Generation of an accessible and versatile micro-hypoxia chamber

Final Report

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Abstract

Heart disease, the number one cause of death in the United States, has the potential to be combated with stem cell therapy. Previous research shows that stem cells can fuse to cardiomyocytes in hypoxic conditions and repair damaged cardiac tissue. In order to study the behavior of the stem cells under hypoxia, Professor Ogle's lab desires a microfluidic cell culture device capable of developing an oxygen gradient and an oxygen sensor to monitor the oxygen concentrations within the device. A cell culture chamber device was fabricated by a previous design team. Our team has selected dichlorotris (1, 10 – phenanthroline) ruthenium (II) hydrate (Rudpp) as an oxygen sensor molecule, designed a PDMS solid state sensor matrix, and tested the response of the sensor in different oxygen concentrations. The sensor shows a linear response to oxygen concentration and improved response in matrix as opposed to in solution. This sensor can also be readily integrated with a variety of microfluidic devices.

Background

Hypoxia and Cardiovascular Physiology

Despite being on a downward trend since the mid-20th century, heart disease is still the number one cause of death in the United States. All cardiovascular diseases contributed to 599,413 deaths in the United States in 2009, 24.6 percent of all deaths that year[1].

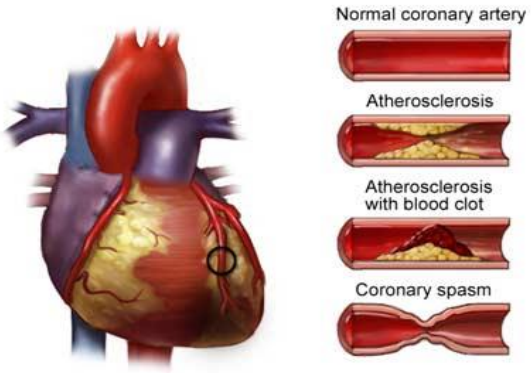

Figure 1: A number of different causes of cardiac ischemia[1]

One of the severe symptoms of heart disease is heart attacks, or myocardial infarctions. A variety of factors can lead to heart attacks, but the primary cause is insufficient blood supply to the heart, or cardiac ischemia (Figure 1), leading to the death or damage of sections of cardiac muscle tissue. The damage occurs because the lack of oxygen, or hypoxia, results in cell apoptosis, or cell death. Immediate treatment of the patient often includes cardiopulmonary resuscitation (CPR) or electrical shock (defibrillation). These treatments are used to prevent fatalities after heart attacks, but do not address the long-term effects of heart attacks[2].

Current methods of long-term treatment include cardiac rehabilitation and lifestyle change in order to improve the function of the failing heart sections, but new experimental methods are being investigated in order to provide a more permanent and effective solution. One such avenue of research is examining the use of stem cell therapy in repairing the damaged cardiac tissue. The hypothesis is that transplanted stem cells will fuse to damaged cardiac tissue and promote growth of new, healthy cardiomyocytes. Similar research using progenitor cells (similar to stem cells) shows the positive rehabilitative effects and potential future in stem cell therapy in treating patients [3]. In order to study the potential use of stem cells in damaged heart tissue, stem cells and cardiomyocytes must be cultured in a hypoxic environment similar to that of the heart, post-myocardial infarctions.

Microfluidics in Hypoxia Research

Two of the primary limitations of basic life science research are cost and resources. Experiments involving cells and other biological materials are expensive and utilize many resources to maintain or conduct experiments. In order to minimize both, the use of microfluidic devices has become popular within the last decade, with an increase of 38 to 1270 published uses of such devices from 2000 to 2010.

Microfluidic devices manipulate minute amounts of liquids and cells in order to study cellular and biological processes at a micro-level. The design and implementation of the device itself greatly varies between research applications because of the specificity of the research that they are used in, but they often share some of the same fundamentals. More than 45% of microfluidic devices published in 2009 used polydimethylsiloxane (PDMS) as the primary material in their composition[4]. PDMS is a particularly desirable material because it is biocompatible, hydrophobic, easily manipulated, optically transparent, gas permeable, and easy to fabricate into micro-scale devices.

A PDMS microfluidic device would be an appropriate candidate for mimicking hypoxic environments. More so than the low cost, the primary characteristic that makes it an ideal candidate is its capability of developing an oxygen gradient. The ability of microfluidic devices to generate laminar flow makes developing this oxygen gradient possible. By introducing oxygen and nitrogen at different ends of a device, the gases can diffuse through the PDMS into the channels to develop a gradient ideal for conducting simultaneous experiments at different oxygen concentrations. In addition, the micro-nature of the device allows the control of oxygen concentration to be more finely tuned and lessens the use of critical reagents, gasses, and cells, minimizing cost. The use of small amounts of oxygen demands that the concentration gradient be monitored precisely, accurately, and in real time in order to confirm that the hypoxic environment is being mimicked properly and as desired.

Current Technology and Existing Devices

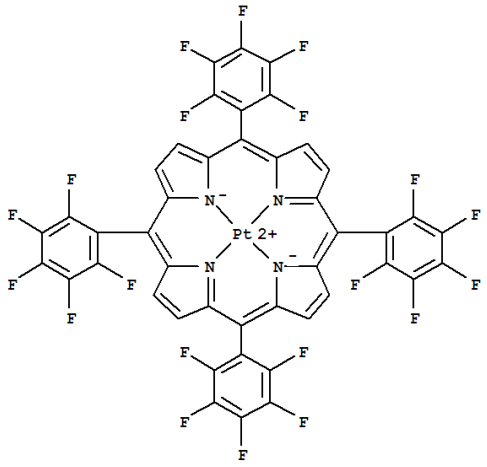

Figure 2: PtTFPP protein

Current methods of monitoring oxygen primarily involve large-scale detection of normoxic and hypoxic conditions. Traditionally and commercially available oxygen sensors include solid-state potentiometric equilibrium sensors, current amperometric sensors, semi-conducting metal oxide sensors, and optical sensors [5]. These devices often consume oxygen and are disruptive in cellular research, which make them non-ideal for the desired application. There are also a number of options that sense oxygen at a smaller scale, but are generally reserved for lab specific applications and experiments. These range from protein sensors to electrical sensors, but there are a few that serve the desired function of monitoring oxygen in a microfluidic device under gradient conditions.

One such oxygen sensor is a non-invasive thin film sensor developed at the University of Maryland. This sensor uses a platinum-based porphyrin sensor (PtTFPP), illustrated in Figure 2, in a PDMS-Teff thin film layer (Figure 3). The sensor molecules are placed into a gas chamber with the microfluidic device, and the experiments are conducted at steady-state conditions. Once the sensor is in contact with oxygen, it can be excited by fluorescent light and will emit a given emission light that is directly proportional to the concentration of oxygen within the measured environment[6]. The incorporation of Teff into the sensor severely limits the gas permeability between the microfluidic device and the sensor, making it non-ideal for the proposed stem cell research.

