Continuous Protein Production and Release via Oscillatory Suicidal Lysis Circuits

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Thesis submitted in partial fulfillment of
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ABSTRACT

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Abstract

Advancements in the biotechnology and pharmaceutical fields have led to the development of an expanding number of applications for certain recombinant proteins of interest. As such, the demand for efficient and cost effective protein production systems is growing. A great deal of research, cost and time goes into improving and optimizing the production of commercially valuable proteins of interest. Many current methods involve growing a culture of cells to its maximum capacity, all of which are producing a certain protein of interest, and then killing off the entire culture to extract the protein. By doing so, regrowth of the entire cell population is required, taking additional time and resources. Controlled lysis could allow for a more continuous protein release through the killing of only a portion of the population and allowing recovery in the exponential growth phase. This study acts as a proof of concept for the implementation of programmable suicidal lysis circuits into bacteria, *Escherichia coli*, being cultured for protein production for the sustained production and release of said proteins.

To test the viability of suicidal lysis as a mechanism for sustained protein release a robust oscillator circuit, ePop, was used. The ePop circuit controls the synthesis of E gene, producing a protein that incites cellular lysis by attacking the cell wall. By culturing cells for long term growth and extracting small volumes of the culture at

various time points for protein quantification, the protein release capabilities of ePop were observed. Protein quantities in the lysates and supernatants of the extractions were determined using SDS-PAGE Coomassi Staining and a Pierce BCA Protein Assay. Also, western blotting was performed on supernatant samples to show the effective release of a specific protein of interest, GFP. The focus was on the presence of protein in the supernatant which is correlated to the release during the lysis cycle of the bacterial population oscillations.

Protein release via the ePop circuit was shown to be effective and robust. The oscillator circuit released measurable quantities of protein in the supernatant of the culture extractions as predicted. The green fluorescent protein of interest used as a pilot protein was effectively released into the supernatant and shown through a western blot with a GFP specific antibody. Population oscillator circuits through cellular lysis were shown to be a viable method for protein release and could be applied to protein production processes as well as other technologies.

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Acknowledgements

The work presented here was made possible by the support and guidance of my advisor, Dr. Lingchong You, and the graduate students and post-docs in the lab. I would specifically like to thank Yu Tanouchi, Robert Smith, Anand Pai, Stephen Payne and Anna Lee for their advice, guidance, and assistance throughout the study. Also, I would like to acknowledge Ying Zhang and Megan Ho of the Dr. Kam Leong lab for their help with the encapsulation and observation of ePop cells.

1. Introduction

The production of proteins is a crucial component of a vast number of biotechnological and pharmaceutical processes with extensive application potential. Protein production is essential for types of cell therapies, drug discovery, vaccine and viral vector production, and biochemical screening. Also, there has been tremendous progress in the design and engineering of protein-based nanomaterials with enhanced qualities and functions [1][3][4]. As such, the ability to produce recombinant proteins efficiently and economically is becoming increasingly expedient. Synthetic biology has enabled protein production capabilities to be greatly accelerated through the engineering of micro-organisms to produce proteins of interest. There is still a great deal of potential for optimizing and improving upon the current methods and production processes to meet the broad domain of development and commercial demands.

For some time now, the augmentation of protein production strategies has been the focus of a substantial amount of research. The most common expression systems for recombinant protein production are procured from bacteria, yeast, and mammalian cell lines [1]. *Escherichia coli* is a popular micro-organism used to facilitate protein expression systems due to its relative simplicity, economical and fast growth dynamics, as well as the exhaustive genetic knowledge and understanding of intracellular mechanisms[2][3].

With this, a broad array of synthetic biology and bioengineering techniques have been implemented and investigated in the pursuit to optimize the production capabilities of such bacteria cell expression systems[1-14]. From host strain and growth conditions down to the specific elements of the plasmid in the expression system, all are deliberately chosen to enhance the production of the specific protein of interest[2-5][13]. Various metabolic factors, protein folding and degradation effectors, as well as cell stress responses must also be carefully considered[3][4][6][7][11][14]. Cells are genetically engineered with the intent of minimizing and eliminating all non-essential cellular processes while maximizing processes and mechanisms that enhance production efficiency[6][8-12].

While methods for boosting protein synthesis and stability are of great importance, other aspects of the production process prove to be extremely significant in the overall yield and cost of protein production as well. Protein isolation and purification are essential yet often difficult and expensive procedures downstream of the initial protein synthesis. When proteins are synthesized, in order to collect them, they must be made available and separated from the cells. In order to do so, a number of methods have been explored. The primary method allows bacteria to grow to a high density in the culture media while continuously synthesizing the desired protein. At a certain point, the cells are harvested and put through a lysis protocol to release all of the proteins, allowing for subsequent isolation and purification of the protein of interest.

