Cellular Model of Hydrogen Peroxide Release: In Preparation for On-Chip Sensor Measurements

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Abstract

Hydrogen peroxide is traditionally associated with cellular damage; however, recent studies show that low levels of H_2O_2 are released by cells as part of normal intercellular communication. The mechanisms of hydrogen peroxide transport, uptake and release, and biological effects are not yet well known but have important implications for cancer, stem cells, and aging. Standard H_2O_2 assays cannot make spatially or temporally resolved quantitative measurements at a cellular scale. Previously we developed a microelectrode array (MEA) and calibration methods for quantifying H_2O_2 gradients in space and time. The sensor was validated using artificial H_2O_2 gradients at subsecond and micrometer scale resolutions. The present study begins cellular work on H_2O_2 release to identify a cellular model system for MEA sensor testing. The morphology and H_2O_2 release from U937 human monocytes were analyzed after stimulation with ionomycin (1.2 ug/mL) and/or phorbol 12-myristate 13-acetate (PMA). Monocytes were stimulated with PMA (10 ng/mL to 150 ng/mL) for six hours. Hydrogen peroxide release was quantified over time using a traditional amplex red flurometric assay method. Mouse pancreatic beta (MIN6) cells were also tested as a negative control. Monocytes stimulated with PMA alone produced, on average, three times more H_2O_2 than those stimulated with ionomycin or a combination. Monocytes without ionomycin released H_2O_2 at 18.34 pmol/min/10^6 cells at 25 ng/mL of PMA. Ten, 25, and 100 ng/mL of PMA produced H_2O_2 significantly faster than the non-stimulated control. No significant difference was seen between PMA concentrations when ionomycin was added. These results indicate that PMA stimulated human monocytes may serve as a good model system for cellular validation of the H_2O_2 MEAs. In the future, biofunctionalization of the electrodes for additional molecular specificity will allow for the expansion of the method to other analytes, giving the sensor potential use in non-traditional lab environments with the ability to perform multiple assays autonomously.

Keywords

Hydrogen peroxide, biosensor, cellular peroxide release, lab on a chip, electrode array, real time sensor

1. Introduction

Hydrogen peroxide (H_2O_2), a type of reactive oxygen species (ROS), plays a well-established role in the killing response of immune cells to microbial invasion [1-3]. Previous research indicated that only phagocytic cells produced ROS and that oxidative stress was responsible for solely negative effects, such as cell damage and disease initiation [4-5]. H_2O_2 is now shown to be an important signaling molecule, indicative of metabolic activity [6]. In eukaryotes, cells

produce concentration-dependent responses to H_2O_2. Hydrogen peroxide has been hypothesized to aid in enzyme activation, to elicit proliferation in response to growth factors, to affect cell shape, immune cell recruitment, and to signal many additional responses [5-6].

With a slightly larger dipole moment than H_2O, simple diffusion through the hydrophobic bilayer is unlikely [6]. Hydrogen peroxide instead travels across cell membranes using water channels, called the aquaporins [6]. With similar dielectric properties, hydrogen bond formation, and dipole moments to water, and with a size of 0.25-0.28 nm, aquaporin (size 0.30 nm) facilitation is a realistic avenue of hydrogen peroxide transportation [7-9].

H_2O_2 was first documented to be a normal product of aerobic metabolism 1970 by utilizing spectrophotometry of catalase Compound I, a product of catalase with H_2O_2 [7]. Today, the main techniques for measuring H_2O_2 also include titrimetry, chemiluminescence, electrochemistry, fluorescence, and electron spin resonance [10]. Standard hydrogen peroxide assays lack the ability to give spatially or temporally quantitative measurements at a cellular scale. Assays measure H_2O_2 secreted into the extracellular fluid by averaging concentrations over large time intervals and cell quantities. Traditional methods are unable to capture the real-time cellular gradients of oxidative bursts. Electrochemical sensors provide an alternative method of quantifying hydrogen peroxide. Sensors, especially those based on microelectrodes, have high sensitivity, fast response, and easy miniaturization [10].

Electrode sensing began with single probe electrodes. By applying appropriate constant potential to the sensor, dissolved hydrogen peroxide oxidizes on the surface of the electrode, leading to an increase in current. Amperometric spikes can be analyzed and mapped to the uptake or release of hydrogen peroxide from cells [11]. Microelectrodes' miniaturized tips produce increased resistance and cause a significant decrease in the signal-to-noise ratio. A technique to improve drift and noise is to oscillate the electrode between two points at a fixed distance from the source. This noise-filtering microsensor technique is called self-referencing (SR). Flux is calculated using Fick's first law of diffusion according to the recorded change in concentration and known distance between the measurement points [12].

The SR technique has a limited temporal resolution as to not disturb the solution by stirring. In addition, single probe electrodes are inherently low throughput and obtaining statistically relevant sample sizes is a challenge, especially when multiple analytes are of interest. Multielectrode arrays provide multiple reference points without movement and increase measurement efficiency [10-13, 14-16].

