MICROFLUIDIC GAS DIFFUSION PLATFORM

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EXECUTIVE SUMMARY

Myocardial infarctions, more commonly known as heart attacks, are responsible for one in four American deaths each year[1]. Ischemia, which is the lack of sufficient oxygen delivery leads to cardiac cell apoptosis. Heart cells are terminally differentiated, so in the event of a mass apoptotic event, the heart is severely weakened. Current research investigates grafting of mesenchymal stem cells to ischemic heart cells to cause fusion and induce the production of new heart cells. The end goal of this project is to develop a next-generation, microfluidic based hypoxia chamber to facilitate studies involving oxidative stress, ischemia, and reactive oxygen species (ROS)-mediated cellular pathways. In order to design this platform, two design components must be considered: microfluidic channel design, and oxygen sensing techniques. The channel design of the microfluidic system was created to generate a spatial oxygen gradient, so that with accurate oxygen sensing techniques, the concentration at which cells begin to exhibit a response to the hypoxic effects can be quantified. Commercial hypoxia chambers exist, but do not yet extend into the micro scale. They are inefficient, in that their equilibration duration is long, and definitive spatial gradients are difficult to establish. Marrying fine gas control with microfluidic channel design may be the answer to eliminating setbacks of macro-scale hypoxia chambers.

The current prototype utilizes a channel design, consisting of eight longitudinal channels, situated orthogonal to two oxygen input lines at one end of the channels. Oxygen gas passes through these input lines, and diffuses down the longitudinal channels from each end, establishing an oxygen gradient between each longitudinal channel. In order to fully quantify this gradient, a fiber optic oxygen probe coated with a fluorescent molecule, ruthenium-tris-4, 7-diphenyl-1,10-phenanthroline $[Ru(dpp)_3]^{2+}$ will be necessary. When the ruthenium fluorophore compound is quenched by oxygen, it does not fluoresce. The resulting fluorescence at the probe's active region is quantified to a percent concentration of oxygen in the cell environment after comparison with a standard curve.

Device validation is twofold employing finite element analysis as well as experimental chemical detection. A simulation of spatial oxygen concentration was created in COMSOL Multiphysics®, taking into account the exact dimensions of our channel design, and flow rates of the input gas. An experimental validation method has been developed which involves the chemical indicator methylene blue $[C_{16}H_{18}N_3SCl]$, and a catalyst titanium dioxide $[TiO_2]$. When methylene blue reacts with oxygen, it changes to its characteristic blue color, and as oxygen levels decrease, the solution becomes clear. The resulting gradient will be compared with a standard curve to quantify the oxygen concentrations. If successful, the heat map generated by COMSOL® should match the experimental gradient found with methylene blue.

Successful completion of this design will allow accurate modeling of in vitro cell behavior at various oxygen concentrations, specifically the threshold at which cells begin to die due to their hypoxic environment. This approach is not only limited to oxygen diffusion, it opens up new possibilities for studying cellular response to a gradient of a variable substance. Other biological applications include, pharmacological drug studies, determination of ideal conditions for stem cell fusion, and in vitro simulation of various physiological organ system

[1] Centers of Disease Control and Prevention. 2012. Heart Disease Facts and Statistics. http://www.cdc.gov/heartdisease/statistics.htm.

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Abstract

Myocardial infarctions, more commonly known as heart attacks, are responsible for one in four American deaths. Ischemia, which is the term for lack of sufficient oxygen supply to cells, leads to cardiac cell apoptosis in the heart. Heart cells are terminally differentiated, so in the event of a mass apoptotic event, the heart is severely weakened. Current cardiac research is focused on grafting mesenchymal stem cells (MSC) to ischemic heart cells to cause fusion and induce the production of new heart cells. It is hypothesized that MSCs are more likely to fuse with cardiac cells under hypoxic conditions, which is an environment in which cells are under stress due to an oxygen deficiency. The goal of this project is to develop a microfluidic-based hypoxia chamber to facilitate studies involving oxidative stress, ischemia, and reactive oxygen species (ROS) in mediated cellular pathways. In designing the device platform, two main components were considered: microfluidic channel design and oxygen sensing techniques. The combination of fine gas control and oxygen sensing techniques with a microfluidic system will simulate an in vivo environment more closely than previous methods.

I. Introduction

The United States Centers for Disease Control and Prevention (CDC) reported that in 2011, 616,000 people died from coronary heart disease in the United States alone. This estimate means that almost one in four American will likely die due to 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 the event of a heart attack, blood flow to the heart is blocked and causes nutrient and oxygen deficiency in the cardiac 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 normally die within three to four hours (Chao, 2002). This is of particular importance in the heart because heart cells are terminally differentiated meaning that they cannot repopulate themselves when they die (Chao, 2002).

Current hypotheses contend 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 by exposing them to hypoxic conditions (Xie, et. Al, 2006). 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.

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.

II. Current Methods

The current methods for testing questions related to conditions of hypoxia are inefficient, and poor models of an in vivo environment. The most commonly used method involves controlling oxygen levels in a macro-scale hypoxia chamber. Commercial hypoxia 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, testing this hypothesis using a commercial hypoxia chamber would require a large amount of time to find the ideal hypoxic conditions at which cell fusion occurs.

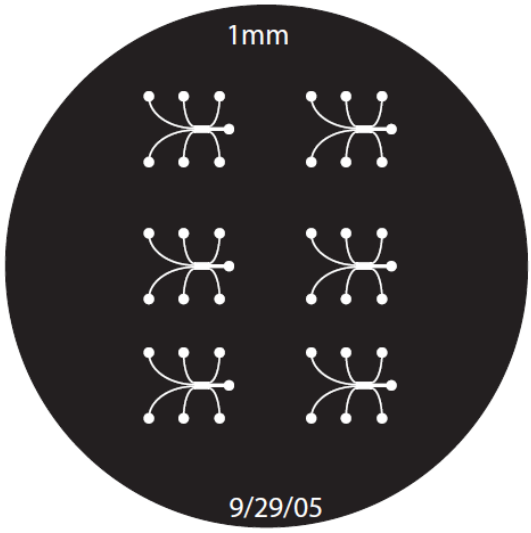


Figure 1: Example photomask design. Design is printed on a transparancy. White areas are clear allowing UV light to pass. (Image courtesy of Professor John Puccinelli, University of Wisconsin-Madison)

Microfluidics, however, has shown to be extremely applicable in this field of research. By creating microenvironments for cells, it is possible to regulate multiple variables per chip thus coining the phrase "lab on a chip."

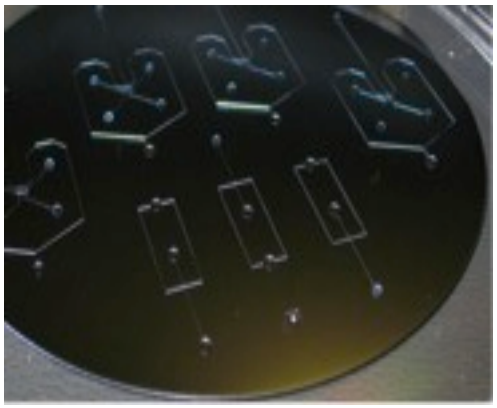


Figure 2: Silicon wafer with SU-8 channel features (Image retrieved from: www.blogs.rsc.org/chipsandtips/2008/04/22/integrated-reservoirs-for-pdms-microfluidic-chips/)

Microfluidics is a relatively new field, with newfound biological applications. Photolithography is used to create a master template of the microfluidic channel design. A viscous polymer, SU-8, is spun onto a silicon wafer to a precise thickness, and is then cross-linked in desired regions when exposed to UV light. The photomask covers the entire silicon wafer, allowing UV light to penetrate and catalyze cross-linking of the intended features **(figure 1)**. After being exposed to UV light, the silicon wafer is washed in a developing solution to remove any SU-8 that was not cross-linked, leaving behind only the intended feature design **(figure 2)**.