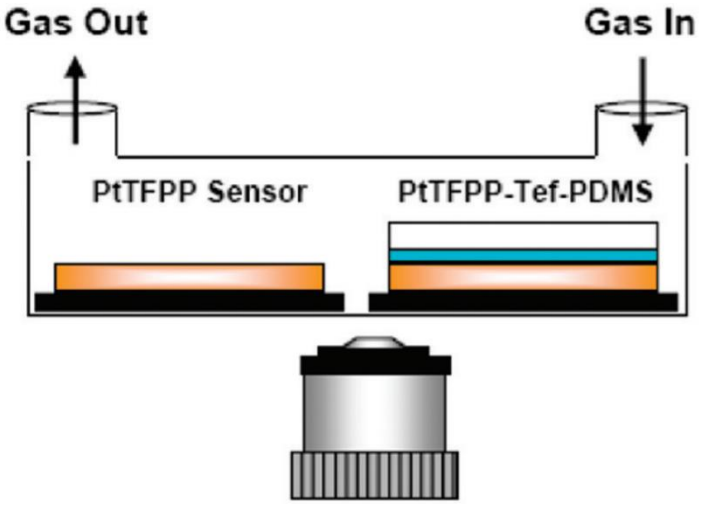

Figure 2: A thin film sensor developed at the University of Maryland

Another similar oxygen sensor is one developed for research at the University of Michigan and the MacDiarmid Institute for Advanced Materials and Nanotechnology at the University of Canterbury. This thin film sensor utilizes a ruthenium tris (2-2' dipyridyl) dichloride hexahydrate (RTDP) oxygen sensitive dye with a PDMS device in order to monitor the oxygen[7]. This sensor measures both lifetime and intensity of oxygen, but using a large scale approach. The sensor is incorporated with the PDMS and measure the oxygen concentration across the entire device, but lacks the ability to differentiate across a gradient. The basis of the sensor is somewhat applicable to the proposed stem cell research, but a solid-state approach would be more suitable.

Previous Work

In previous semesters, a number of steps have been taken to develop a microfluidic device and sensor for use in studying stem cell fusion with damaged cardiac cells in hypoxic conditions.

In the first semester of the project (spring 2012), the design team worked largely on designing and fabricating the microfluidic hypoxia chamber. The master template for the device, as pictured in Figure 4, was successfully designed for repeated construction of PDMS microfluidic devices. The device consists of a number of lateral micro-channels where cells will be cultured in passively pumped media, and two longitudinal channels where oxygen and nitrogen gas will be passed in order to develop the oxygen gradient. The entire process for building the PDMS device from a master template consists of an approximately 45-minute production protocol followed by a four-hour heating process. The spring 2012 team also did some preliminary testing with methylene blue to attempt to determine if their device adequately and appropriately developed oxygen gradients. Although the results were promising, they weren't entirely conclusive because methylene blue is a nucleus stain with non-ideal oxygen-sensing capabilities.

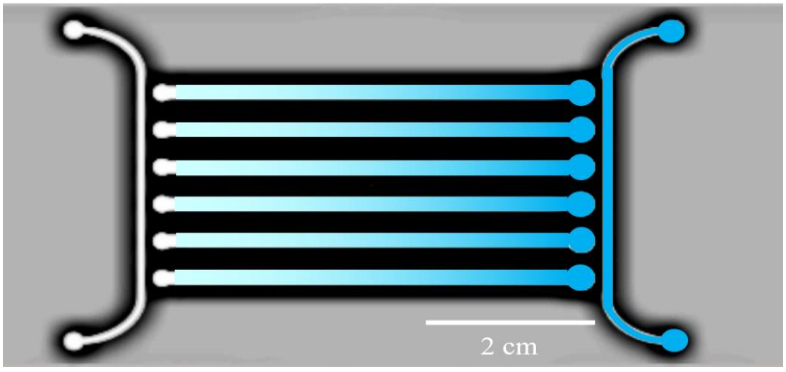

Figure 4: The design of the microfluidic device. Oxygen (blue) flows through the right-hand channel and diffuses to the left, creating a decreasing gradient. Nitrogen (white) flows through the left-hand channel.

In the second semester of the project (fall 2012), the design team worked on the selection and implementation of an oxygen sensor for real-time monitoring of the oxygen concentrations across the device. The sensor needed to be compatible with the previously designed microfluidic device, accurately sense the oxygen, and be non-cytotoxic. The team selected a platinum-based porphyrin sensor (PtOEPK) and began preliminary testing in order to develop a standard curve for the sensor. They incorporated the PtOEPK into a toluene-dissolved polystyrene matrix and constructed a thin film from that matrix. The team tested for the excitation of the molecule in wells, but failed to validate the sensor effectiveness due to time constraints and errors in their testing protocol. Also, the grade of polystyrene used did not contain polymerization inhibitors, causing the matrix to become viscous. Additionally, polystyrene was not tested against other matrix materials.

Problem Statement

High accuracy oxygen detection has become a critical step in understanding various physiological effects of hypoxia. Previous work on this design project has produced a functioning microfluidic hypoxia culture device chamber and preliminary work on the oxygen sensor. A real-time oxygen sensor is needed to monitor the oxygen gradient across the channels of the microfluidic device without inhibiting or interfering with the cellular processes of interest.

Design Criteria

Of the requirements outlined in the product design specifications (Appendix A) for the oxygen sensor, there are four principal components that our team focused on.

The first is the simplicity, repeatability, and time efficiency of the protocol to fabricate the sensor. We believe that a simple protocol would help maximize the effectiveness of the Ogle lab's research, as well as allowing it to be more broadly applied to other areas of microfluidic research. A lack of complexity saves time, energy, and money by minimizing the possibility of errors on the part of the researcher. Ideally, the overall microfluidic device and sensor construction should, together, take less than a day so that the experiment is ready to begin the following day.

The second focus is non-cytotoxicity. The sensor design must not utilize any materials that compromise the life of the stem cells or cardiomyocytes. In addition, they should not interfere with the cellular processes within the culture channels and not consume any oxygen.

The sensor must be accurate to within 2% of actual oxygen concentration. A lack of accuracy would compromise the experimental validity and hinder the ability of the researcher to correlate a certain fluorescence to a specific oxygen concentration.

Lastly, the sensor must be compatible with both the microfluidic device and confocal microscopy. The method of integrating the sensor with the microfluidic device must not compromise any the functions of the device. Additionally, the sensor and the method of attachment must be translucent to allow the microscope to view the cells through the sensor material. Otherwise the ability to observe cell behavior will be compromised.

Design Alternatives

Alternative Design 1: Oxygen-Based Luminescent Sensor

There are many oxygen-based luminescent sensors available for detecting oxygen concentration. These sensors fluoresce differently according to different oxygen concentrations, observable under a microscope. The team investigated two sensors which are commonly used.