We have previously proposed a novel three-layer scheme, using electroactive polymer nanocomposites precisely placed onto the electrodes of a fabricated Pt/Ir microelectrode, for the measurement of glucose transport [12-14, 17]. New microelectrode sensors, fabricated without enzymatic polymer layers, have shown subsecond and five micron scale resolution when characterized with artificial H_2O_2 gradients. The present study selects and characterizes a cellular model of H_2O_2 release for further sensor validation. Extracellular H_2O_2 concentration was fluorescently quantified from basal and stimulated mouse pancreatic beta cells (MIN6 cells) and human monocytes (U937 cells). The two cell types were chosen in order to compare their relative H_2O_2 production. Beta cells form 65-80% of pancreatic islets; they store and release insulin in response to glucose [17]. There is limited knowledge of H_2O_2 involvement in beta cell function [18]. Conversely, H_2O_2 production during monocyte differentiation and phagocytosis is established. Monocytes are large, non-adherent leukocytes that travel through the blood stream. Upon stimulation from pathogens, foreign irregularities, or cytokine signals, monocytes differentiate into macrophages. Macrophages adhere to the blood vessel wall, eventually embedding into the surrounding tissue and phagocytosing the invasion. Beta cells and monocytes

were observed in basal and stimulated environments against two experimental protocols. Quantification of H_2O_2 release using a traditional assay will provide comparative results for future sensor work. Development of the sensor can provide efficient, real-time, and noninvasive quantification of cell populations, which is a major technological improvement.

2. Materials and methods

2.1 Chemicals and reagents

Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit was purchased from ThermoFisher. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich. Medias and FBS (fetal bovine serum) were purchased from Life Technologies.

2.2 Cell culture

All cells were cultured in T75 tissue treated flasks, incubated at 37oC, and provided fresh media every other day. MIN6 cells were detached from flask when 80% confluency was reached using 0.05% trypsin/EDTA solution. U937 cells were removed without 0.05% trypsin/EDTA solution due to their nonadherence. After centrifugation, the cells were suspended in fresh media and transferred to a new flask. MIN6 cells were purchased from ThermoFisher and fed with DMEM (high glucose) media supplemented with 15% FBS (fetal bovine serum). U937 cells were purchased from ATCC and supplemented with 10% FBS. Cells were counted using Trypan Blue staining and a Countess Automatic Cell Counter (Invitrogen) prior to each experiment.

2.3 Quantification of hydrogen peroxide

2.3.1 Amplex Red Kit

The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit quantified extracellular H_2O_2. All assay components were obtained from the kit, including but not limited to hydrogen peroxide for the standard curve, sodium phosphate reaction buffer, dimethylsulfoxide (DMSO), and the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine). The Amplex Red reagent, in the presence of horseradish peroxidase (HRP), reacts with H_2O_2 in a 1:1 stoichiometry and is oxidized to resorufin. Resorufin was incubated at room temperature for 30 minutes and its fluorescence read at 545/590 nm Ex/Em. All fluorescent readings were taken with a BioTek Synergy NeoB plate reader. Resorufin slowly undergoes a second oxidation to a non-fluorescent product. All assay plates were therefore read same day as the Amplex Red mixture was added.

For experiments 1 and 2, a standard curve was developed from 20 uM of H_2O_2 to 1.1 uM using 75% serial dilution steps, plus a 0 uM blank. Each well contained 50 uL of H_2O_2 and buffer and 50 uL of Amplex Red reagent. The fluorescent value corresponding to each known H_2O_2 concentration was used to map the unknown H_2O_2 concentration in cellular studies. During experiment 3, a standard curve from 2 uM to 0.13 uM was developed using 50% serial dilution steps, plus a 0 uM blank. This more accurately modeled the H_2O_2 concentration ranges seen in cellular studies. Due to Amplex Red's continual development, each group of cellular fluorescent time points were modeled with their corresponding standard curve time point (Figure 1).