Following fabrication of the master mold, the microfluidics platform is made by pouring liquid poly(dimethylsiloxane) (PDMS) or another gel forming polymer onto the master mold. After removing all gases from the mixture using a vacuum, the PDMS block is baked causing it to solidify. The PDMS is then adhered to a glass slide following treatment with a plasma wand. In order to add liquid or gas to the channels, small holes are 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)**.

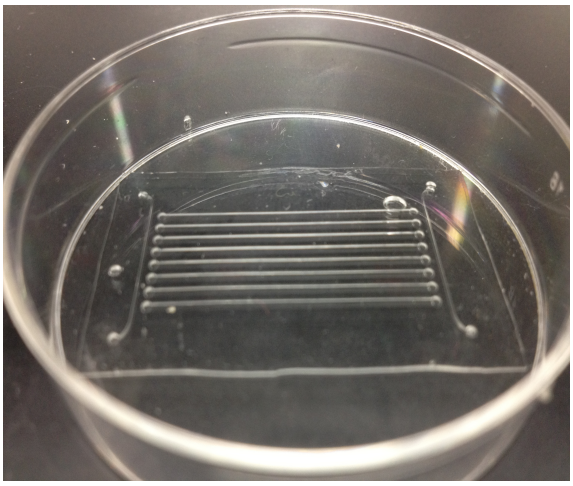


Figure 3: PDMS polymer adhered to glass slide.

Microfluidics has however not yet been applied to study ischemia in the heart, but numerous studies involving microfluidic platforms have conducted to generate oxygen gradients in one system (i.e. Li et. al 2011, Lo et. al 2010, and Lam et. al 2009).

III. 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.

IV. Design Specifications

Several critical design specifications must be met in order for the device to be used in experimentation. First, the channel design should be capable of producing an oxygen gradient ranging from 1% to 21% oxygen (concentration in ambient air). The materials used to construct the platform cannot interfere with cell culture; therefore, all materials used must be biocompatible and non-cytotoxic. While there are multiple options for materials to be used for construction of the channels, the use of PDMS in the design is critical for two reasons. PDMS is gas permeable, which allows for diffusion of oxygen into the cell culture media at various concentrations, thus establishing a gradient. It is also the most commonly used material in cell-based microfluidic assays, allowing for comparison with other studies. Additionally, the platform must be functional inside of a cell culture incubator at 37° C and 5% CO_2 concentration – the standard conditions of cell incubators. Constraining this design to work under these stipulations increases its desirability for use and study in other laboratories, as the materials are common, inexpensive, and widely used. Lastly, the channels in the platform need to be between 250µM to 750µM by 250µM - 500µM (W x H). The width is critical as the average cell is 10µM wide, thus allowing for cells to easily enter the channel. Distortions in the channel geometry can lead to variance in resistance to gas flow, making it difficult to predict the formation of an oxygen gradient. Meeting these design criteria will be the first steps in successful, and reproducible simulation of in vivo ischemia.

V. Preliminary Design Options

Preliminary design options for both of the critical aspects of the project: platform design and oxygen detection were considered. Three designs for each were created and evaluated using a design matrix.

Platform Design

As previously mentioned, one of the two critical aspects to this design is the PDMS platform design. In order to determine the optimal design conditions, three platform designs: parallel flow model, two-channel model, and oxygenator model were designed and considered for the final 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

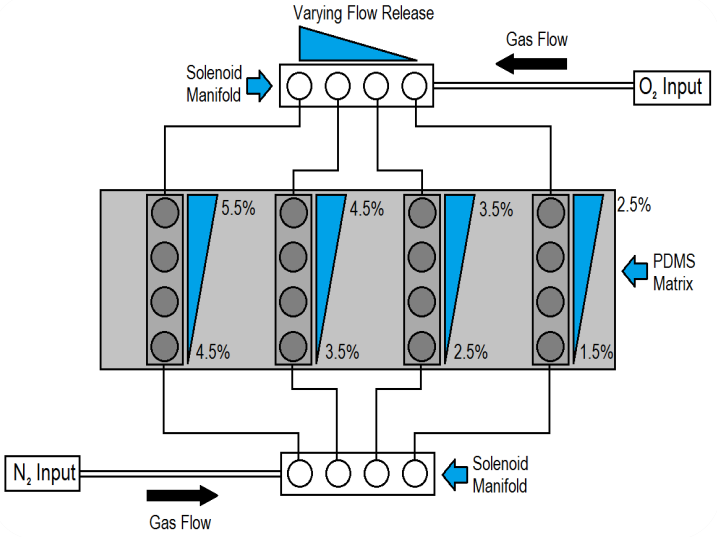


Figure 4: Block diagram of the Parallel Flow platform.

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.

Two-channel

Relying on 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 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.

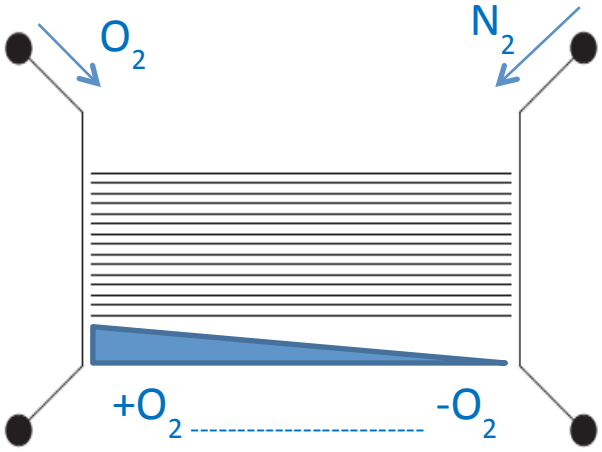


Figure 5: Two-channel platform design schematic.

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.

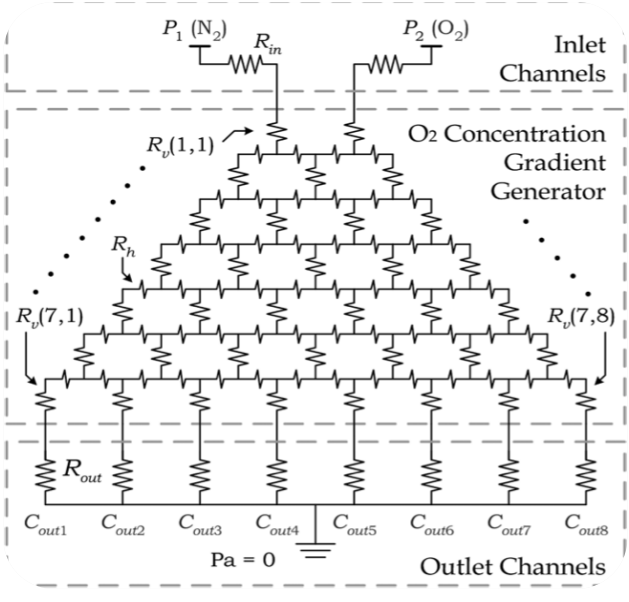


Figure 6: Oxygenator platform design.

Platform Design Matrix

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

Table 1: Platform design matrix.

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**

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 one for cost, while the two-channel and oxygenator received comparable scores of six and seven. 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 eight 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 eight 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 two. 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 nine, 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 four, 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

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.

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 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).