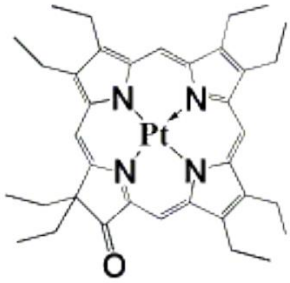

Figure 5: PtOEPK protein

Platinum (II) octaethylporphyrinketone (PtOEPK), shown in Figure 5, is a fluorescent porphyrin molecule. It was used in a previous semester's work with a polystyrene matrix as a solid state oxygen detector. It acts as a hydrophobic dye with excitation wavelengths of 408 nm and 601 nm and an emission wavelength of 791 nm.

The PtOEPK sensor has a relatively high sensitivity to oxygen with high photo-stability compared to the other methods that were considered. It also introduces a relatively low cost as the thin film sensor could be reused for multiple experiments, over the course of a few months. However, its biological nature leads to variability during detection. The two step interaction between molecule and oxygen and molecule and microscope leads to this stochastic nature. As it does not provide direct measurement, there might be variation between the actual oxygen concentration and the fluorescence the microscope reads. Moreover, due to its non-standard excitation and emission wavelengths, it is not compatible with the microscopes available to the design team. Extra filters would have to be purchased for future work with this sensor, albeit at relatively low cost[8, 9, 10].

Dichlorotris (1, 10-phenanthroline) ruthenium (II) hydrate (Rudpp FIG) has a linear response to oxygen concentration. This sensor gives high intensity data in low oxygen concentrations, which is relatively error free because of its high signal to noise ratio, a desired property in hypoxia measurement. It also has standard excitation and emission wavelengths, which are 455nm and 613nm, respectively, allowing easy and convenient data collection with typical filters for microscopes. Moreover, Rudpp molecules have a long duration before bleaching, providing a promising service life for research despite its disposable nature. In addition, it is relatively cheap compared to other sensors.

Alternative Design 2: Bead Injection Spectroscopy (Liquid Sensor)

Bead injection is a recently developed technique where a specific volume of bead suspension is injected and acts a sensing layer, which is disposable. As an oxygen sensor, the beads would be bonded non-covalently to oxygen phosphorescent probes. The beads are then trapped next to the cells and placed under a non-destructive spectroscopic detector (Figure 6). The fluorescent signals from the beads would then be picked up and measured.

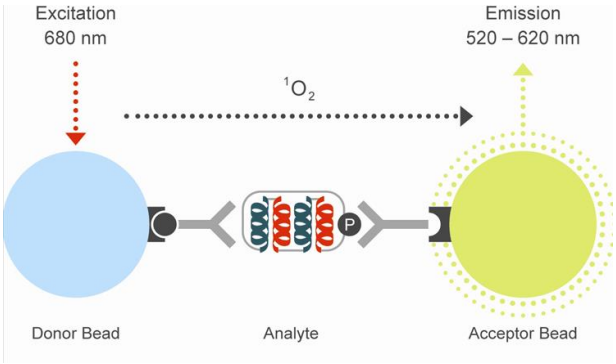

Figure 6: a typical bead injection system

In particular, the acid form of Pt-tetra (paracarboxy-phenyl) porphyrin tetraethyl ester, Pt-(p-COOH)4PP was used as the oxygen-dependent quenching phosphorescence. The excitation wavelength used was 509 nm and emission wavelength was 550 nm.

With the BIS method, the beads can be placed proximal to the cells and measurements can be made at that highly specific location. It also allows a time scale of minutes for oxygen detection. Moreover, it avoids the problem of sensor surface degradation that other sensor devices may have. However, the biggest drawback of the BIS method would be the consumption of oxygen during detection, which would not be desirable as this can significantly skew the accuracy of the oxygen concentration measurement. Additionally, there is only a limited amount of literature available on the success of this method, so its accuracy and viability has not been verified[12, 13].

Alternative Design 3: Electronic Sensor

The electrical sensor (Figure 7) makes use of the energy generated during the collision of oxygen molecules and a fluorescent chemical in its excited state in order to measure oxygen concentrations. An optical fiber carries a light at around a 475 nm wavelength to a probe. There is a layer of hydrophobic sol-gel at the tip of the probe, which is excited by the light from the fiber and emits energy at around 600 nm. The presence of oxygen molecules generates excess energy, which is then collected by the probe and sent to the spectrometer.

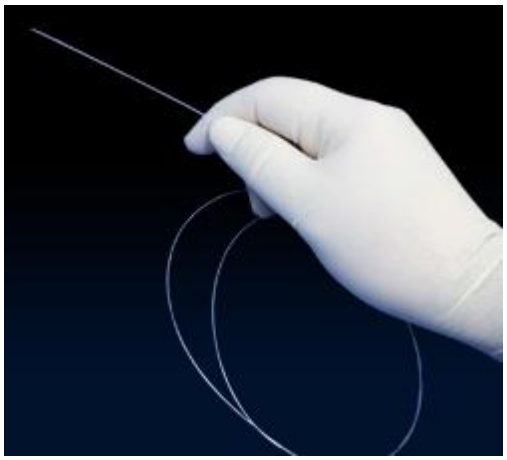

Figure 7: a typical optical sensor

This method is relatively accurate compared to the other sensors due to its strong basis in fiber optics. It also produces discrete quantitative data, unlike the other sensors which rely on color detection. Moreover, it is reusable for multiple experiments. On the other hand, it requires an array of individual probes to provide a complete curve of data, and would therefore be a large initial investment. Also, potential changes are needed for the microfluidic device designed by the previous teams to adapt to the size of the sensor and prevent interference with the cells[11].

Design Matrix

Category	Oxygen-Based Luminescent Sensor	Bead-Injection Spectroscopy	Electronic Sensor
Sensitivity (25%)	4	2	4
Accuracy (25%)	3	2	4
Integration (15%)	4	3	2
Ease of Fabrication (10%)	4	5	1
Response Time (10%)	4	4	5
Cost (15%)	3	3	2
Total (out of 100)	72	56	64

Table 1: Design Matrix detailing category weights and scores for the methods considered.

Observing Table 1, sensitivity and accuracy are the two most heavily weighted categories. Accuracy is a measure of how close a measurement is to the true value. Sensitivity (more commonly understood as its equivalent: precision) is a measure of how closely grouped a number of measurements are to a given value that may not necessarily be the true value. Something that is both accurate and sensitive will not only give a measurement close to the true value, but will also remain close to that value over a number of measurements. An oxygen sensor necessarily must be able to satisfy both of these functions. The electrical fiber optic sensor scored best overall for the two categories combined because

according to the supplier, it provides consistent measurements. Errors in sensor systems generally appear when one signal has to be translated into another type of signal. While electronic sensors require only one signal conversion, both BIS and luminescent sensors require at least two conversion steps. Protein sensors are subject to degradation, mishandling, or improper procedure when making them, and hence may lose their accuracy. Bead injection Spectroscopy (BIS) consumes oxygen during operation, earning it the lowest score for sensitivity and accuracy because this consumption could significantly skew the measurements. In addition, bead injection spectroscopy (BIS) is currently under development and there is disagreement concerning its actual sensitivity and accuracy.