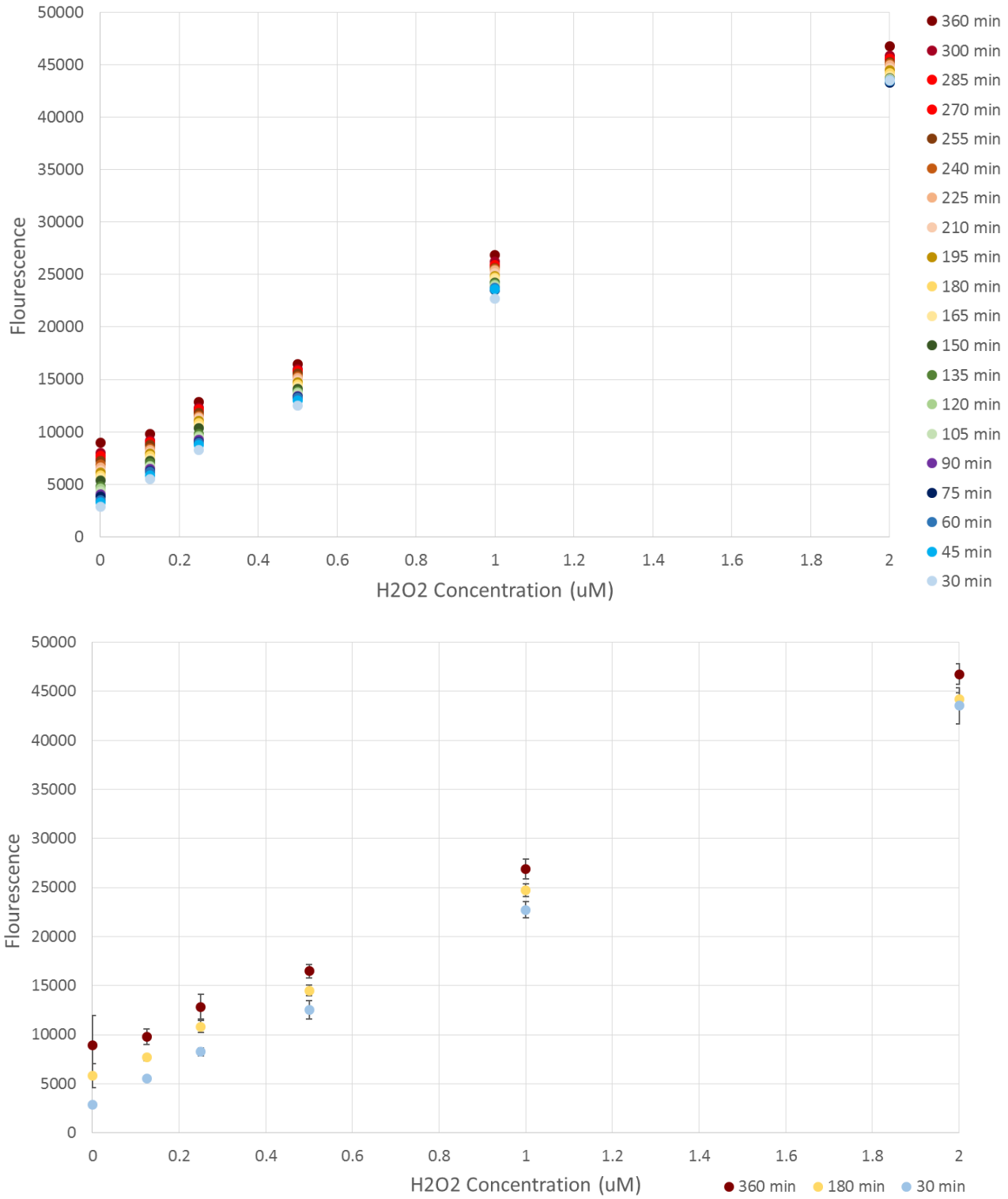


Figure 1. The standard curve. The fluorescence of resorufin, the oxidative product of the Amplex Red reagent, has an incubation time of 30 minutes. It continues to develop over time, however. The fluorescence-to-H_2O_2 concentration mapping is shown for every time point (top) where each time point is marked with increased fluorescence. The first, half-way, and last time point (bottom) more easily demonstrate this development, with ± one standard deviation. A standard curve mapping was uniquely created for each time point to minimize errors in H_2O_2 development.

2.3.2 Experiments 1 & 2: Transferring Buffer

Pancreatic beta cells were observed over five days in flat bottom ultra-low adhesion 6-well plates (50,000 cells/well) and 96-well Spheroid Microplates (10,000 cells/well). On day five, the media was removed and replaced with 0.05M sodium phosphate buffer. H_2O_2 release was monitored over two hours in basal (buffer only) and high glucose (16.7M) environments. At each time point, 50 uL of buffer was transferred from the cellular environment to a flat bottom 96-well assay plate. Following the last time point, 50 uL of Amplex Red reagent was added to each assay well and the fluorescence read (Figure 2).

H_2O_2 release was monitored from monocytes in flat bottom 96-well plates over three days, their typical macrophage differentiation period. Monocytes were tested in basal (buffer only) and stimulated environments. Cells were stimulated with 1.2 ug/mL of ionomycin and 10, 25, 50, 75, 100, or 150 ng/mL of PMA. At each time point, 50 uL of buffer was transferred to a flat bottom 96-well assay plate. Between time points, the assay plate was frozen. Following the last time point, 50 uL of Amplex Red reagent was added to each assay well and the fluorescence read (Figure 2). All samples were taken in triplicate.

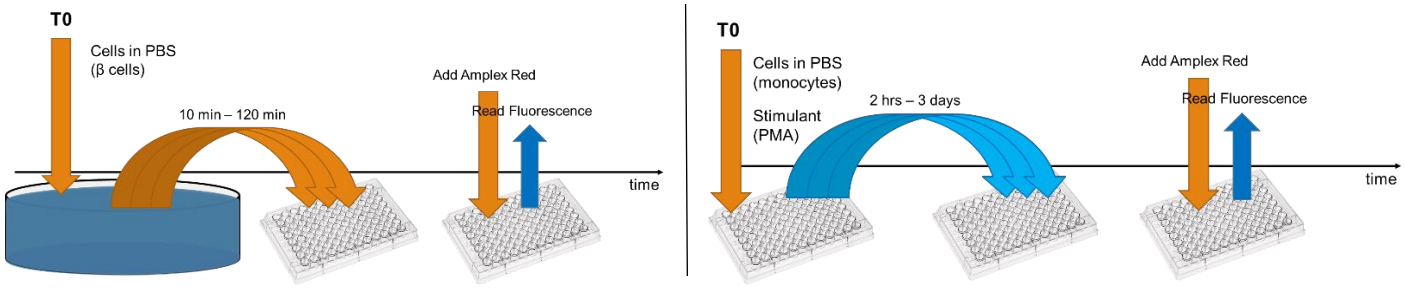


Figure 2. (Left) Protocol 1 for the pancreatic beta cells. Cells were incubated in buffer or a high glucose environment up to 120 minutes. Buffer was transferred into a 96-well plate in triplicate at time points starting from 10 minutes. After the last time point, Amplex Red reagent was added to each well, incubated for 30 minutes at room temperature, and fluorescently read. (Right) The protocol was updated for monocyte studies in which each time point occupied a well of a 96-well culturing plate. This removed error in later time points due to the accidental removal of cells in the buffer transfer. Transferring buffer from the culture plate (cellular environment) to an assay plate (without cells) allowed the quantification of time points below 30 minutes and above one day for the beta cells and monocytes respectively.

2.3.3 Experiments 3: Cellular Readings

Following centrifugation, cells were suspended in 0.05M sodium phosphate buffer, obtained from the Amplex Red Assay Kit. 25 uL of pancreatic beta cells (75,000 cells/well) or monocytes (10,000 cells/well) in buffer, 25 uL of stimulant (or buffer in basal environments), and 50 uL of Amplex Red reagent were added to individual flat bottom 96-well plates. Pancreatic beta cells were stimulated with high glucose (20M). Monocytes were stimulated with PMA only or PMA and 1.2 ug/mL of ionomycin. PMA was tested at 10, 25, 50, 75, 100, and 150 ng/mL. The 96-well plate was fluorescently read at multiple time points. H_2O_2 release was monitored from pancreatic beta cells for up to four hours and from monocytes for up to six hours (Figure 3). All samples were taken in triplicate.

