quantify the amount of dissolved oxygen within the system without altering its concentration within the media. Thus, the fluorescent nanoparticles are scored the highest for their novel capability of detecting oxygen from within the extracellular fluid. The microelectrode probe scored very low in this category because of its oxygen consuming properties. In the realm of biocompatibility, all of the designs scored fairly well. The fluorophores used in the oxygen quenching methods are non-cytoxic under normal conditions; however the detection nanoparticles can become cytoxic at high concentrations which is why it scored slightly lower than the thin-sensor film format. The O_2 probe scored comparably to the thin sensor because it is also bio-compatible under normal conditions.

Given the weights and scores determined by the team, the most effective way to quantify and validate the oxygen gradients produced by the gas diffusion platform is to incorporate a thin sensor film in conjunction with an epifluorescence microscope.

Preliminary Design

After analyzing our two design matricies, the two-channel design and the thin-film sensing method were picked. The two-channel device will be first created into mask as shown in figure 11 using Adobe Illustrator. This mask will then be used to create the silicone master for the device through UV-rays as previously explained. Once this master of SU-8 is created, the PDMS can then be poured onto the master allowing for different thinknesses depending on the amount poured. The mold of the master can be made and used as a final device such as in the 3-D solidworks rendering in figure 12**.**

A certain level of oxygen will be pumped through one of the channels while the other pumps nitrogen. This will then form a gradient in the middle chambers where the cells would then be placed in DMEM, though the addition of cells is not needed in this design. The higher gradient would occur in the chamber closest to the input of oxygen and decreases both across the chamber, as well as, down the oxygen channel. This is due to PDMS being oxygen permeable and the diffusion rate at different areas across the channel.

To be able to measure the oxygen gradients formed by the device, the thin film detection method was choosen (figure 8). This sensing method will help maintain biocompatabilty because it will be placed on the surface of the two-channel design therefore not interfereing with the cells. This will still be able to give accurate oxygen readings based on the fluoresence of the image corresponding to the different oxygen concentrations. A epifluorescent microscope provided by the client will be used to watch these transitions.

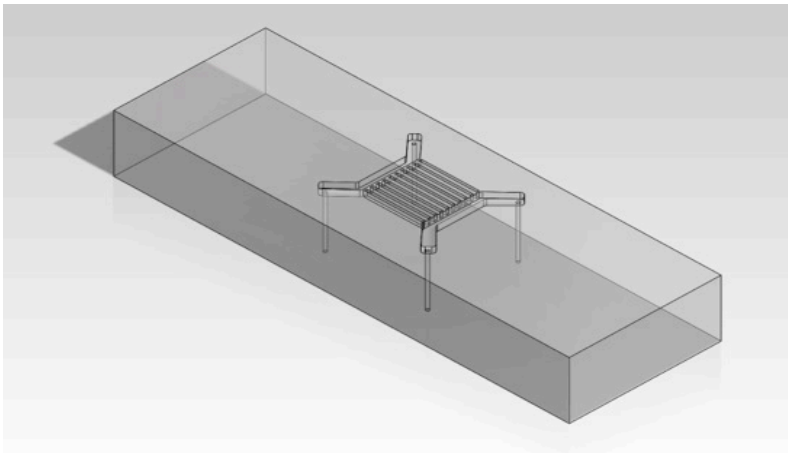

Figure 12: A 3-D Solidworks rendering of the 2-Channel design

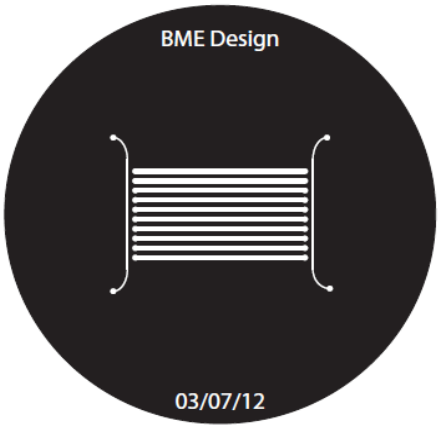

Figure 11: The mask used on the silicone wafer, which allows UV-rays where the channels will be.