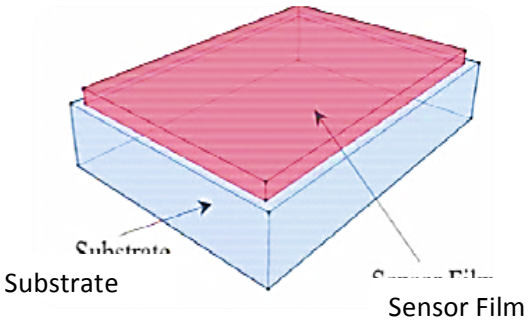


Figure 8: Thin film sensor composed of fluorophore embedded within a semi-porous silicone matrix. Oxygen diffuses through matrix to quench the fluorophore (Grist, et. Al 2010).

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.

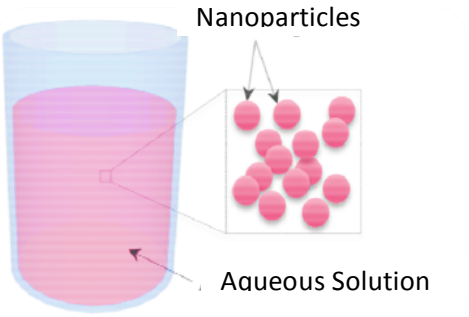


Figure 9: Representation of PDMS coated nanoparticles in solution (Grist, et. Al 2010).

M*icroelectronic Probe*

The use of a microelectrode array **(figure 10)** for measuring dissolved oxygen concentrations has been proposed by Lim *et al.* (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 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).

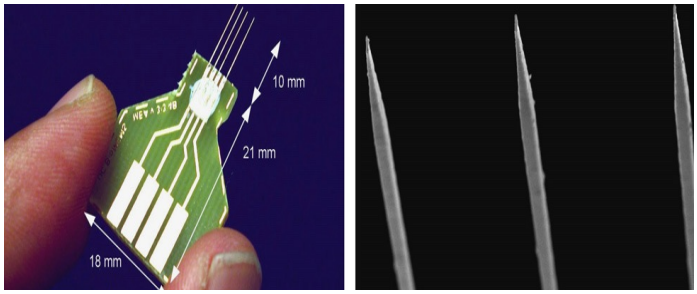


Figure 10: Dissolved oxygen microelectrode millimeter dimensions shown (Left) and dissolved oxygen sensing tips (Right) (Lim, et. Al 2009).

Gas Detection Design Matrix

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

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**

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 4. 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-cytotoxic under normal conditions; however the detection nanoparticles can become cytotoxic at high concentrations, which is why it scored slightly lower than the thin-sensor film format. The oxygen probe scored comparably to the thin sensor because it is also biocompatible 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.

VI. Final Design Selection

After analyzing the two design matricies, the two-channel model and the thin-film sensing method were picked as the final design components. However, prior to integrating the final design components, the ability of the platform design was tested by using COMSOL® to model the oxygen gradient created in the channel. Additionally, the thin-film oxygen detection method was determined to be too expensive for an initial proof-of-concept of the design, thus an alternative method was developed using methylene blue. Finally, the future work outlines a plan to integrate the results from the COMSOL® modeling, oxygen detection method, and microfluidics platform design are presented. Successful integration of these components will ultimately lead to the implementation of the final design using the two-channel model and thin-film sensing method as outlined above.

VII. Simulation of Oxygen Concentration Gradients

Using the basis of the two-channel design, but including oxygen in both input channels as opposed to nitrogen and oxygen, the final channel design specifications were validated through finite element analysis in COMSOL Multiphysics®, and a full system response was obtained for both stationary and time-dependent studies. Inside of these simulations, the global ambient temperature was set to 310 Kelvin to best match current experimental conditions that strive to mimic an in vivo environment. In the simulation geometry, each gas channel was modeled as being 260 μm wide and 14,500 μm long. Each gas flow channel was modeled as being 750 μm wide and 31,000 μm long. After being drawn in 2-D the geometry was extruded to 3-D having a height of 250 μm to match the actual SU-8 thickness fabricated through soft lithography. The simulation was simplified to only allow diffusion from gas channels, into the thin layer of PDMS between gas and cell channels, into the cell channels, and out the other end in reverse order. Although the current prototype does not include a mechanism for preventing diffusion between cell channels or between the device itself and ambient air, several design possibilities have already been proposed and will be discussed later.

The flow of oxygen gas into the device was simulated using the Incompressible Navier-Stokes model which is included in the COMSOL® Microfluidics Module **(1.0).** All initial gas velocities were set to zero and no slip boundary conditions were assumed for all walls. Gas flow through the device was induced by creating a pressure differential between the inlet and outlet boundaries of 2,500 Pa resulting in an average flow velocity of 25.569 m/s at the center of the channel. This pressure differential also results in a pressure gradient along the edges of the gas flow channels, which is important for the creation of an oxygen gradient on the device.

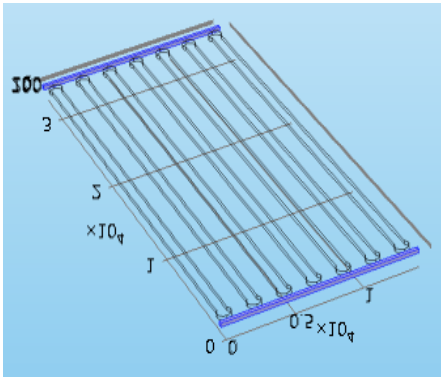


Figure 11: Gas Flow Domains: The domains used for modeling the gas flows were modeled as containing pure diatomic Oxygen gas at a pressure parameter set by the user. (COMSOL®)

$$\rho\frac{\partial u}{\partial t}+\rho(u\bullet\nabla)u=\nabla\bullet[-pI+\mu(\nabla u+(\nabla u)^{T})]+F \qquad \textbf{(1.0)}$$

The paths of oxygen diffusion were modeled using the COMSOL® Convection and Diffusion model **(2.0)**.

$$\nabla\bullet(-D_i\nabla c_i)+u\bullet\nabla c_i=R_i \qquad \textbf{(2.0)}$$

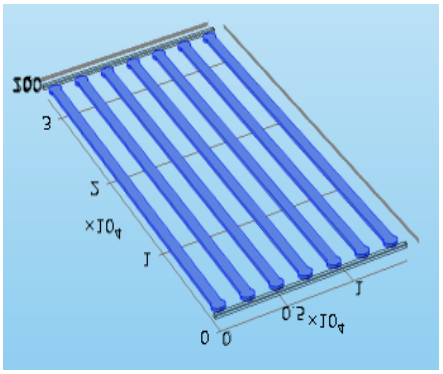


Figure 12:Cellular Culture Channels for Oxygen Diffusion: Thee longitudinal channels of the device were modeled as containing pure water and an initial oxygen concentration of 0 mol/m3. (COMSOL®)

The oxygen diffusion coefficients of interest are 3.4E-9 m^2/s for PDMS and 2.2E-9 m^2/s for water. It was assumed that whatever concentration of oxygen on the cell side of the PDMS would become equal to the concentration on the gas side in a comparatively short amount of time, and thus the PDMS was removed from the model for greater simplicity. The domains containing oxygen gas **(figure 11)** were set as containing the chemical allowing the software to load its physical properties from the COMSOL® materials library. All domains not containing oxygen were given the initial concentration of 0.0 mol/m^3. For the purpose of estimation, each longitudinal channel was modeled as containing pure water in lieu of cells and DMEM **(figure 12).** Additionally, cellular oxygen consumption is a factor to be considered in future models, but was not included in the current simulation. The boundary concentration of each longitudinal channel is directly proportional to the pressure exerted by the

$$PV = nRT \qquad \textbf{(3.0)}$$

oxygen gas on the opposite edge of the PDMS. Hence, the boundary oxygen concentrations were related to the gas pressures via the ideal gas law **(3.0).**

Rearranging equation 3 yields:

$$\frac{n}{V}=\frac{P}{RT} \qquad \textbf{(4.0)}$$

which can be further adapted to give the boundary concentration of oxygen at the edge of the PDMS between the gas channel and a specific cell channel **(5.0)**. The cells are numbered in increasing order from right to left starting with channel 1 which is located directly adjacent to both

$$\frac{n[mol]}{V[m^3]} = \left[\frac{14,500 - ((channel\#) \bullet 1,803.57)}{14,500}\right] \bullet \frac{P[Pa]}{R\left[\frac{J}{K \bullet mol}\right] \bullet T[K]} \qquad \textbf{(5.0)}$$

of the gas inputs. Thus, the greatest concentration of dissolved oxygen is expected in channel #1 with the lowest concentration of dissolved oxygen in channel #7.