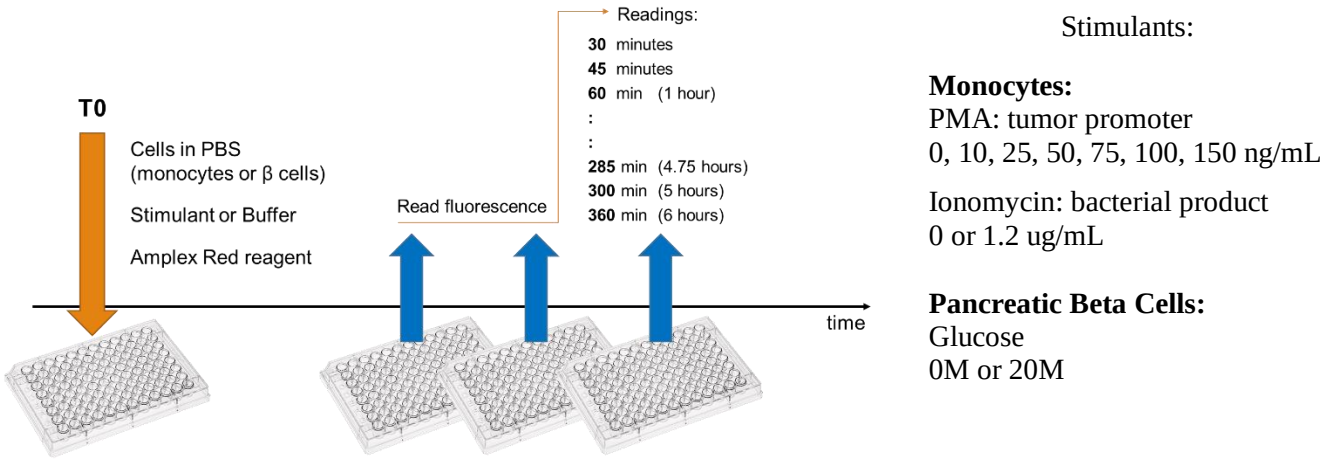


Figure 3. In protocol 2, cells, stimulants, and Amplex Red reagent are all added to wells of a 96-well plate at T0. Monocytes were studied without stimulation, stimulated with PMA only (10-150 ng/mL), with PMA (10-150 ng/mL) and Ionomycin stimulation (1.2 ug/mL), and with only ionomycin stimulation (1.2 ug/mL). Beta cells were studied without stimulation and with a high glucose environment (20M). The plate was read at fifteen minute increments between 30 and 300 minutes, and was read a final time at 360 minutes.

3. Results and Discussion

3.1 Experiments 1 & 2 Results

3.1.1 Pancreatic Beta Islet Formation

Beta cells in Spheroid Forming wells formed 3D, non-adherent spherical islets within one day of culturing. After five days, each islet (10,000 cells) was approximately 400-500 micrometers in diameter. Beta cells in ultra-low adhesion flat bottom 6-well plates (75,000 cells) took five days to form a complete network of non-adherent, branching, pseudo-islets (figure 4).

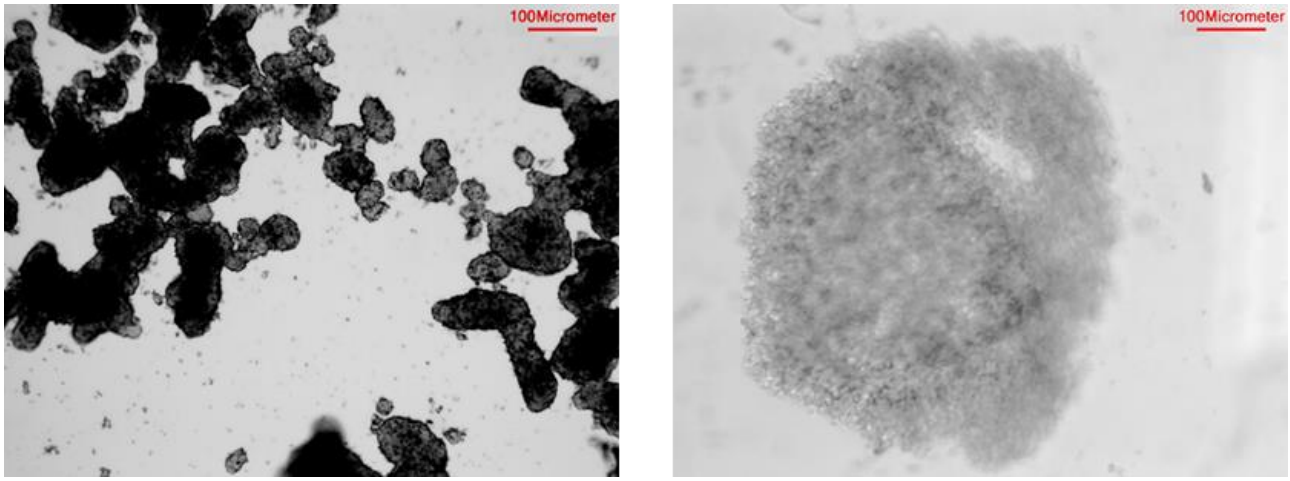


Figure 4. Pancreatic beta cells after five days of culture in ultra-low adhesion flat bottom 6-well plates (left) and Spheroid Forming 96-well plates (right). Cells in the flat-bottom wells took five days to network and formed large, branching pseudo islets. There was irregularity between groups of pseudo-islets and between wells. Cells in Spheroid Forming plate formed more-uniform islets. Islets were formed within 24 hours and continued to become more dense until experiments at day 5.