While this method is adequate, it involves the complete extinguishing of the whole bacteria population requiring an entire new population to be grown up again with each discrete lysis cycle.

The following research investigates the viability and efficiency of transforming a population oscillator circuit into a protein production system. The gene circuit, identified as ePop, harnesses quorum sensing to incite population level oscillations via cellular lysis. The cell lysis that occurs at high cell densities would serve to release the synthesized proteins serially, thus providing an alternative to the standard complete population extinction method. Sustained oscillations would give rise to continuous protein production capabilities and eliminate the need for a total bacterial regrowth period.

1.1 ePop Gene Circuit

The design of the ePop circuit involves two separate modules under the control of two different promoters[15]. The first module is responsible for sensing the local cell density (quorum sensing). The module is under control of a $P_{lac/ara-1}$ promoter which can be induced by Isopropyl β-D-1-thiogalactopyranoside, IPTG. There are two genes in the module downstream of the promoter, a luxR gene and a luxI gene. The luxI protein is a synthase of the auto-inducer signaling molecule acyl homoserine lactone, AHL[15]. The AHL signaling molecule is able to diffuse freely through the cell wall and enter the

surrounding media. At sufficient concentrations, AHL is able to bind to the transcription factor, LuxR, produced by the luxR gene. Upon binding, the species form an activation complex[16] capable of activating the P_{luxI} promoter of the second module.

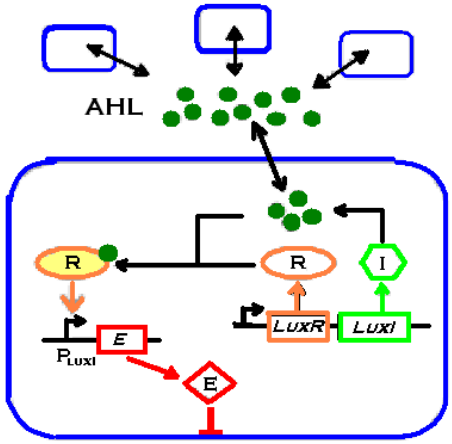

Figure 1 : Illustration of the modules of ePop and its function was designed. The first module contains two genes, luxR and luxI. LuxI is a precursor to the signaling molecule AHL which is able to freely diffuse beyond the cell wall (blue) and into the surrounding environment. At a critical concentration, AHL can bind to LuxR and the complex is then able to induce synthesis of the E gene via the pLux promoter. The E protein then attacks the cell wall leading to lysis.

The P_{luxI} promoter controls the expression of the lysis gene, E gene. The E protein induces cellular lysis by inhibiting MraY, an instrumental enzyme involved in *E. coli* cell wall synthesis[15]. Thus, once a sufficient local cell density has been reached, AHL will effectively bind to the luxR transcription factor activating E protein synthesis leading to cell lysis. As the cell density decreases due to a portion of the population committing suicidal lysis, the subsequent AHL concentration falls before the bacterial population is

able to recover again and again begin E protein production. Through the repeating pattern of growth and lysis, oscillations are observed. The plasmid employs a chloramphenicol resistance gene for a selective marker and the ColE1 origin of replication[15].

In the currently deployed plasmid, a mutation in the luxR gene of the construct abolished the designed quorum sensing mechanism. However, oscillations were still generated through a different mechanism, fluctuations in plasmid copy number. While the promoter controlling the E gene can be induced by the luxR/AHL dimer, there is a basal level of E protein synthesis even in the absence of the dimer. So the number of ePop plasmids within an individual cell has a substantial effect on the overall E protein production rate. As stated above, the ePop plasmid has a ColE1 origin of replication. One quality of this origin is that it codes for two RNA types, RNAI and RNAII. RNAII possesses the ability to anneal to the ColE1 origin and act as a primer for newly synthesized DNA, leading to replication of the plasmid. RNAI, on the other hand, is very prone to binding to RNAII, inhibiting it from annealing to the ColE1 origin. RNAI is transcribed at a much higher rate than RNAII such that under normal conditions, the vast majority of RNAII is bound by RNAI and very minimal plasmid amplification takes place. However, when cells experience stressful conditions, such as nutrient scarcity or starvation, cells are unable to produce sufficient amounts of amino acids for protein