Equation **(5.0)** can be used to compute the expected oxygen boundary concentration as a function of the applied input gas pressure and the specific channel being measured. Both time dependent and stationary studies were performed on the model using an extremely fine physics-controlled mesh consisting of 648,323 nodes for finite element analysis. The steady state results shown in **Figure 13** indicate that the device will create seven discrete oxygen concentrations decreasing from some maximal value determined by the oxygen input pressure. It follows that this microfluidic channel configuration is fully versatile and can be used to create a seven-segment gradient of any range of oxygen concentrations by simply varying the input pressure.

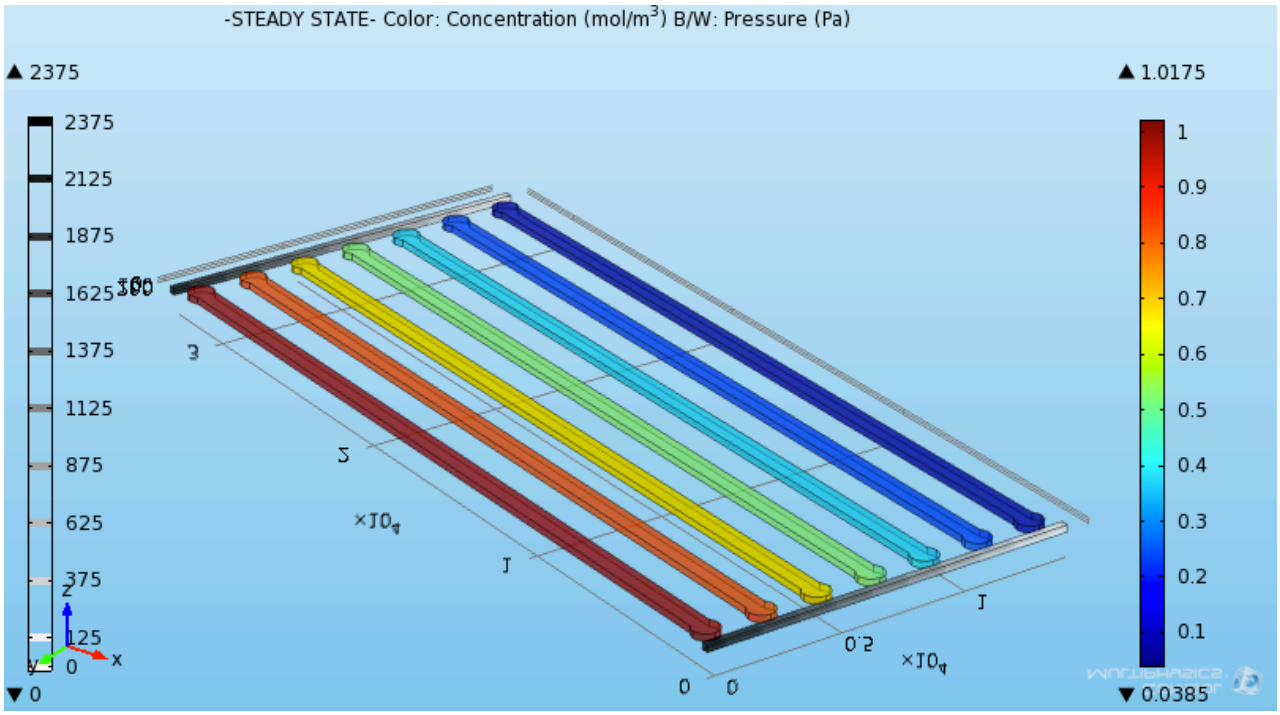


Figure 13: Steady State Results of COMSOL® Multiphysics Finite Element Analysis: The oxygen flow channels are colored in grayscale with contoured bands indicating the pressure of oxygen at a given point along the direction of flow. Gas inlets are on the left side of the figure, with an input pressure of 2,500 Pa and an outlet pressure of 0 Pa. The oxygen concentration in each channel is measured in mol/m^3 and increases linearly as a function of the pressure in the adjacent O_2 gas channel. Seven discrete concentration graduations are created at this input pressure, ranging from 0.0385 mol/m^3 (3.2% saturation) to 1.2 mol/m^3 (oxygen saturated). (COMSOL®)

VIII. Current Platform Design

Mastermold design

Based on the results of the oxygen gradient simulation, the final master transparency designs were verified. The results of the modeling verified that microfluidics is a field concenrned with the finest of detail in dimension and fluid flow rate and indicated the importance of precise channel design Minute changes to the preliminary channel design in COMSOL® resulted in a successful model of an oxygen gradient **(figure 13)**.

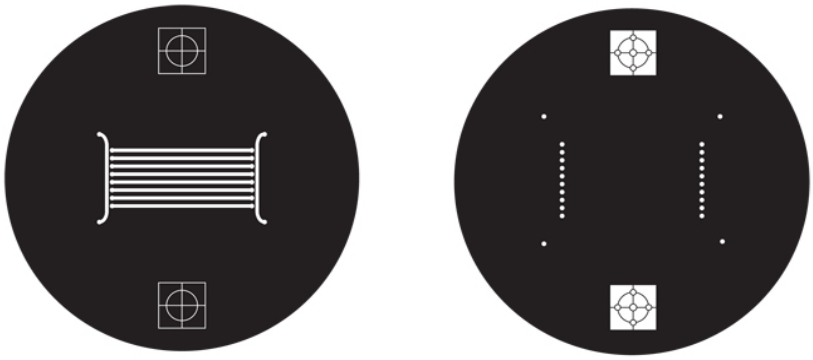


Figure 14: Top (Right) and bottom (Left) layer photomasks.

In addition, continued research into the methods of photolithograpy resulted in the addition of a second layer **(figure 14)**. A second photomask was incorporated to increase the tolerance for the gas line inputs. **Figure 14** shows cross-hair alignment aids that allow for precise positioning of the second layer with the help of a microscope.

Based on the successful COMSOL® model and the continued design research above, the final dimensions of the longitudinal channels to house the cells of 31 x 0.75 x 0.25 mm (LxWxH) and the 770 μm membrane separating the gas channels from the cell culture channels were confirmed to be able to establsih a gradient.

Master Tempate Fabrication

Based on the design of the master mold outlined above, the fabrication of master mold was completed. Fabrication of the master template involves a series of baking intervals, exposure to UV light under a photomask, and a developing solution wash.