3.1.2 Buffer Transfer

Buffer was transferred from pancreatic beta cell studies to obtain time points less than 30 minutes. Issues arose in the accidental removal of cells which compounded and reduced the accuracy of following time points. The protocol was revised for monocyte studies such that each time point was an individual well. Buffer was again transferred to overcome Amplex Red reagent time limitations. Both buffer-transferring protocols, however, resulted in low fluorescent values and large noise (figure 5). Any upward trend in monocyte H_2O_2 concentration is likely due in combination to H_2O_2 production and to reduced sitting time before fluorescent quantification and reduced H_2O_2 degradation. These effects cannot be separately resolved and are further complicated with potential cell transfer. Buffer transferring, in either derivation, is therefore not an appropriate protocol.

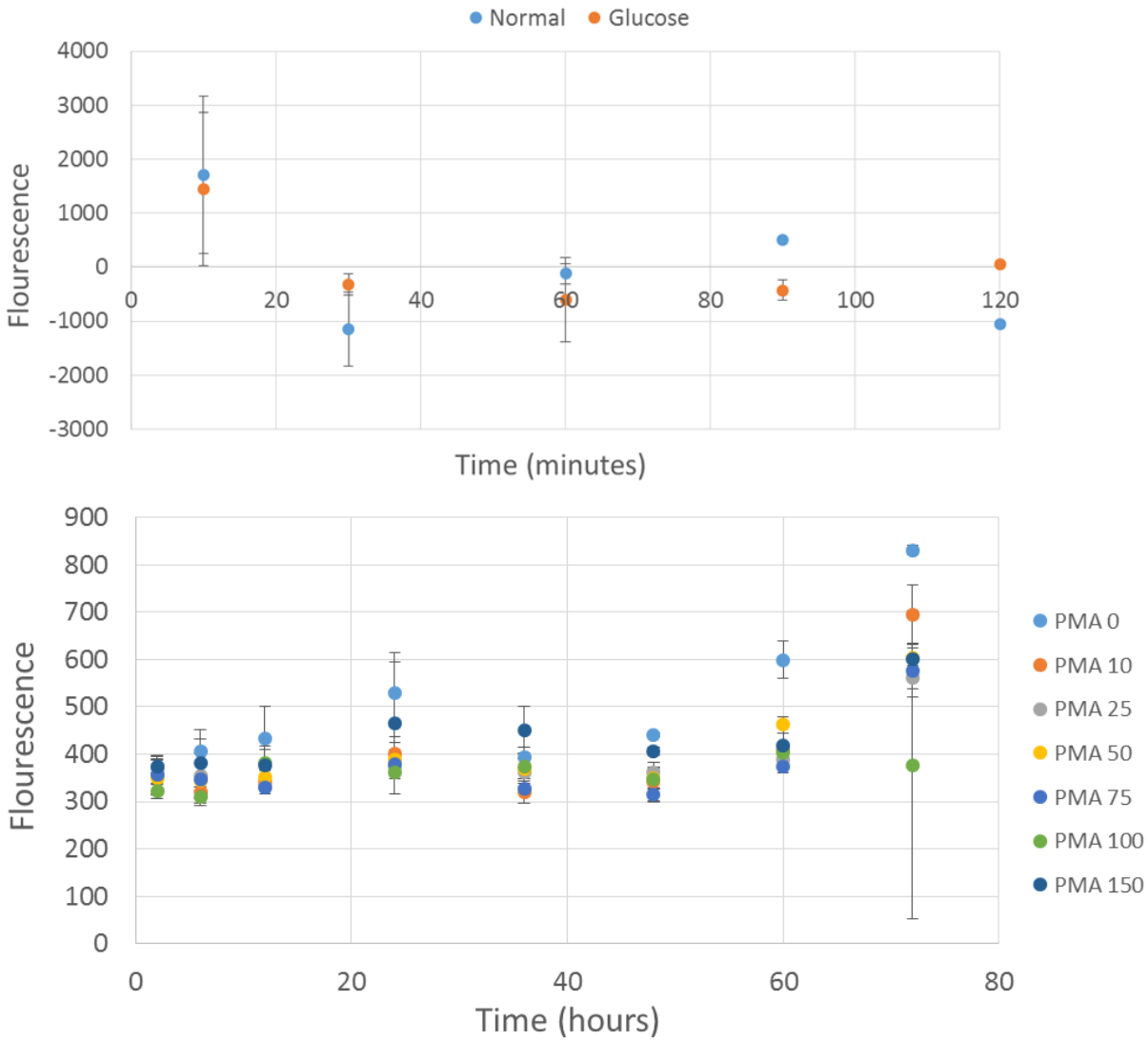


Figure 5. Fluorescent results from pancreatic beta cells (top) and monocytes (bottom). Results indicate low fluorescent values and large noise in both buffer-transferring protocols. In most cases, fluorescent values read below the standard curve blank (0 uM H_2O_2) and show no reliable trend in H_2O_2 production. Due to accidental cell transfer, repeated freezing and thawing, and H_2O_2 degradation in assay plate among varied waiting times (before Amplex Red is added), buffer transfer is not a reliable protocol.