production and respond by entering the stringent response. During this time there is a shortage of amino acids readily available for transfer RNAs, tRNAs, to bind to. At this point synthesis of the spoT gene catalyzes the production of guanosine tetraphosphate (ppGpp), a nucleotide effector[17]. The accumulation of ppGpp leads to a decrease in ribosomal synthesis, by binding and altering RNA polymerase, reducing its affinity for stable RNA promoters[17]. When unbound, tRNAs are uncharged and actively degrade RNA molecules and are more prone to degrading RNAI than RNAII. This active degradation of RNAI leads to a relative increase in the RNAII concentration, allowing for plasmid amplification. In this way, local environmental conditions, greatly impacted by cell density, are able to dictate fluctuations in plasmid copy number within individual cells. Once the plasmid copy number reaches the number necessary for a threshold quantity of E protein production, cells will begin to lyse. The lysis of a percentage of the cell population allows for the local nutrient availability to increase allowing for the cell population to recover again. The repetition of this cycle leads to the observed oscillations in the bacteria population[15].

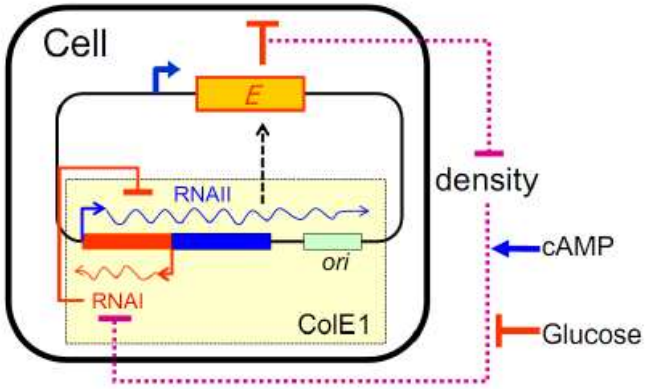

Figure 2 : Diagram of the interactions causing cell density oscillations via fluctuations in plasmid copy number. Increases in cell density lead to a scarcity of nutrients leaving tRNAs uncharged. The ColE1 origin codes for two RNAs, RNAI & II. Uncharged tRNAs actively degrade RNAI causing a relative increase in the amount of RNAII which has the ability to act as a primer for the ColE1 origin, causing plasmid replication.

Even with the unintentional mechanism controlling the oscillatory behavior, the oscillations have proved to be very robust and reproducible[15]. As such, for a proof of concept, the current construct can be used, though the fully functional ePop gene circuit can be re-introduced to optimize or more tightly control the production method if necessary.

1.2 Population Growth Dynamics

To confirm the ePop population dynamics, the time course experiments of Marguet in "Oscillations by Minimal Bacterial Suicide Circuits Reveal Hidden Facets of Host-Circuit Physiology" were re-performed. Absorbance measurements exhibited the dynamics of ePop to be robust and reproducible. MC4100Z1 cells with ePop displayed

oscillatory growth dynamics that were abolished by increasing the glucose concentration in the media. The exact role that glucose plays in inhibiting oscillations remains unclear. Glucose could impact the circuit interactions in a number of ways: through the population growth rate, ppGpp production, degradation of SpoT, etc.[15]. Before repeating the time course experiments discussed in the paper above, the ePop plasmid was sequenced through Eton Bioscience to ensure there were no mutations from the published version. The sequencing confirmed the ePop sequence with the expected mutation within the luxR gene, abolishing the initially designed quorum sensing. The ePop plasmid was then transformed into MC4100Z1 cells and plated on an LB agar plate containing chloramphenicol to ensure that only cells experiencing successful transformation could form colonies. The plates were incubated at 37°C for 12 hours to allow for colony formation. For the time course experiments, eight colonies were selected from the agar plate and inoculated in LB and MOPS media buffered to 7.0pH along with chloramphenicol and incubated in a 37°C shaker (250rpm) for 12 hours in order to reach a sufficient cell density. A 48-well BD Falcon Tissue Culture Plate was then prepared with 500μL of fresh LB and MOPS media and chloramphenicol in each well. Each of the six rows of the plate contained a different concentration of glucose in the fresh media: 1%, .5%, .25%, .125%, .0625%, 0%. Each well was then inoculated with 50μL from the growth cultures. Each of the eight columns of wells on the plate received

one of the eight clone growth cultures. 250μL of mineral oil was added to the top of each well to prevent evaporation of the media below. Cell growth was observed using a Perkin Elmer Victor3 plate reader at 37°C. Absorbance measurements were taken from each well every 15 minutes using 600nm light. Oscillations were observed in wells containing low levels of glucose. However, the oscillations dissipated as the glucose concentration was increased.