First, a dime sized amount of a SU-8 2100, was applied to a silicon wafer. This was then spun up to 1500 rpm to spread the SU-8 coat over the wafer at a consistent thickness of 250 μm. Upon completion of the spin cycle, the wafer is heated for approximately two hours, ramping from 65°C (10 min) to 95°C (90 min) causing the SU-8 to harden.

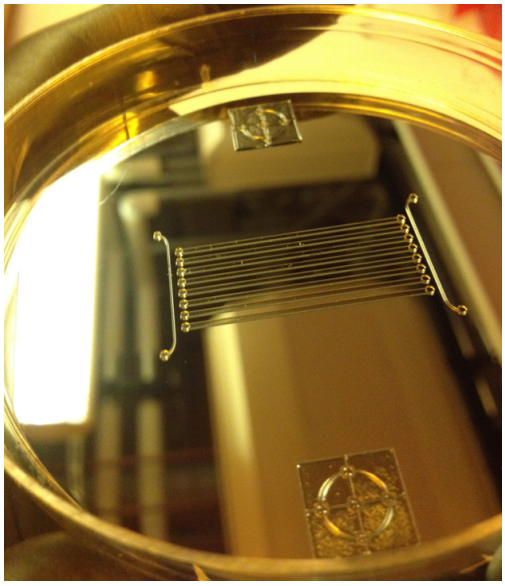


Figure 15: Final master template design.

After the wafer cools to room temperature, the wafer is exposed to UV light with the photomask applied, exposing only the desired features to the light source. During exposure, the UV-light reacts with the SU-8 catalyzing the formation of an acidic product. The wafer then goes through a post-bake cycle, at the same temperatures as the pre-bake, 65°C (5 min) and 95°C. The post-bake completes crosslinking of the UV exposed regions,

rendering it insoluble.

This device required a second layer to increase the tolerance for the gas input lines. The same protocol used for the first layer was followed, and a new photomask was applied. Precise alignment of the second photomask was required, so that the features of the second layer lined up with the features of the first layer.

Following the post-bake of layer two, the master is ready to be developed. For approximately 30 minutes, the wafer is bathed in a developing solution, which removes any of the remaining, soluble SU-8. Upon completion, only the areas that were exposed to UV light remain **(figure 15)**. The finished master template can then be used to mold a PDMS block. A lab protocol for this procedure can be found in Appendix B.

PDMS Block

In order to fabricate the PDMS block, the master is placed in an aluminum foil boat to contain the liquid PDMS. The wafer was adhered to a PMMA disk to avoid breakage of the silicon wafer. The PDMS gel is then poured on the device to a thickness defined by the user. It is then placed in a vacuum chamber for 15 minutes to remove any gases from the gel. The device is then placed on a hot plate for four hours at 80°C with a weight on top to ensure consistent thickness. This bake cycle renders the PDMS solid. Once completed the PDMS can be separated from the wafer and then placed on a glass slide yielding finished mold **(figure 3)**. A lab protocol for this procedure can be found in Appendix C.

IX. Current Oxygen Detection Method

The second major aspect of this project is the ability to measure oxygen content in the microfluidic channels. This quantification is necessary in order to determine at what level of hypoxia causes the most efficient fusion of cardiomyocytes with mesenchymal stem cells. Previous research has used three effective methods for quantifying oxygen: ruthenium-based thin film sensors, ruthenium-based beads, and micro-oxygen probes (Grist et al. 2010). The basic method of quantifying each of these techniques is outlined in the preliminary design oxygen detection method section.

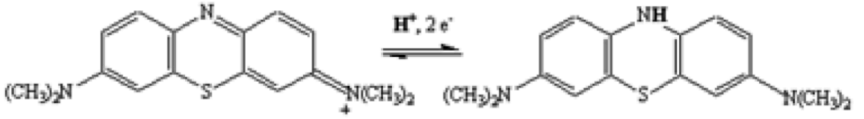
Initially the decision was made to pursue the ruthenium-based thin film sensors as produced by Ocean Optics Inc. Ocean Optics was able to provide glass slides with the thin film sensor for $350.00 per slide (Ocean Optics Inc. 2012). The other option that was extensively researched was a micro-oxygen probe. The final cost of the micro-oxygen probe was $4170.00 (Unisense 2012).

As a proof of concept to determine if the final PDMS design was capable of creating a sufficient oxygen gradient, it was determined that both of these detection methods were beyond the reasonable price range. This meant that a more cost effective method of oxygen detection would need to be used in order to determine the effectiveness of the system.

One option for such detection is methylene blue. Methylene blue is reagent that is most commonly used in histological analysis to make nuclei more visible by staining them blue (Bruckner 2012).

However one of the unique features of methylene blue is its ability to convert from its normal blue form to its colorless, leuco form (Lee and Mills 2003).

The conversion of methylene blue to leuco-methylene blue is accomplished through a reducing reaction in which methylene blue is reduced to its leuco-methylene form **(figure 16)**(Lee and Mills 2003). A number of mild reducing agents are commonly available and capable of driving the reducing reaction; primarily these are glucose (dextrose) and ascorbic acid (Lee and Mills 2003).



Methylene Blue (Oxidized) **Leuco-Methylene Blue (Reduced)**

Figure 16: Methylene Blue reduction reaction. Methylene blue molecule is reduced to leuco-methylene blue which is clear (Lee and Mills 2003).

$$2H_2O + Glu + MB \xrightarrow{NaOH} LMB + Glu_{ox} + O_2$$

Figure 17: Chemical reaction showing the oxidation-reduction of methylene blue. Oxidized methylene blue (left side of equation) is blue in color, reduced methylene blue (right side of equation) is clear (Lee and Mills 2003).

One of the more unique features of the reaction however is that when it is in an alkaline solution, such as sodium hydroxide, the available dissolved oxygen is capable of serving as a oxidizing agent and quickly reversing the leuco-methylene blue form to its oxidized blue form (Lee and Mills 2003, Mills and Wang 2009).

What this chemistry basically concludes is that when methylene blue is mixed with glucose in an alkaline solution, it will form its clear leuco-methylene blue form, but when dissolved oxygen is present, it will oxidize to its blue methylene blue form **(figure 17)**.

The theoretical validity of this reaction was tested experimentally and was determined to occur as expected. In order to determine the effectiveness of this method to provide verification of the ability of the microfluidic device to generate an oxygen gradient, a standard curve was generated by exposing three identical 30mL methylene blue samples to three concentrations of oxygen, 0%, 21%, and 95%. Details for the composition of the methylene blue samples is included in **table 3**.

Table 3: Reaction conditions for color change of methylene blue under various oxygen concentrations.

Reactant	Amount
Methylene Blue	7.5µg
Glucose	0.5g
NaOH	0.333M

A **B** **C**

Figure 18: Methylene blue solution in the presence of (A) 0% O_2 gas, (B) 21% O_2 gas, (C) 95% O_2 gas

The test tubes were exposed to the various oxygen concentrations by inserting the gas source directly into the solution and observing the color change. After each test, each sample was allowed to equilibrate back to its normally reduced state

before the next gas source was inserted. Pictures of each tube were taken and the color change observed **(figure 18)**.

Each picture was then analyzed using ImageJ software to measure the color intensity from each sample. The intensity was measured and averaged for all three color channels combined (Red, Green, and Blue). The images were also separated into individual color channels and each was analyzed for color intensity. The results from each sample when exposed to the same treatment were averaged and the standard deviation determined for each separate channel. These results were graphed and the trends observed **(figure 19)**. Additionally, single factor ANOVA analysis was conducted to determine if the light intensity was significantly different among the three oxygen concentrations used. This was conducted separately for each of the color channels and yielded statistically significant results for each color channel **(table 4)**. Ultimately however, the graphed results **(figure 19)** indicated that the blue color channel would not be ideal for determining the oxygen concentration as it is

Table 4: P-value results of light intensity measurements for all three O_2 concentrations and for each of the four color channels.