3.2 Cellular Readings

3.2.1 Validation of Differentiation and H_2O_2 production from Monocytes

Tumor promoter, PMA, and bacterial product, ionomycin, initiate monocyte differentiation into macrophages. Monocytes became adherent within two hours after stimulation. Within 5.5 hours, stimulated cells are visibly darker than non-stimulated monocytes (figure 6). The physiological changes in stimulated environments validate differentiation into immature macrophages.

Monocytes display a basal H_2O_2 release rate of 12.20 pmol/min/10^6 cells. This production is verified as real when compared to beta cell results. Beta cells display no H_2O_2 production in basal or stimulated environments (figure 7).

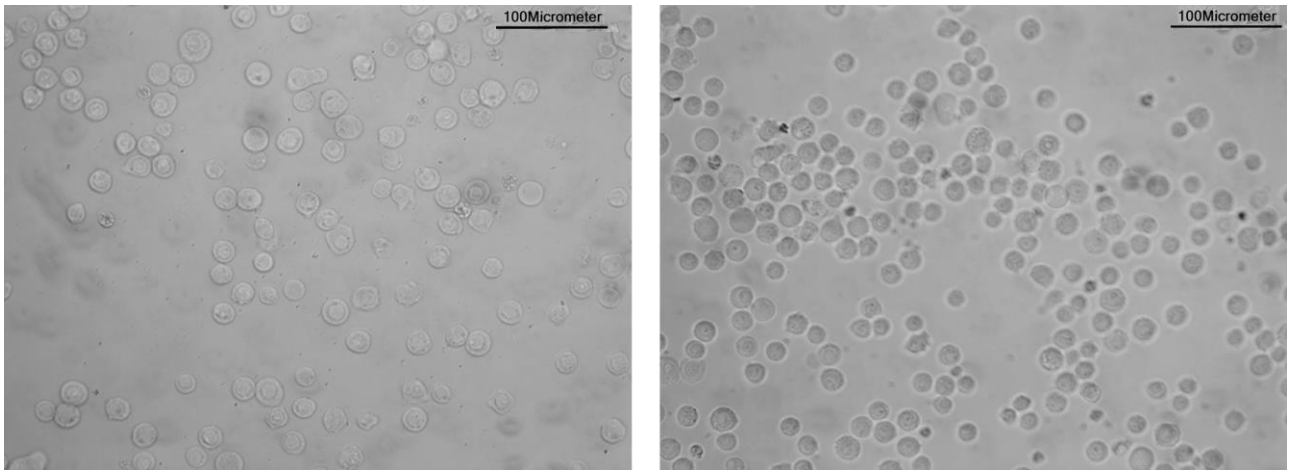
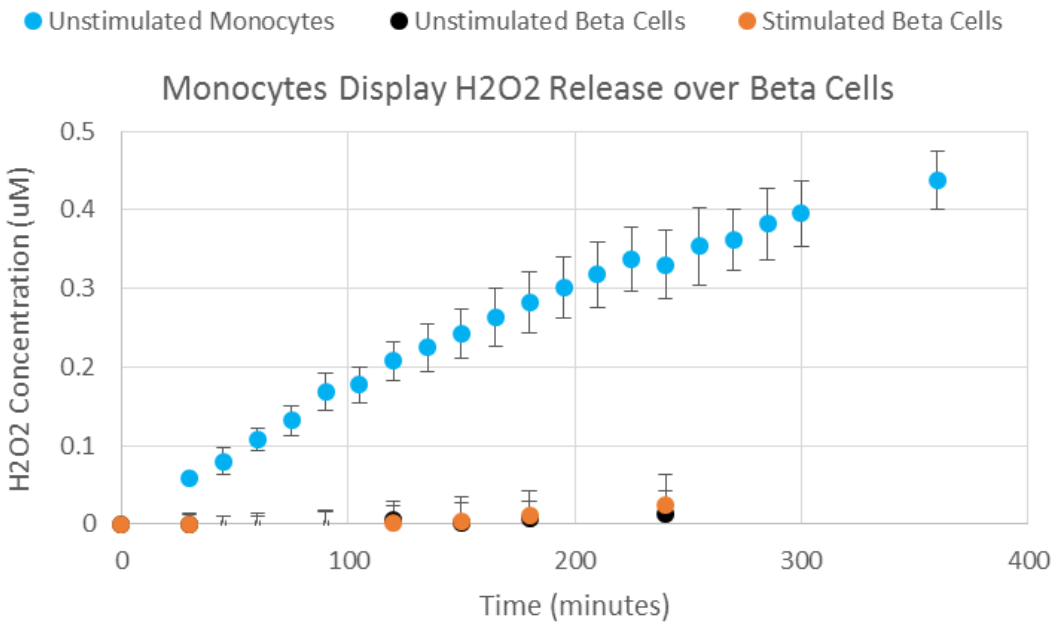


Figure 6. Bright-field images of monocytes taken after 5.5 hours in PBS buffer (left) and PMA stimulation (right). Stimulated cells show phenotypic changes indicative of differentiation into macrophages, such as adherence and darker appearance.