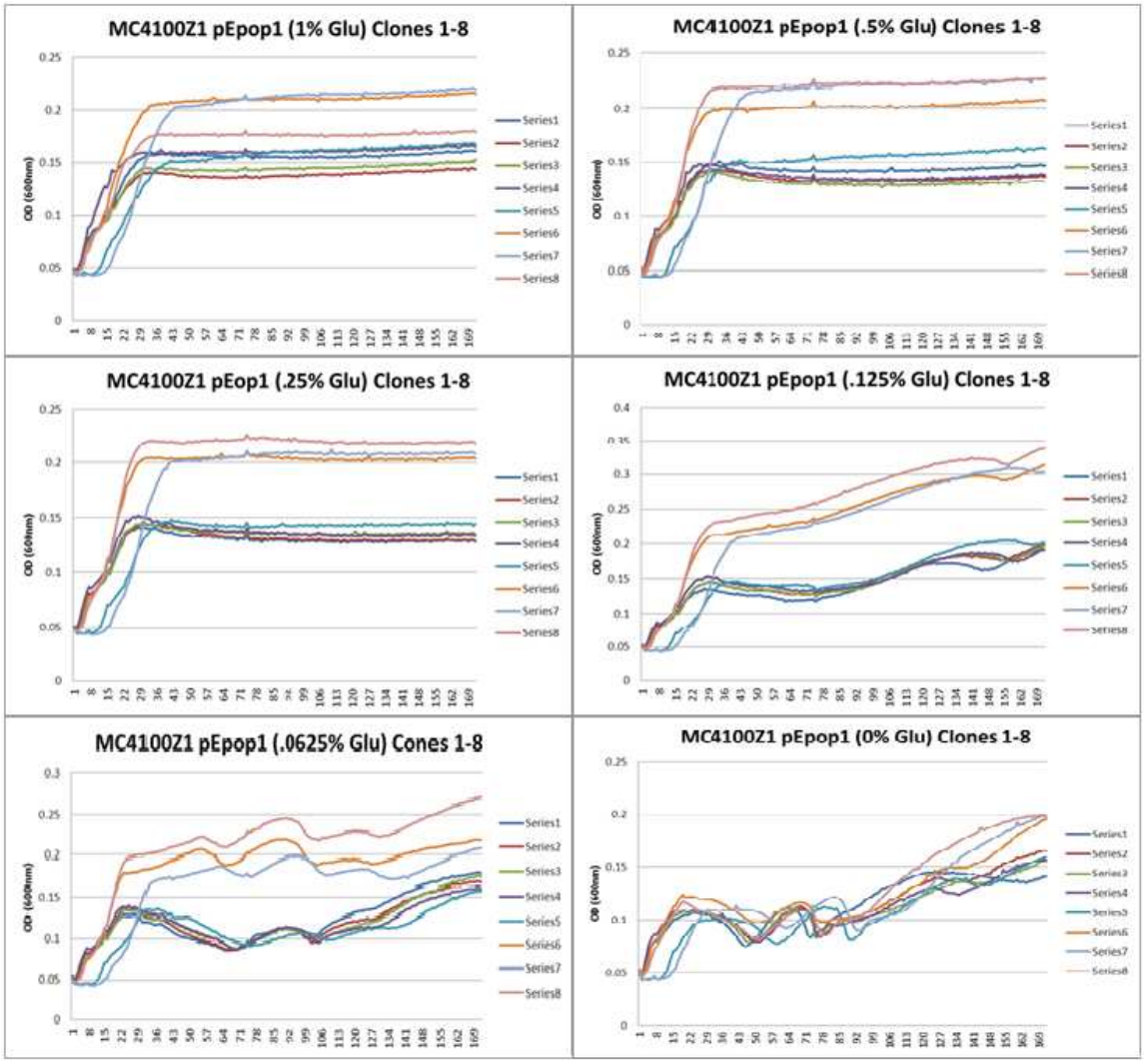

Figure 3 : Time course absorbance plots with varying levels of glucose in the media. Eight clones of MC4100Z1 cells with ePop were inoculated in LB and MOPS with chloramphenicol with glucose concentrations of: 1%, 0.5%, 0.25%, 0.125%, 0.0625%, and 0%.

In order to test if fluorescence could also be used as a metric to quantify cell population density, a second plasmid was transformed into MC4100Z1 cells with ePop.