Integration is a "measurement" of the ability of the sensor to be integrated with the current channel design. Our design must be compatible with the cell culture microfluidic device that creates the hypoxia condition. Luminescent sensors scored highest in this category because they can be fabricated into a thin sheet which can be easily attached onto the top of the culture device. Utilizing BIS or an electrical sensor would require modification of the channel and/or cell culture protocol to align with the sensor's method of oxygen measurement.

Ease of fabrication is a measure of how simple the device would be for the design team, or a lab associate, to create. This is a relatively important standard to consider because as a research tool, new sensors will be made frequently when needed in experiments. The electrical sensor scored the lowest in this category because these fiber optic devices involve complicated and sensitive fabrication methods that would be difficult for the design team to reproduce. A system of the electronic fiber sensors could be purchased, but this would be outside of budget. BIS scored the highest in this category because the liquid sensor is available for use as it comes, only requiring simple dilution, and does not require an extra physical sensor matrix as is necessary for the luminescent sensors.

Response time is a measure of how quickly the sensor adjusts to and reports changes in oxygen concentration. The oxygen-based luminescent sensors will report changes in oxygen concentration by exhibiting a color change, which is relatively fast, on the order of seconds to minutes. Depending on the sensor, the electronic system that will be used to analyze the signal may have fast response times on the order of milliseconds. Another factor that can affect response time is the calibration of the sensor and the software and analysis tools. These tools have a relatively small impact on the time for response, but can contribute to errors[14].

Cost is relatively important to this project. Although it is not as important as some of the more technical requirements, it is an aspect. None of the options are especially cheap, but the electrical sensors border on $600 per sensor. Our applications would require an array of sensors, multiplying the cost. A 10mg vial of PtOEPK costs around $235, a 1 g vial of Rudpp costs $85, and 10 mg of BIS would cost $250 ~$300. Although the electronic sensor can be easily used for up to a year, the PtOEPK and Rudpp sensors can also last on the order of months[15, 16].

As can be seen in Table 1, luminescent sensors scored the highest by a fair margin, justifying their use as the preferred option for sensor fabrication. The final design section further describes how this protein sensor will be implemented and made compatible with the current channel design.

Design Development

PtOEPK vs. Rudpp

Studies have shown that both PtOEPK and Rudpp have the potential to be used as oxygen-based luminescent sensors. In addition, there has been success in fabricating both of them into solid-state sensors. This made it difficult to judge the feasibility of their use based solely on literature. Here we conducted a series of experiments that aimed to compare the effectiveness of PtOEPK and Rudpp as oxygen sensing molecules.

The excitation and emission wavelengths used for PtOEPK were 398 nm and the emission length 760 nm respectively. This combination of wavelengths is non-standard and creates difficulties in using this molecule. Specifically, the 760 nm emission requires a filter that is difficult to obtain. In addition, the bandpass/tolerant lengths of the emission waves are very narrow and consequently make it harder to observe.

The Rudpp sensor has a common DAPI excitation wavelength (455 nm) and a standard Texas-Red emission wavelength (613 nm). In addition, both the emission and absorption wavelengths are in a wide bandpass range and hence allowing easier observation and data collection.

In the first experiment, PtOEPK sensor was fabricated according to a protocol optimized by a design team in 2012. PtOEPK was dissolved in toluene with 7% PS for structural support. It was then delivered into a 96-well plate to dry overnight while being covered with foil to protect it from light. Once the sensor was dry, it was placed into an oxygen chamber and tested under several different concentrations of oxygen. The intensity data was collected via fluorescent microscopy based on averaging the florescent emission in a pre-defined area of interest (AOI). Intensity data was then plotted against the concentration of oxygen and statically analyzed.

Similar experiments were conducted on Rudpp. First, the Rudpp was diluted in ethanol to make a 50uM solution. The solution was then placed into a 96-well plate and tested under several different concentrations of oxygen. The intensity data was collected by florescent microscopy based on averaging the florescent emission in a pre-defined AOI. Intensity data was then plotted against the concentration of oxygen and statically analyzed.

Intensity: As can be seen from the curve, the PtOEPK intensity does not change significantly when oxygen concentration changed from 20% to 5%. The Rudpp sensor, however, showed a significant change with respect to oxygen concentration changes.

Compatibility: it is worth mentioning that when fabricated according to the pre-optimized protocols, the PtOEPK sensor has a non-transparent purple color that will strongly affect the microscopy of the cells (Figure 8).

Conclusion: Given the experimental results, we conclude that the Rudpp is easier to fabricate, has more significant change in emission when oxygen concentration changes and is ultimately more reliable as the sensor molecule for the purposes of this project.

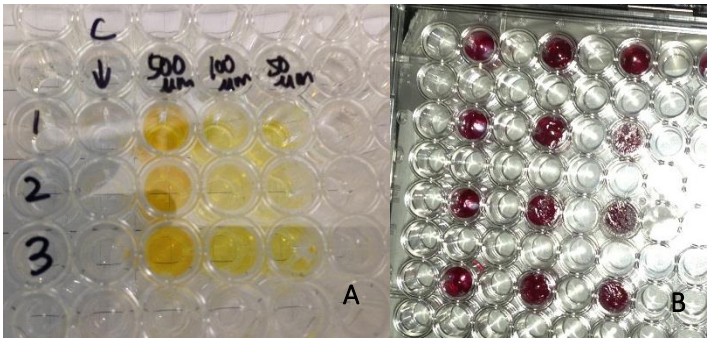

Figure 8: bright field picture of Rudpp (A) and PtOEPK (B) showed that Rudpp sensor is more microscopy-friendly

PS vs. PDMS

Polystyrene and PDMS are both classic biomaterials that are used in cell culture and microfluidic devices. Studies have also shown success in both polystyrene and PDMS as solid-state sensor matrixes. PS and PDMS, however, have significant differences in their properties and are optimized for different applications. Considering the simplicity of incorporating the molecule onto the matrix material, PS is hydrophilic, which made it hard to coat molecule onto. PDMS, however, is not only very hydrophobic but also absorbs small molecules. Also, as a supporting matrix, a thin film of PS is more brittle and hard than PDMS. PS is also not gas permeable while PDMS is completely gas permeable. To compare the two materials and choose an optimized matrix for this project, we conducted experiments using both PS and PDMS.

Both of the experiments were conducted using Rudpp molecules since it has a better response curve when tested alone. Sensor matrices of PS and PDMS were each fabricated and then coated with Rudpp solution. The uniformity of the coating is observed and compared.