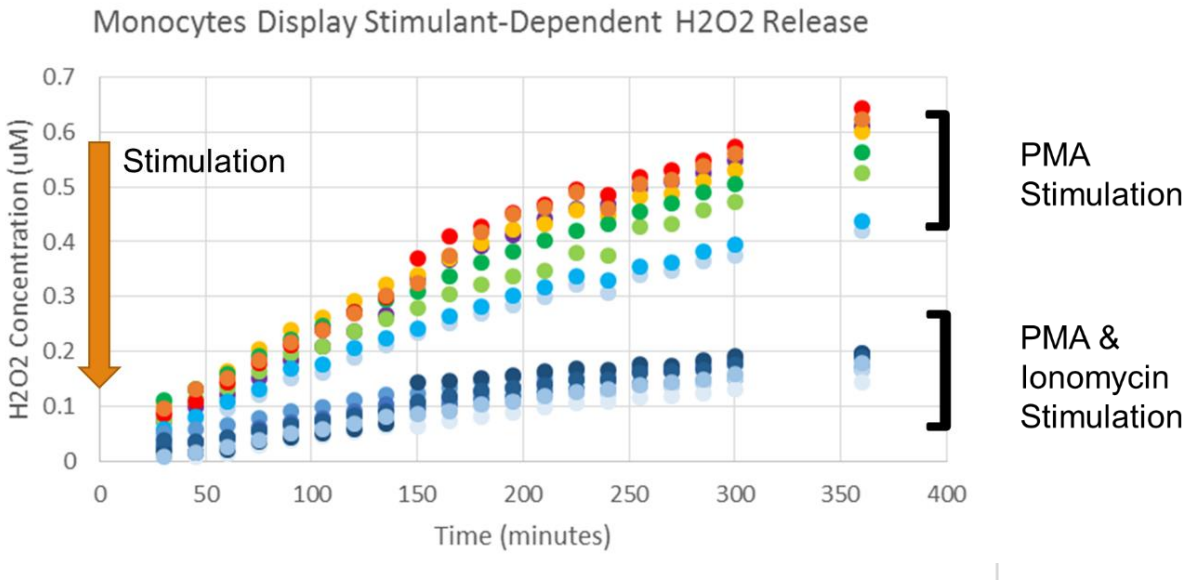
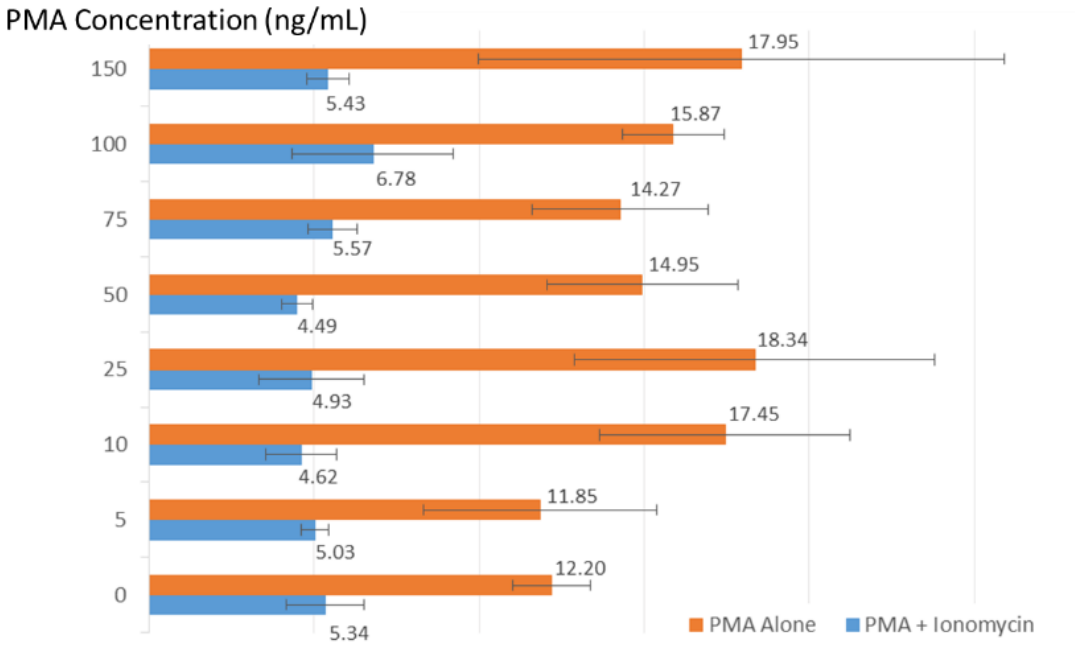


Figure 7. Monocytes release H_2O_2 at a basal rate, indicated in both charts (top and bottom) in true blue. A lack of H_2O_2 production in unstimulated and stimulated beta cells acts as a negative control, verifying the increase as a cellular relationship and not a byproduct of the assay used (top). Ionomycin reduces H_2O_2 release in all trials, including without PMA (bottom). Error bars have been excluded to show concentration-level changes, but are reported in tables 1 and 2.

3.2.2 Stimulant dependent H_2O_2 Production

Ionomycin significantly reduced H_2O_2 release in monocytes in all cases (figure 7). Most interestingly, monocytes stimulated with ionomycin (with and without PMA) reduced H_2O_2 release to 43% of the basal rate (without stimulation). H_2O_2 release rates are, on average, 3.06 times larger without ionomycin among varied PMA concentrations (table 1 & figure 8). PMA-ionomycin combined stimulation is common in cytokine production studies [19]. H_2O_2 release via ionomycin stimulation, however, is much less studied than PMA stimulation, and no comparison has yet been made with combined stimulation. Both ionomycin and PMA produce spikes of ROS generation in rat peritoneal macrophages [20]. PMA-induced ROS, however, was composed of mainly (61-70%) superoxide, while ionomycin-induced ROS was not [20]. The superoxide radial stabilizes directly to H_2O_2, while other ROS (such as the hydroxyl radical) can stabilize to water without producing H_2O_2 (figure 9). Our focus on H_2O_2 production instead of total ROS production likely plays a role in the seemingly negative effects of ionomycin stimulation.