Light Intensity Channel	P-Value
RGB	0.0000444*
Red	0.00000195*
Green	0.0000909*
Blue	0.000513*
*p<0.001	

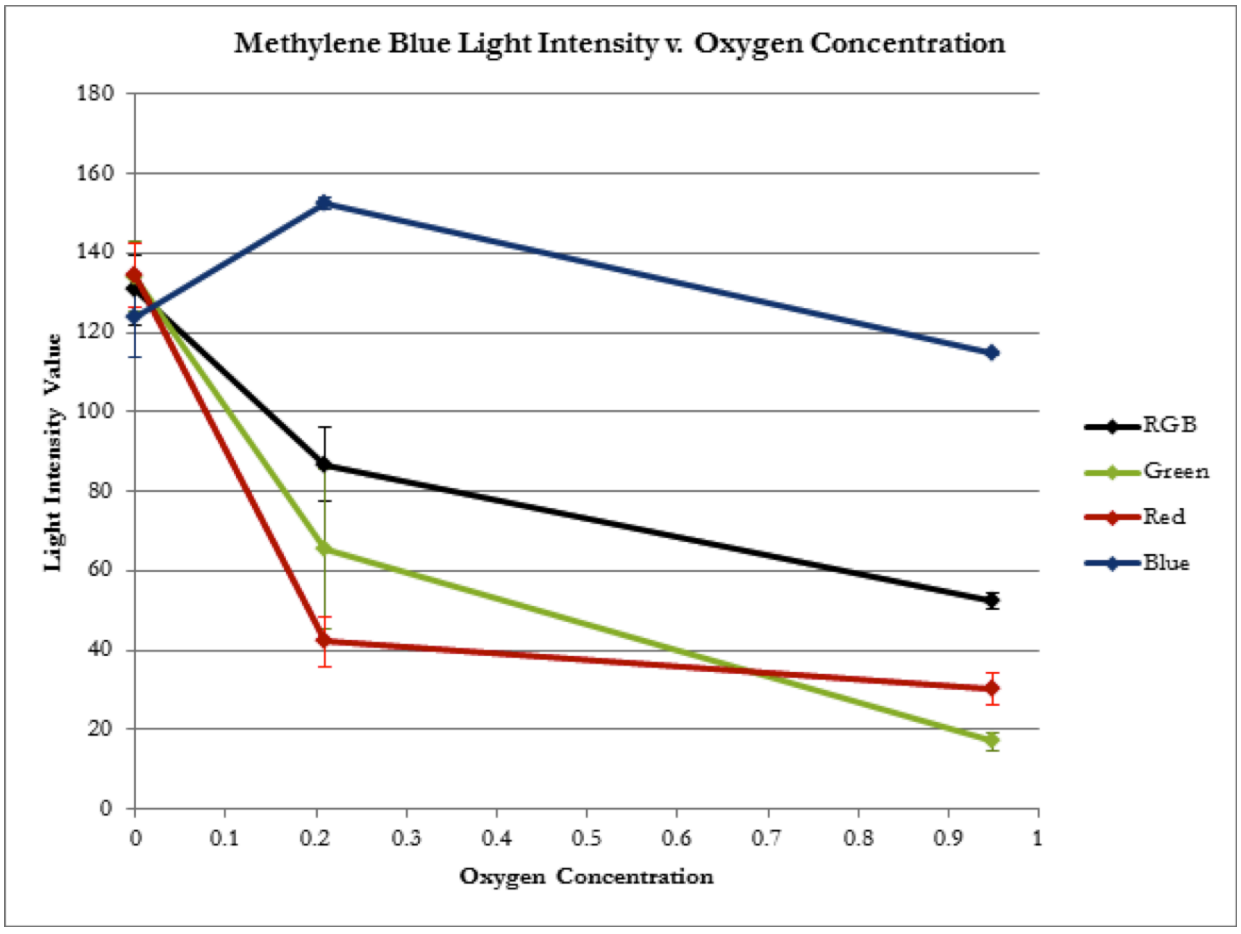


Figure 19: Color intensity for RGB average and green, red, and blue channels for 0%, 21%, and 95% O_2 concentrations. Each oxygen concentration was tested with three replicate. Error bars represent standard deviation.

observed have a parabolic nature, with the color intensity increasing at 21% oxygen but then decreasing at 95% oxygen. Therefore, the blue channel is not an ideal measure of oxygen concentration. The other channels however, did indicate a biologically significant change in color intensity from 0% to 95% with the green channel displaying the greatest change. Therefore, the green channel is the most likely candidate for use as a determinant of oxygen concentration.
The next step for this detection method would be to test it in a hypoxia chamber at more controlled oxygen levels, from 0% to 50% in 10% concentration intervals. This will allow the creation of a true standard curve. After obtaining a standard curve, the solution will be put into each of the microfluidic channels with oxygen gas connected. Once the system has equilibrated the microfluidic device will be photographed and the intensity measured using ImageJ, which will then be compared to the results of the standard curve.

Ultimately, however, because methylene blue is used as a nucleus stain it will not be ideal for use as an oxygen detector in a cell culture system (Bruckner 2012). The final experimental design will need to incorporate the more expensive oxygen detection methods previously mentioned in order to maintain ideal cell culturing conditions.

X. Budget

The overall cost of the project was $48.16, which was within the proposed budget of $500.00 - $1,000.00. The budget includes all materials that were used to create the master templates, PDMS blocks, and those used for oxygen detection **(table 5)**.

Table 5: Overall budget for the construction of the current prototype.

Date	Item	Part Number	Company	Quantity	Unit Cost	Total Cost	Order Total
4/22/12	Methylene Blue	N/A	N/A	1	Donated	Donated	$0
4/24/12	SU-8 2100	SU-8 2100	Microchem	1	Donated	Donated	$0
4/25/12	Silicon Wafers	N/A	WRS Materials	2	$16.00	$32.00	$32.00
4/27/12	3.175mm Tygon Tubing	5554K42	McMaster Carr	7.62m	0.85/m	$6.50	$16.16
	3.175mm to 6.35mm Tube Fittings	5779K251	McMaster Carr	2	$4.83	$9.66	
							$48.16

XI. Safety and Ethics

Safety
Safety as far as microfluidics is concerned, deals mostly with the chemicals that are involved during fabrication, as well as, the chemicals involved in the sensing methods. It is important for those, who are handling these chemicals, to be trained in their proper usage. In addition, common lab safety procedures such as long pants, close-toed shoes, gloves, and goggles should be worn at all times. Aside from this, the dangers of microfluidics fabrication is not beyond the scope of any other standard lab bench work.

Ethics

While the device fabrication has very few ethical concerns associated with its design and fabrication, the ultimate end goal of the device must be kept in mind. The device will ultimately incorporate both cardiomyocytes and mesenchymal stem cells into a culture system. While the use of an immortalized cardiomyocyte cell line does not represent an ethical concern, the use of stem cells does.

The use of stem cells in biomedical research offers incredible promise to the medical field, such as this particular application of potentially regenerating damaged heart tissue (Fischbach and Fischbach 2004). However, thus far in this new scientific field, stem cells have yielded no concrete results despite the great potential they are believed to have (Fishbach and Fischbach 2004). This great potential however needs to be balanced with the ethical concerns of obtaining stem cells. The most publically known method of obtaining stem cells is through human embryos in which the stem cells are removed before they can differentiate and the embryo is sacrificed (Fischbach and Fischbach 2004). The ethical debate surrounding this method has been so heated that the United States government banned any federal agency from funding embryonic stem cell research 2001 (Fischbach and Fischbach 2004). Despite the ban being lifted in 2009, the debate still persists (CNN 2009).