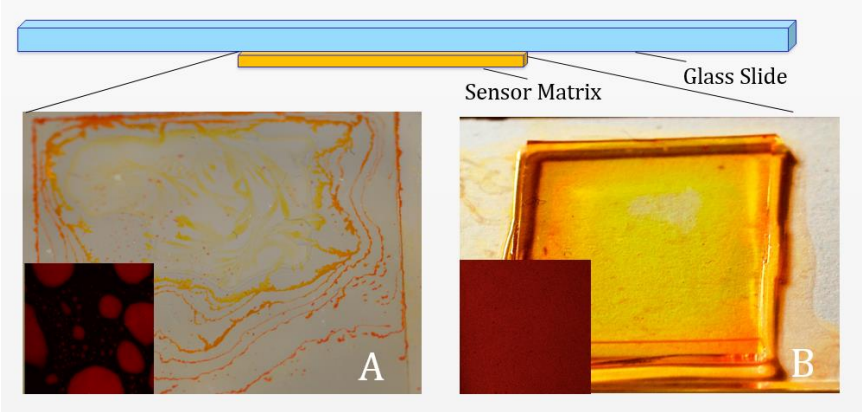

Figure 9: Image A is the Rudpp sensor incorporated (via soaking) onto a polystyrene matrix and Image B is the Rudpp sensor incorporated onto a PDMS matrix.

As can be seen from the Figure 9, the PS coated sensor is not uniformly distributed while the PDMS matrix shows a more uniform distribution. Therefore, the PDMS is a more feasible matrix material for this sensor. However, considering the fact that PDMS is gas permeable, an additional layer of a different material is required to prevent gas exchange between atmosphere and device, which could dramatically alter the results in a real experiment.

Final Design

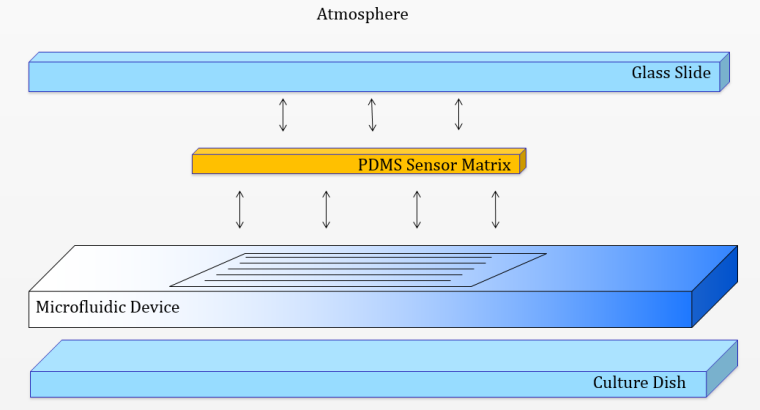

Figure 10: Final design layout. The culture dish layer may be replaced with a cell culture glass slide

The final design of the device, illustrated by Figure 10, involves three distinct components. In the cell culture compartment developed by a design team in 2012, there are eight parallel micro-channels for cell movement and two perpendicular gas channels on either end to develop an oxygen/nitrogen gradient. The final design of the sensor involves a PDMS film coated with Rudpp, which has a linear response to oxygen concentration. With the previously developed microfluidic device attached on a glass slide, the

sensor matrix is placed on top of the channels to monitor the oxygen concentration. Another glass slide is used as an anchor to cover the sensor matrix and prevent gas exchange between the microfluidic device and the atmosphere.

Cost Analysis

Although cost was not a principle design criterion, the final design is very cost-effective. The material components, glass and PDMS, can be bought in bulk. The total cost for those components is approximately $2.00 The Rudpp sensor molecule is also cost-effective. If bought individually, (from a retailer like Sigma Aldrich) it is between $50.00 - $100.00 for one gram. This sensor can also be bought in bulk or for research discount and will amount to approximately $1.00 per sensor. The total cost, per sensor, is approximately $3.00, making it a viable option for the Ogle Lab as well as other labs that may choose to use the sensor in the future.

Testing and Results

Sensor molecule testing & concentration

To optimize the concentration of Rudpp sensor molecule and test the sensor's response curve, Rudpp was made in 500uM, 100uM, and 50uM solutions. Each concentration was tested at 20%, 15% and 8.5% oxygen and the results were analyzed.

	Control average	500uM	Error (STDV, Control)	Error (STDV, 500)
20%	341.44	525.91	0.21	41
15%	343.03	573.67	0.08	34
8.5%	343.57	882.86	0.17	47

Table 2: sensor molecule response data

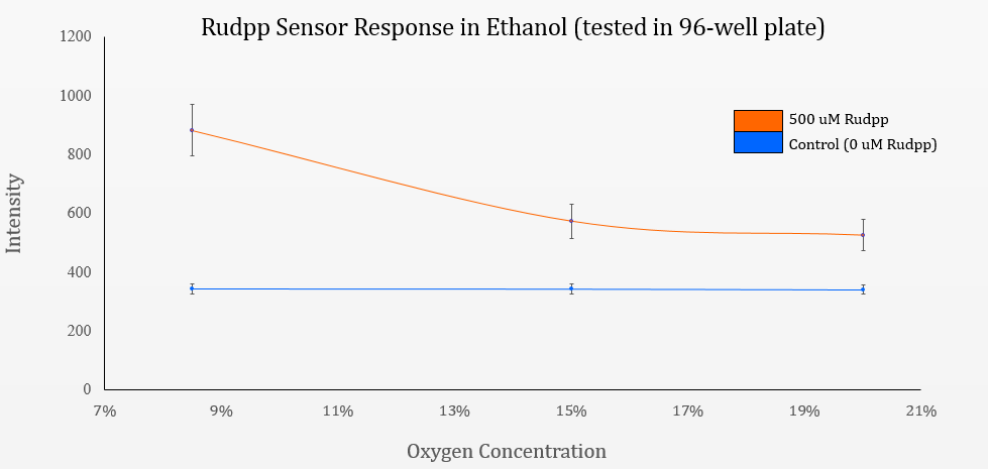

Figure 11: Sensor molecule response curve, best fit curve

Data interpretation

Shown above are data from three independent trials at the 500uM concentration. Although the sensor curve has a reasonable trend at lower concentrations, some nonsense data was observed in each concentration. Considering the coating method requires higher concentration to function, the final decision of the sensor molecule concentration was 500uM.