[PMA] (ng/mL)	H_2O_2 Rate of PMA-Stimulation (pmol/min/10^6 cells)	H_2O_2 Rate of PMA-and-ionomycin Stimulation (pmol/min/10^6 cells)
0	12.20 ± 1.17	5.342 ± 1.17
5	11.85 ± 3.55	5.029 ± 0.405
10	17.45 ± 3.79*	4.621 ± 1.07
25	18.34 ± 5.45*	4.928 ± 1.58
50	14.95 ± 2.89	4.492 ± 0.475
75	14.27 ± 2.66	5.568 ± 0.748
100	15.87 ± 1.54	6.780 ± 2.43
150	17.95 ± 7.96*	5.428 ± 0.632

Figure 8 and Table 1. Figure 8 (left) pictorially demonstrates the change in H_2O_2 release rate between PMA stimulation and PMA-and-ionomycin stimulation. Table 1 (right) indicates each rate in pmol/min/10^6 cells with one standard deviation. Asterisks indicate statistically increased rates from each groups' 0 ng/mL PMA basal rate.

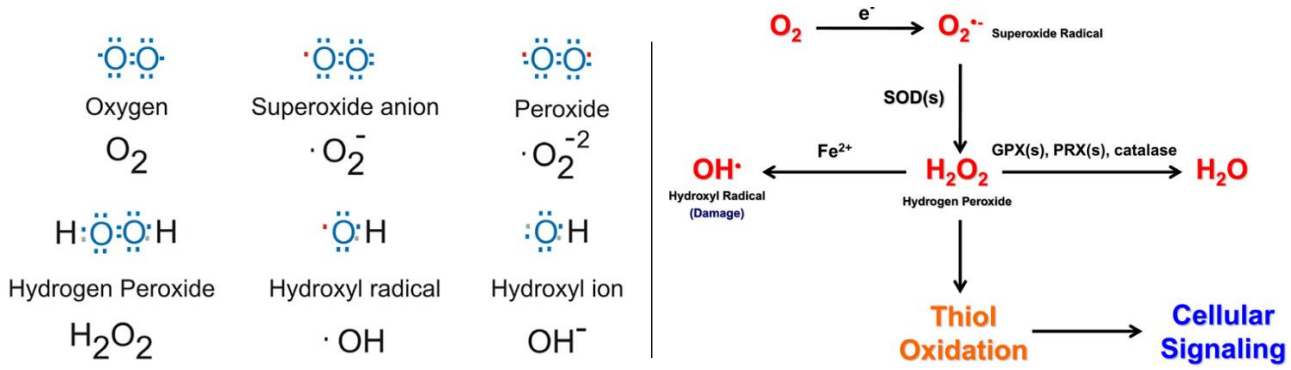


Figure 9. Of the reactive oxygen species (pictures left), the superoxide radical most commonly stabilizes to H_2O_2. H_2O_2 is released or broken down by certain cells into H_2O or, if left untreated, can become the hydroxyl radical can cause macromolecule damage. Because PMA produces mostly superoxide, a large efflux of H_2O_2 is expected from the monocytes. If Ionomycin produces mostly other ROS, a reduced H_2O_2 release rate is likely expected. Figures from http://www.biotek.com (left) and L.B. Sullivan, N.S. Chandel, "Mitochondrial reactive oxygen species and cancer." www.cancerandmetabolism.com/content/2/1/17 (right).

3.2.3 PMA Concentration dependent H_2O_2 Production

Results indicate possible concentration dependent H_2O_2 production rates among various PMA environments. When paired with ionomycin, no significant differences in H_2O_2 rates are seen between PMA concentrations. In the PMA-only stimulations, however, 10 ng/mL, 25 ng/mL, and 100 ng/mL of PMA produce statistically greater H_2O_2 release from the basal rate ($p < 0.05$) (table 1). Concentration-dependent responses to PMA agree with previous literature, which found an optimal stimulation at 10 ng/mL of PMA when testing ranges from 0.01 to 1,000 ng/mL [21]. Larger sample sizes and more advanced statistical analysis will better uncover a potential relationship and optimal concentration between PMA stimulation and H_2O_2 release rates.

3.2.4 Rate Comparisons

The largest H_2O_2 production rate of 18.34 pmol/min/10^6 cells is seen in 25 ng/mL PMA-only stimulation of monocytes. The current MEA has a resolution of 32 pmol/cm^2/sec. Given an average U937 cell line diameter of 12.5 uM [22] and an immature macrophage diameter of approximately 30 uM (obtained from figure 6), and assuming a perfect circle, the MEA would need a resolution between 0.25 and 0.043 pmol/sec/cm^2, respectively.

5. Conclusions and Future Work

This study compares the hydrogen peroxide release rate from mouse pancreatic beta cells (MIN6) and human monocytes (U937) under two experimental protocols and several stimulants to develop a cellular model. The cellular model can then be used for future characterization and physiological tests of our microelectrode array (MEA). We have found a lack of hydrogen peroxide release in control and glucose-stimulated beta cells. We additionally found a stimulant-dependent H_2O_2 response in monocytes which favors PMA over ionomycin. A relationship between H_2O_2 release rates and PMA concentration is likely following significant increases at 10, 25, and 150 ng/mL of PMA over basal rates. While the H_2O_2 release rates from monocytes are smaller than the resolution of the currently-characterized MEA, future sensor improvements and more monocyte stimulation studies may elucidate the U937 line as an ideal cellular model of

hydrogen peroxide release. In addition to larger sample sizes, future studies can look at additional stimulants which alter the phenotype of the differentiated macrophage [23]. Ultimately, physiological verification of the MEA will allow real-time spatial resolution of analytes released or consumed by cells. Electrode sensing can provide more detailed understanding of H_2O_2 spatial signaling, more efficient analysis of cell populations, and higher throughput of multiple analytes via biofunctionalization, advancing cellular sensing.

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