This debate however, is a little less controversial for the end goal of our device. Our device will be meant to create a co-culture system using cardiomyocytes and adult mesenchymal stem cells obtained from adult bone marrow. This means that embryos have not been sacrificed in obtaining these stem cells and the debate around the sanctity of human life is lessened. There is however an ethical concern because the adult stem cells still must be obtained from human subjects. However, by taking all proper precautions and ensuring the donor understands the risks and is willing, the ethical concerns of using of adult mesenchymal stems is minimal.

XII. Future Work

Though the current design has been modeled to establish the desired gradient, there are still aspects to improve upon. Plans for improvement include channel design modification, implementation of the current oxygen detection method, and incorporation of cells within the device. With the current channel design it takes roughly an hour to establish a steady state gradient. To decrease gradient formation time, the thickness of the PDMS membrane separating the gas input channel from the longitudinal channels can be decreased, allowing for oxygen to diffuse more rapidly.

The diameters of the inputs to the gas channels are currently too small to fit a syringe down without the help of a micromanipulator. Increasing this diameter would facilitate easier attachment of the gas lines.

In addition to the alteration of channel dimensions to improve gradient formation, the channels can be treated with a parylene solution to inhibit cross channel diffusion. Parylene is a general name for a variety of chemical vapor deposited poly(p-xylylene) polymers used as moisture and dielectric barriers. Minimizing cross channel diffusion will allow for a more, repetitive, consistent gradient in the steady state.

The methylene blue oxidation – reduction with glucose has been used as a proof-of-concept, as it is relatively inexpensive and yields quantifiable results. This process needs to be tested inside the microfluidic device to experimentally validate the COMSOL® modeling. But in order to implement,

an interface between the oxygen gas source and the device must be purchased. An Airgas, two-stage oxygen specific regulator was sought out, but not yet purchased. Clear tygon tubing was purchased from McMaster Carr, having dimension 3.175mm outer diameter by 1.5875mm inner diameter. The tubing was connected to the regulator using female, quick connect fittings purchased from McMaster Carr. Lastly, a connection between the tubing and microfluidic platform needs to be determined. After collecting experimental data using the methylene blue method, confirming the ability of the device to generate a gradient, more accurate methods of detecting oxygen can be sought out for purchase. These alternative methods include the ruthenium based thin sensor strips, and the oxygen probe from Unisense or Ocean Optics Inc.

When it is confirmed that the device is generating an oxygen gradient, cells can then be cultured in the longitudinal channels, allowing for study of the behavior of MSCs and cardiac cells under hypoxic conditions.

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APPENDIX A: PRODUCT DESIGN SPECIFICATIONS

Function: The purpose of this device is to create a microfluidic environment in which cells (cardiomyocytes and mesenchymal stem cells) can be cultured and exposed to varying oxygen concentrations, and the reactions of the cells to the various oxygen concentrations can be observed and quantified.

Client Requirements:

1. Device will be a microfluidics system
2. Device should be made using a master device out of SU-8 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. The device needs to incorporate an oxygen detection method to allow for the determination of oxygen concentration at specific points in the cell culture system.
6. All components should be biocompatible with cell culture and non-cytotoxic to cells
7. The design should be as simple as possible.
8. 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. The device needs to be able to measure oxygen concentration while having no adverse effects on cultured cells.
    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. The oxygen detection system needs to operate within a range of +/-1% oxygen concentration.
    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°C, 5% CO_2 dark incubator and will have standard cell culture media (DMEM) inside the device. While culturing, the device will be exposed to 2500 Pa in the gas channels which will diffuse into the device. When cells are being observed they will be placed onto a

fluorescent microscope in the dark and exposed to light to cause the oxygen detection system to fluoresce.

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 (75mm by 25 mm). The cell channels 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. The oxygen detection system will be a ruthenium based thin film. Oxygen detection will be conducted using 3.175mm OD Tygon tubing and a fine gas regulator.
k. *Aesthetics, Appearance, and Finish*: Each device should be made cleanly, but aesthetics is not especially important for this design.

2. Production Characteristics:

a. *Quantity*: One master template. Up to 100 devices created from the master template. One thin film oxygen sensor per device. One set of oxygen tubing and fine gas regulator.
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. Oxygen dection must be accurate to +/-1% oxygen concentration.
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.

Appendix B

Greg Czaplewski **Last Updated 7/30/11**

First layer (5um height):

- Program spin coater:
  - ramp from 0 to 500 in 5 seconds and stay at 500 for 10 seconds
  - ramp from 500 to 2800 in 5 seconds and stay at 2800 for 10 seconds
  - ramp from 2800 to 2900 in 5 seconds and stay for 30 seconds
  - stop wafer in 1 second
- Take clean wafer from packaging and center it on the chuck of the spin coater
  - Make sure there is no debris or particles on the wafer (extremely important for thin layers)
- Pour su-8 (5 series) onto the center of the wafer
- Connect vacuum, turn it on, turn on lab air, and press start
  - After spin, make sure there's no bubbles or debris on wafer
- Place wafer on center of hot plate –must be balanced! – and pre-bake:
  - 65°C for 2 minutes
  - Ramp up to 90°C for 7 minutes
  - Ramp down to 65°C for 1 minute
    - Slow cool down of wafer prevents brittleness and cracking
- Expose wafer to UV light:
  - Fiber optic cable should be 12 inches above sample (make sure it exposes the whole sample to UV light)
  - Hook up vacuum to platform and place wafer on top
  - Tape photomask of first layer to glass (USE SAME SIDE CONSISTENTLY FOR ALL STEPS!!!) whether its black side down or up (preferably down)
  - Center photomask over wafer using movable stage
  - Expose wafer to roughly 9 mW/cm^2 of light for 5 seconds
    - Repeat 5x with breaks of about 30 seconds between each repetition
- Place wafer on center of hot plate and post-exposure bake (PEB):
  - 65°C for 4 minutes
  - Ramp up to 85°C for 8 minutes
  - Ramp down to 65°C for 2 minutes
    - Slow cool down of wafer prevents brittleness and cracking
- Develop wafer:
  - After wafer cools, place in new developer solution for 3 minutes (no agitation)
  - Rinse with new developer, then IPA
    - Quickly dry with nitrogen gas after applying IPA (prevent streaks)
    - White stuff on wafer means there's some su-8 left (rinse again)
- Look at wafer under microscope to confirm distinct features and no cracking

2nd layer (200um – 250um):