Final Design testing

After the final concentration of the sensor was decided, the final design was fabricated and tested to obtain another response curve. Three independent trials were tested each under three oxygen concentrations (20%, 15% and 8.5%) with a confocal fluorescent microscope. The results are analyzed and displayed below:

	20%	15%	8.30%
Experimental Group 1	1067	1115	1219
Experimental Group 2	833	922	1057
Experimental Group 3	670	803	836
Average	856.6666667	946.6666667	1037.333333
STDV	162.936252	128.5621337	156.9762898

Table 3: final design response data

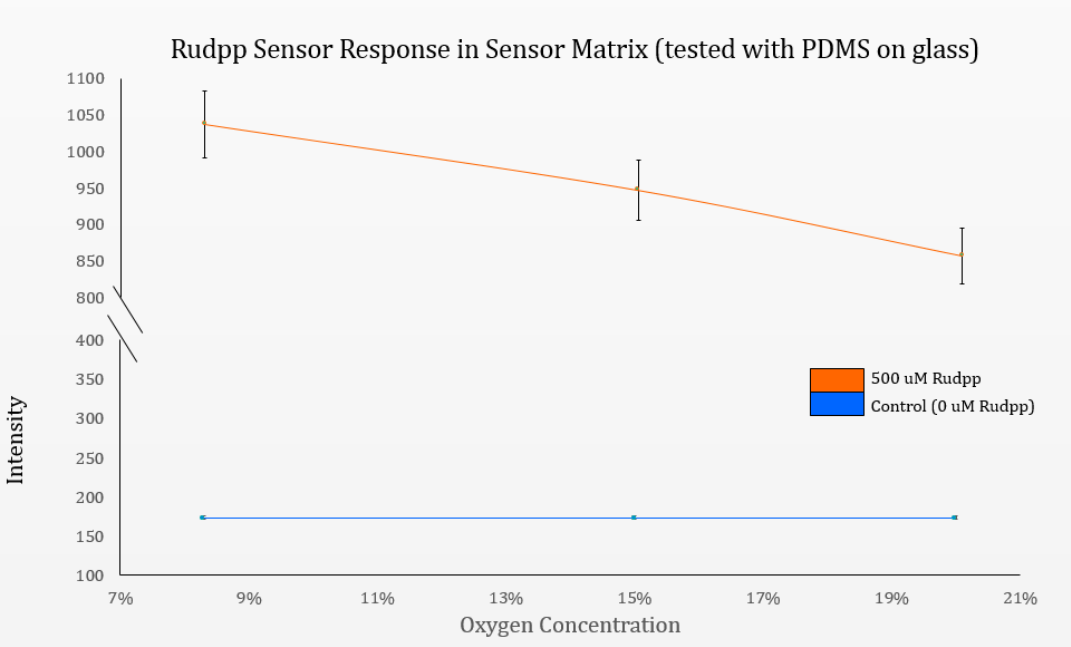

Figure 12: Sensor molecule response curve, best fit curve

Data Interpretation

As can be seen from Figures 11 and 12, when dissolved in solution, the Rudpp response curve "quenched" at ~15% oxygen, whereas matrix-incorporated Rudpp displayed a linear decrease.

This is due to both the nature of the sensor molecule and the design set-up. The Rudpp sensor molecule itself has a relatively long time response (~ 1 minute), which means that once the oxygen concentration in the surrounding environment changes, it takes about a minute for the molecule to change emission intensity. The fact that the sensor was in solution in a 96-well plate with only limited surface area contacting the air made this response time even longer, highlighting another reason that the matrix design was used.

When conducting the experiment, we started at 20% oxygen, lowered to 15% (takes only around two minutes), and took a picture immediately when oxygen concentration arrived at 15%. Then we continued to lower to 8.5% (which took more than 10 minutes), and again took the pictures immediately. Together with the curve, this experiment showed that the time to change oxygen from 20% to 15% is shorter than the required time for the sensor to change emission, but the time to go to 8.5% oxygen is much longer. This explains why the curve is not linear in solution in the 96-well plate. When coated onto the surface of the matrix, the sensor is fully exposed to the air in a much larger area and consequently we were able to significantly reduce the response time. This is supported by the results in that: 1) the graph is more linear, 2) the 20% to 15% oxygen region saw a significant increase in intensity once the sensor

was coated onto the matrix, and 3) the intensities at 8.5% and 15% oxygen are higher in the matrix condition than in solution.

Taken together, the sensor matrix not only prevents gas exchange between the device and the atmosphere (along with the glass slides), but also improved the Rudpp molecule response by making the response more linear and decreasing the response time.

Future Work

With the oxygen sensor design completed, future work will focus on further validating the design and then integrating the oxygen sensor with the PDMS microfluidic device created by the Spring 2012 Design Team. The sensor needs to be tested with a wider variance of oxygen concentrations. The microscope setup we used could not attain oxygen concentrations lower than about 8%. Validation of the sensor at even lower oxygen concentrations will ideally show the flexibility of the sensor. Additionally, the response time of the sensor needs to be validated. Although we expect it to be longer than PtOEPK due to observations during testing, determining precise response times is useful for constructing experiment protocols.

Once these characteristics of the sensor have been experimentally validated, the original micro-hypoxia chamber needs to be verified. Although seemingly well-designed, the Spring 2012 design team had no way to see if their device developed a gradient appropriately because they lacked the oxygen sensor that we now possess. Using our sensor, we need to make sure that the device develops a smooth and dependable oxygen concentration gradient and can appropriately house cells. The sensor and microfluidic device will be integrated, as detailed in the *final design* section, and these concerns will be addressed assuming that the sensor can still be appropriately calibrated once integrated with the microfluidic device. Due to the gas-permeable nature of PDMS, few problems are anticipated with calibration.

Lastly, it is potentially relevant to explore the capabilities of the oxygen sensor we created outside the realm of research on stem cells in hypoxic conditions. It is possible that there are other areas where a fluorescent oxygen sensor could prove useful. In fact, our sensor is not limited to the “micro” condition that this project dictates. A sheet of PDMS onto which we coat the sensor molecule could potentially be much larger, expanding the possibility of relevance to other research areas.

Conclusion

The primary goal of this project was to create an oxygen sensor for use with a microfluidic cell culture chamber. The main requirements for the sensor were accomplished through sound testing and reliable research. The journey to the final design involved redesigning and re-evaluating. Some events, however, stand out as highlights in the creation of the final design. In the beginning of the semester, the team was testing PtOEPK as the sensor material, but preliminary tests showed that PtOEPK did not meet the requirements of the design criteria. After further research and consultation, the team began investigating Rudpp as the sensor material, which served the needs of the sensor much better than PtOEPK. Additionally, PS was the original matrix material, but testing showed that this material caused

non-uniform distribution. After further research and re-evaluation, PDMS was selected due to its gas-permeability and the ability to support a uniform coating of the sensor material. The validation of the sensor and matrix showed that the design requirements were largely met.

The Rudpp sensor matrix, with its linear intensity response to oxygen concentration, satisfies the four principal design criteria that the team chose to emphasize as especially important to the efficacy of the sensor. The linear trend and strong intensity response make the Rudpp sensor highly accurate; the oxygen concentration measured is well within two percent of actual. Research has determined that Rudpp is non-cytotoxic, making it safe for use with stem cells and cardiomyocytes. The research team developed a protocol that allows for both the sensor and the microfluidic device to be prepared in less than a day, lessening the "busy work" of this research. The experimental preparation protocol is simple enough to be completed by a relatively inexperienced undergraduate, although using the microscope will require someone with training. Lastly, although not backed by concrete testing, full compatibility is expected between the sensor and the device due to the gas-permeable nature of PDMS.