Future Work

Moving forward in the design process requires the members of this group, first obtain chemical safety certification in order to work in the client's lab. Once this process is fulfilled work can begin to manufacture our intial design, the two chamber, following the steps outlined in Current Methods. The thin-film sensor will be calibrated by placing a small sample of the fluorophore-polymer matrix into a full-sized hypoxia chamber at a known concentration of oxygen. After performing this test an appropriate number of times, a standard curve for concentration of oxygen vs fluorescent intensity will be produced. Only once the oxygen detector has been calibrated will the PDMS platform be mated with the detector.

After the platform has been attached to the detector, each of the microwells in the PDMS will be cultured with cardiomyocytes. Oxygen and Nitrogen flow through the device will be established and fluorescent imaging will be used to calculate the present levels of oxygen within the device.

Contingent upon the success or failure of the device at this stage, the team will alter the channel design or the detection method such that it meets the needs of the client as well as the aforementioned design specifications.

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Appendix A: Product Design Specifications

Function: The purpose of this device is to create a micro-environment in which cells (heart and stem cells) can be cultured and exposed to varying oxygen gradients, and the reactions of the cells to the various oxygen concentrations can be measured.

Client Requirements:

1. Device will be a microfluidics platform
2. Device should be made using a master device out of SU-8 silicone so multiple devices can be constructed from the master template.
3. Device should be made using poly(dimethylsiloxane) (PDMS) as it is oxygen permeable and widely used as a microfluidics platform material
4. Oxygen concentration in platform should vary from 21% O_2 (ambient concentration at room temperature to 1% O_2.
5. All components should be biocompatible with cell culture and non-cytotoxic to cells
6. The design should be as simple as possible.
7. Price range of $500-$1000 for the entire process.

Design Requirements:

1. **Physical and Operational Characteristics**
    a. *Performance Requirements*: The master device template must be reusable for the creation of many devices, each device itself will be used only once for one experiment and contain no bubbles.
    b. *Safety*: Production protocols will be followed including all safety requirements. The device itself should be made of materials that are safe for handling under basic laboratory safety procedures. All team members involved in device production will receive chemical safety training.

c. *Accuracy and Reliability*: The master device needs to reliably allow the creation of each new device to the exact same specifications. This will allow for replicate data to be collected and tested.

d. *Life in Service*: The life in service of each device will be one experiment lasting no more than two weeks but likely on the scale of three to four hours, the amount of time required for hypoxic conditions to be evident in cardiomyocytes. The master device should last indefinitely until the end of the experiment (up to a few years).

e. *Shelf Life*: Each device should last after production until it is used. Once in use the device must maintain integrity throughout the entire experiment and not degrade in the presence of standard cell culture media or cells. The master device should last indefinitely.

f. *Operating Environment:* Each device will be maintained in a 37^{o}C, 5% CO_2 incubator and will have standard cell culture media (DMEM) inside the device.

g. *Ergonomic*s: The most important aspect of ergonomics is in creating each device from the master template. Each replicate from the master device should be identical to allow it to be used in laboratory experiments.

h. *Size*: Each device should be able to be placed on a standard glass slide. The chamber in the device itself should be 250-500μM tall and 250μM-750 μM wide to facilitate cell attachment.

i. *Weight*: The overall weight should not exceed a few grams, but a specific weight of the device is not critical.

j. *Materials*: The master device will be made out of a solid silicone plate with SU-8 cross-linked to its surface. Each device will be made out of PDMS which will be formed to the master device. The PDMS will then be cross-linked to a glass slide.

k. *Aesthetics, Appearance, and Finish*: Each device should be made cleanly, but ergonomics is not especially important for this design.

2. Production Characteristics:

a. *Quantity*: One master template, up to 100 devices created from the master template.

b. *Target Product Cost*: $500-$1000.

3. Miscellaneous:

a. *Standards and Specification*: Each device created from the master template must be identical to all previously made devices.

b. *Customer*: Dr. Brenda Ogle and Brian Freeman.

c. *Competition*: Similar designs using oxygen control have been created by the Eddington Lab at the University of Illinois at Chicago, however there is no analogous competition for our specific problem.