- Program spin coater:
  - ramp from 0 to 500 in 5 seconds and stay at 500 for 10 seconds
  - ramp from 500 to 1300 in 5 seconds and stay at 1300 for 10 seconds
  - ramp from 1300 to 1400 in 5 seconds and stay for 30 seconds
  - stop wafer in 1 second
- Take wafer from 1st layer process and center it on the chuck of the spin coater
  - Use nitrogen gas to remove any particles or contaminants (be gentle!)
- Pour su-8 (2100 series) onto the center of the wafer
- Connect vacuum, turn it on, turn on lab air, and press start
  - After spin, make sure there's no bubbles or debris on wafer
- Place wafer on center of hot plate –must be balanced! – and pre-bake:
  - 65°C for 7 minutes
  - Ramp up to 90°C for 55 minutes
  - Ramp down to 65°C for 2 minute
    - Slow cool down of wafer prevents brittleness and cracking
- Develop off alignment marks so 2nd layer can be aligned with 1st layer
  - Stand wafer up on end in (new or used) developer solution so that only alignment marks are being developed off (could develop off two sides for better alignment)
  - Takes about 15-20 minutes to develop each side
- Expose wafer to UV light:
  - Fiber optic cable should be 12 inches above sample (make sure it exposes the whole sample to UV light)
  - Hook up vacuum to platform and place wafer on top
  - Tape photomask of 2nd layer to glass (USE SAME SIDE CONSISTENTLY FOR ALL STEPS!!!) whether its black side down or up (preferably down)
  - Center photomask over wafer using movable stage
  - Align photomask to alignment marks that can be seen through microscope
  - Expose wafer to roughly 9 mW/cm^2 of light for 20 seconds
    - Repeat 3x with breaks of about 2 minutes between each repetition
- Place wafer on center of hot plate and post-exposure bake (PEB):
  - 65°C for 5 minutes
  - Ramp up to 85°C for 55 minutes
  - Ramp down to 65°C for 3 minutes
    - Slow cool down of wafer prevents brittleness and cracking
- Do not develop off this layer
  - Because it's difficult to get a smooth layer to spin on top with the tall features in the way (also causes a lot of air bubbles)

3rd layer (200um – 250um):

- Program spin coater:
  - ramp from 0 to 500 in 5 seconds and stay at 500 for 10 seconds
  - ramp from 500 to 1300 in 5 seconds and stay at 1300 for 10 seconds
  - ramp from 1300 to 1400 in 5 seconds and stay for 30 seconds
  - stop wafer in 1 second
- Take wafer from 1st layer process and center it on the chuck of the spin coater
  - Use nitrogen gas to remove any particles or contaminants (be gentle!)
- Pour su-8 (2100 series) onto the center of the wafer
- Connect vacuum, turn it on, turn on lab air, and press start
  - After spin, make sure there's no bubbles or debris on wafer
- Place wafer on center of hot plate –must be balanced! – and pre-bake:
  - 65°C for 7 minutes
  - Ramp up to 90°C for 55 minutes
  - Ramp down to 65°C for 2 minute
    - Slow cool down of wafer prevents brittleness and cracking
- Develop off alignment marks so 3rd layer can be aligned with 2nd layer
  - Stand wafer up on end in (new or used) developer solution so that only alignment marks are being developed off (could develop off two sides for better alignment)
  - Takes about 15-20 minutes to develop each side
- Expose wafer to UV light:
  - Fiber optic cable should be 12 inches above sample (make sure it exposes the whole sample to UV light)
  - Hook up vacuum to platform and place wafer on top
  - Tape photomask of 3rd layer to glass (USE SAME SIDE CONSISTENTLY FOR ALL STEPS!!!) whether its black side down or up (preferably down)
  - Center photomask over wafer using movable stage
  - Align photomask to alignment marks that can be seen through microscope
  - Expose wafer to roughly 9 mW/cm^2 of light for 20 seconds
    - Repeat 3x with breaks of about 2 minutes between each repetition
- Place wafer on center of hot plate and post-exposure bake (PEB):
  - 65°C for 5 minutes
  - Ramp up to 85°C for 55 minutes
  - Ramp down to 65°C for 3 minutes
    - Slow cool down of wafer prevents brittleness and cracking
- Develop wafer:
  - After wafer cools, place in new developer solution for 20-30 minutes (moderate – heavy agitation)
  - Rinse with new developer for 15 seconds, then IPA

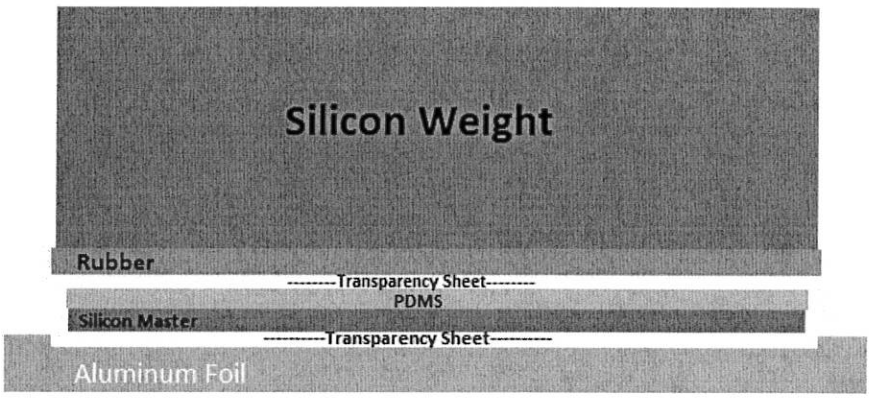
        - Quickly dry with nitrogen gas after applying IPA (prevent streaks)
        - White stuff on wafer means there's some su-8 left (rinse again)
            - More likely to happen when applying thicker layers
- Look at wafer under microscope to confirm alignment of all layers, that features are distinct, and that there is no cracking or peeling

Silanization

- Place wafer in desiccator (vacuum chamber) along with about 50 uL of trichloro(octadecyl)silane
    - Apply vacuum and let sit for 3 hours

PDMS Mold Fabrication:

- Mix 5 parts PDMS elastomer base with 1 part curing agent
    - 15 grams is more than enough for 2 masters
- De gas PDMS in desiccator for about 20 minutes



- Set up aluminum foil and transparency sheet layer and put silicon master on top
- Pour PDMS on top of silicon master
- Degas again for 5-10 minutes
- Place transparency sheet, rubber sheet, and silicon weight on top of silicon master
    - Place on hot plate for 4 hours at 80°C
- Take off of hot plate, let cool
- Cut around devices with a razor blade but stay inside the alignment marks
    - Trying to peel off the alignment marks causes the PDMS film to rip/tear

- For subsequent PDMS mold fabrication, make sure to cut off all cured PDMS that might create a gap between the freshly poured PDMS and the transparency sheet that goes on top.
    - Gaps cause PDMS to cover the inlet and outlet ports so holes don't get punched

Appendix C

Marika Xydes-Smith Last Revised 7/30/11

Microfluidic device making

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Weigh out 15 grams base and 3 grams curing agent on weigh balance. (1-2% error in weighing is OK) 18 grams total. Mix thoroughly with a fork for about 1 minute.

10-15

Remove bubbles from PDMS for about 20 min in dessecator. (pump at ~8 kpa)
When opening valve, do so very slowly.

Pour liquid PDMS onto molds. Use about 70% of total liquid. Keep weigh boat edge in liquid when pouring to avoid adding in air bubbles on accident.
Rotate plates around until all devices covered with PDMS. De-gas in desiccators for 10 min. If dimples or bubbles on devices, add more PDMS and more degassing.

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Once all bubbles gone, cut out overhead plastic sheet to fit devices (must be smaller than whole plate but still more than cover all devices. Carefully and very slowly press down plastic over PDMS. Lay down rest of plastic overhead sheet over both devices.

up

Add rubber squares directly above devices dirty side down.
Add weights. Incubate on hot plates for 4 hours at 80 degrees F.

When done, remove weights and plastic, cleaning as necessary.
Remove plastic cover and then plastic disks covering device. Gently pry off with razor's edge and slowly pull away from PDMS, otherwise will rip the PDMS.

Outline the devices with razor, pull on corners with tweezers until loose. Very slowly pull off PDMS covering devices and place upside down in ¼ inch 70% ethanol in petri dish.

Before use, dry with kim wipe and clean with 70% ethanol. Using tweezer points, carefully punch out inlets and outlets for all devices and check under microscope for validity.