Our work this semester certainly has potential to be applied elsewhere in research involving oxygen concentration measurement. The PDMS matrix can be cut to much larger sizes than that used for this project, expanding the possibilities beyond the "micro" level. Its thinness allows it to be easily adhered to whatever surface necessary for the experiment. Also, the sensor and microfluidic chamber we created does not have to be limited to stem cells and cardiomyocytes. The sensor and device could be used to study other cells in hypoxic conditions as well.

This project emphasizes the value of re-evaluation and adaptation to the design process. The design process is not merely following one step after the other. It involved going "back to the drawing board" as necessary in order to end up with the best possible product. This semester's work reiterates that the design process is dynamic, not linear.

Stem cell fusion in hypoxic conditions has the potential to profoundly change the discussion of cardiomyocyte regeneration and healing. This technique could help prevent future heart problems for those who have experienced them before, which is a powerful possibility given that heart conditions usually degenerate until death. If this technique is to ever see use in real patients, it must be well understood in ideal *in vitro* conditions first. Our work would prove highly useful to the end.

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Appendix

Appendix A: Product Design Specifications

Development Oxygen Sensor for Microfluidic Hypoxia Chamber

Product Design Specifications

February 6, 2013

Client: Professor Brenda Ogle, PhD

Advisor: Professor John Puccinelli

Team: Sarvesh Periyasamy, Roland Pomfret, Lok Wong, Jiaquan Yu

Function:

High accuracy oxygen detection has become a critical step in understanding various physiological effects of hypoxia. The purpose of this project is to test, redesign and produce an oxygen sensor that can be used with the microfluidic-based hypoxia chamber. Previous work on this design project has produced a functioning microfluidic-based hypoxia chamber and preliminary work on the oxygen sensor. This semester will focus on testing the hypoxia chamber, re-examining possible oxygen sensors, and implementing the two systems together. After confirming the hypoxia chamber and oxygen sensor have been assembled properly, the device will be used in experiments involving oxidative stress, ischemia, and reactive oxygen species (ROS)-mediated cellular pathways.

Client Requirements:

- Test the reliability and accuracy of the current preliminary oxygen sensing design using a 48 well plate.
- Improve the system so that it can detect the oxygen concentration from 100% oxygen to 1% oxygen with an accuracy of at least 2%.
- All components of the device must be compatible with the microfluidic device (made with PDMS).
- The device must not have any cytotoxic effects on the cells being cultured, which might be in close contact with the device.
- The sensor may be placed on the top or bottom of the microfluidic device without affecting microscopy.
- The protocol should be easily repeatable and the fabrication should not be time consuming as new sensors have to be made for each experiment.

Design Requirements:

1. Physical and Operational Characteristics

    a. Safety: The oxygen sensor must be safe for individuals to handle in a laboratory

setting. The device must be sterile in accordance with the cell culture protocols that the device will be used with. The materials used should not leak any toxic residue in high temperature situations (such as when autoclaved).

b. Accuracy and Reliability: The accuracy and reliability of the sensor will depend greatly on the fluorescent dye and microscope. We are aiming for a less than 2% error rate within each sample and less than 5% variation between samples.

c. Life in Service: The device and the oxygen sensor for it are of disposable nature. After each experiment, which can last up to approximately seven days, both the device and the sensor will be disposed of and new ones will be made for the next experiment.

d. Shelf Life: Since a device is made as needed, shelf life is not a significant factor in this project. We propose a one month functional storage time.

e. Operating Environment: The oxygen sensor should be stable in the incubator (37 C 5% C02). The sensor will be in close contact with and should not be affected by cell culture media, serum, and other chemicals. The sensor should also give a reliable fluorescent signal in a room temperature environment for an hour in order to provide enough time for microscopy.

f. Ergonomics: The device should be easy to use by a variety of researchers. It should be small enough to hold in the hand and be simple enough to be operated by inexperienced users.

g. Size: The dimension of the sensor should be made according to the dimension of the device so that the sensor can be assembled to the device. The device has a six-inch diameter.

h. Weight: Weight is not a critical design constraint at this time. The weight of the device should allow it to be carried easily in the hands of the researcher. We aim to keep the mass under 50 grams.

i. Materials: The materials used should be standard materials used for solid state sensors. Possible materials include fluorescent dye, PDMS, polystyrene and other standard cell culture materials.

j. Aesthetics, Appearance and Finish: Since the sensor may be placed over the microfluidic device, the sensor area above the culture should be transparent and clear so that microscopy is not affected. The sensor should have sufficient sealing with the microfluidic devices.

2. Production Characteristics

a. Quantity: There will be one oxygen-detecting unit produced for one micro-hypoxia

chamber.

b. Target Product Cost: The proposed budget for this semester is $500.

3. Miscellaneous

a. Standards and Specifications: The product is not drug related and does not require any FDA approval. Neither human nor animal testing is required so there are no concerns for approval. The only protocol that the device must adhere to is the mammalian cell standard operation procedures and specifications.

b. Customer: The device is a custom design for Dr. Brenda Ogle and graduate student Brian Freeman. Eventually, other members of the Ogle lab will be utilizing the device and, if applicable, other labs doing similar research at other institutions.

c. Patient-related Concerns: The device will be used in a purely research setting so there are minimal concerns regarding patients. There is no patient data or other sensitive information at risk.

d. Competition: Most of the competing devices are from various other labs and universities and are therefore optimized for their own research projects. The University of Michigan created a well-known thin-film sensor which is too expensive for the Ogle Lab. The Microtechnology Medicine Biology Lab at the University of Wisconsin-Madison also created an oxygen sensing system which is not optimized for Dr. Ogle's purposes.

Appendix B: Original PtOEPK Sensor Matrix Testing Protocol

1. Fabrication:

After the design is optimized, the PtOEPK sensor protein will be added to the matrix for sensitivity testing. Fabrication protocol will be decided by testing and optimizing alternative designs. To achieve the best model of actual experimental conditions, the sensor will be integrated onto the cell culture device during the testing.

2. Material & Methods:

Sensors made in the first experiment will be dissolved into toluene and diluted into different concentrations. New material will be used to make exact concentration of PtOEPK sensor.

Oxygen concentration will be manipulated and intensity of emission light will be recorded accordingly. The device, with and without media, will be tested to examine the effect of cell culture media on sensor function. A calibration curve of emission intensity versus oxygen concentration will be plotted. Each condition will be tested on both 405 and 601nm wavelength of excitation and will be measured in 791 nm emission wavelength. The results between different excitation wavelengths will be compared and analyzed.

3. Conditions tested:

3.1: concentration of PtOEPK

0.1% w/w; 1% w/w; 2% w/w; 5% w/w; 10% w/w;

3.2: time in oxygen condition

We will adjust the concentration of oxygen surrounding the sensor environment and let the sensor stay in the condition for 1, 3, 5 minutes before measuring intensity. This will give us information regarding the response time of the sensor.

4. Procedures:

4.1 Sensor fabrication: will follow optimized sensor matrix fabrication protocol (to be decided) with each concentration in 3.1 added. Three copies of each condition of sensor in 3.1 will be fabricated.

4.2 Sensor liquid will be placed and dried out overnight.

4.3 Sensor will be placed into the oxygen chamber, oxygen concentration will be adjusted and intensity will be measure at different time point (3.2)

4.4 Each sensor will be tested using both 405 and 601nm wavelength of excitation and will be measured in 791 nm emission wavelength. The results between different excitation wavelengths will be compared and analyzed.

4.5 Measured intensity will be plotted vs. oxygen concentration in each condition.

Appendix C: PDMS matrix and Rudpp Sensor Protocol (Final)

1) PDMS Sensor Matrix preparation:

a. Weigh 20 g of PDMS
b. Add 2 g of curing reagent (1:10 ratio of curing reagent : PDMS)
c. Mix the gel and curing reagent until it forms a uniform gel
d. Put into vacuum chamber to de-gas for at least 60 minutes
e. Check to make sure there are no air bubbles in the solution (it should be transparent)

2) Sensor Matrix Spin Coating:

a. Open Spin coating machine
b. Put a 6 inch silicon wafer on the spinner and make sure it is centered and leveled
c. Set spin coating parameters to be “program 2”, 100 rpm, 5 rpm/sec rate of increase and 60 seconds.
d. Carefully pour 20 g of PDMS on the middle of the wafer.

e. Start the machine and complete the spinning process.

3) Sensor Matrix Fabrication:

a. Carefully move the silicon wafer and PDMS onto a hot plate, level the hot plate, and remove any air bubbles in the PDMS

b. Bake PDMS for 4 hours at 80 ^{o}C

c. Carefully remove the PDMS from the hot plate (after the hot plate cools back to room temperature)

d. Carefully remove the PDMS from the silicon wafer and measure height (should be around 600um)

e. Cut the PDMS into desired dimensions (corresponding to device dimension)

f. Soxhlet extraction at 90°C in 100% ethanol for 3.5 hours (4 cycles) to remove uncross-linked PDMS and increase adsorption

g. Carefully remove the sensor matrix out of the Soxhlet extractor after it cools down and keep sensors in sterilized container

h. [Optional] You may autoclave the PDMS sensor to ensure it is sterilized

4) Rudpp Sensor Coating

a. Take a 5 ml centrifuge tube and dissolve sensor molecule (Rudpp) in ethanol to make 50 µM solution for 2ml [Note: this process needs to be conducted in cell culture hood]

b. Submerge the sterilized PDMS matrix into Rudpp sensor solution

c. Seal the 5 ml tube with Parafilm, set overnight (covered with aluminum foil to protect from light) to allow full coating of the PDMS [Note: this process needs to be conducted in cell culture hood until covered with aluminum foil]

d. Take the PDMS matrix out of the solution and attach to a glass slide [Note: this process needs to be conducted in cell culture hood]

e. Remove excess sensor liquid on the glass, flip the sensor, and put on top of the cell culture device. (PDMS sensor should seal with PDMS device with no bubbles in between. Gas channel inputs and outputs should not be covered by the glass to allow tubing connections.)

Appendix D: Executive Summary

Heart disease has been the number one cause of death in the United States for a number of years. One of the major causes of heart failure is the massive death of smooth muscle cells due to hypoxia. Although there are temporary surgical and therapeutic solutions to restore oxygen levels, the damage to the smooth muscle cells is permanent. One proposed treatment for the permanent damage to the cardiac tissue is the use of stem cell fusion. Publications have revealed that once fused with damaged tissue, stem cells can differentiate and help regain tissue function. Although this phenomenon has

previously been studied, there is no *in vitro* method that probes the stem cells' ability to "fix" the cardiomyocytes under quantifiable hypoxic conditions.

Current devices developed by various labs are proficient in studying stem cell behavior, but lack the integration of an oxygen sensor. Labs at the University of Wisconsin and at the University of Michigan have developed thin film oxygen sensors that serve monitoring functions similar to the requirements of this project, but have never been tested for cell culture purposes. The experimental demands of studying stem cells in small and differentiated oxygen concentrations requires a sensor that can monitor oxygen concentrations from 0% to 20% and can be integrated with a microfluidic device in a unibody design. One microfluidic device that is capable of creating an oxygen gradient will be able to provide continuous data under many different concentrations of oxygen.

The final design of the microfluidic hypoxia cell culture device involves a polydimethylsiloxane (PDMS) culture compartment and a dichlorotris (1, 10-phenanthroline) ruthenium (II) hydrate (Rudpp) sensor component. The cell culture compartment was developed by a design team in 2012 and is being improved and integrated with the sensor design by the current team. In the microfluidic device, there are eight parallel micro-channels for cell movement and two perpendicular gas channels on either end to develop an oxygen/nitrogen gradient. The most challenging element, the sensor, is a polystyrene film coated with the ruthenium-based fluorescent molecule which has a linear response to oxygen concentration. This sensor gives high intensity data in low oxygen concentrations, which is relatively error free because of its high signal to noise ratio, a desired property in hypoxia measurement. The final design is comprised of a bottom layer of cell culture dish, a middle layer of the PDMS culture chamber, and the oxygen sensor-coated glass slide on top of the channels of the chamber. This design minimizes gas exchange between the PDMS device and the atmosphere.

Although PDMS microfluidic devices have been well validated for cell culture, testing was required for the sensor. Looking at the fluorescent intensity response of the Rudpp molecule, as oxygen concentration decreased, the mean intensity increased. In addition, the fluorescent intensity difference between the dissolved Rudpp molecule and the control without Rudpp was significant. Preliminary experiments also showed that coating Rudpp onto polystyrene will not adversely affects its ability to detect oxygen. The team plans to integrate the sensor with the microfluidic device to validate the function of the entire device in actual experimental conditions. With the sensor calibration complete, the team will ensure that it maintains its functionality when integrated with the device to measure an oxygen gradient in real-time.

The sensor fulfills the oxygen sensing requirements set forth by the client. The design of the microfluidic device should allow the client to develop and sense hypoxic conditions necessary for experiments that observe the interactions between stem cells and cardiomyocytes. The microfluidic technology developed in this project will allow the client to conduct the necessary experiments in providing a more in-depth understanding of stem cell behavior and can be used to investigate stem cell therapies for heart diseases in a physiologically relevant setting.