The green fluorescent protein plasmid used was pZS31GFP. However, in order to ensure a successful double transformation the plasmids had to have different restriction markers. Thus the chloramphenicol selection marker had to be excised from the pZS31GFP plasmid and replaced with a kanamycin selection marker from another plasmid. The plasmid that was chosen for its kanamycin selection marker was pPROLarA. A restriction digestion was performed on both plasmids using two enzymes, SacI and AatII, due to their corresponding restriction sites closely flanking the desired inserts. Gel electrophoresis was performed on the results of the digestions and the correct bands were excised and recovered using a Zymoclean Gel DNA Recovery Kit. Ligation was then performed on the recovered pZS31GFP vector and the kanamycin insert from the pPROLarA plasmid. The resultant plasmid was termed pZS31GFPk. The gfp gene on pZS31GFPk is under the control of a P_{Tet} promoter, which is repressed by tet repressor (tetR). Because tetR is constitutively expressed in the MC4100Z1 strain, the P_{Tet} promoter in this construct must be induced by anhydrotetracylcine, aTc, which binds tetR with a high affinity, thereby preventing tetR repression. The glucose time course experiments were then re-performed with the two plasmid system and in the presence of aTc within the media.

While absorbance readings exhibited the expected oscillatory behavior, the fluorescent signal did not. The plate reader GFP readings were found to be the same for

cultures with and without aTc in the media. This was due to a high amount of auto-fluorescence exhibited by the LB media. When using an M9 minimal media, significant fluorescent differences between aTc+ and aTc- cultures could be observed with plate reader GFP measurements. MC4100 cells with ePop were able to produce the same oscillatory behavior in M9 media as observed with the MC4100Z1 strain and M9 media was used from this point on.

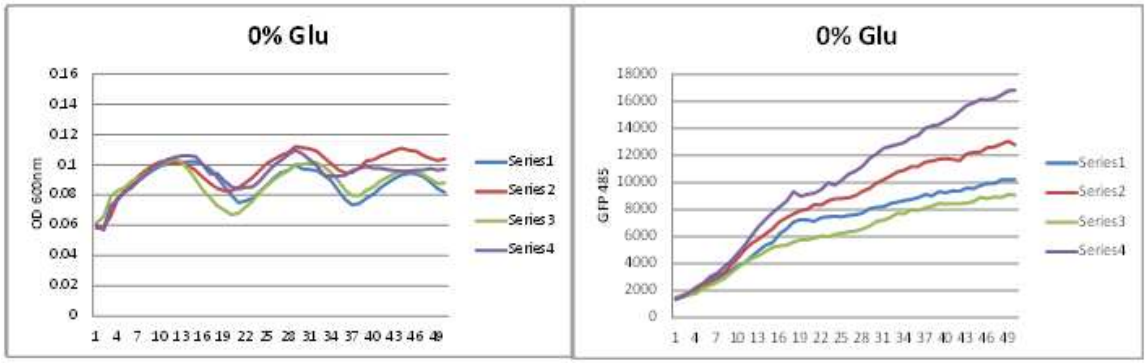

Figure 4 : Time course absorbance and fluorescence plots of MC4100 cells in M9 media with 0% glucose added. The four clones of MC4100 cells with ePop + pPROTetGFP exhibited the desired oscillations in the absorbance plot (left) but steady growth in fluorescence (right).

Performing the glucose time course experiments again in M9 and with aTc revealed an interesting outcome. While absorbance levels produced oscillations, the fluorescent signal grew with the initial cell growth but did not oscillate as the cell population did. Instead the fluorescent signal seemed to reach a high steady state level which was maintained for the remainder of the experiment. Due to the low degradation rate of the green fluorescent protein, proteins released into the media after cell lysis

continued to contribute to the GFP reading though they were no longer representing live cells. The ability of the bacteria to produce and release functional proteins in a consistent and pseudo-continuous manner could be an advantageous mechanism to be implemented into protein production systems to streamline protein extraction steps.

2. Protein Production

To test the protein production capabilities of the ePop system, the following research design was implemented. First, a green fluorescent protein was selected as the protein of interest (POI) to be used as a proof of concept in part because it is well understood and readily available, but also because its release had already been observed. The GFP protein used is a variant, GFPmut3, which was inserted into a backbone vector with a kanamycin resistance gene and a p15A origin of replication. The gfp gene is under the control of a pTet promoter. The p15A origin was selected to make the resultant plasmid, pPROTetGFP, a high-copy-number plasmid leading to higher synthesis rates for the protein of interest. This plasmid was transformed into MC4100 cells along with the ePop plasmid creating a two plasmid system. MC4100 cells were chosen over MC4100Z1 because they do not synthesize a Tet repressor and therefore do not require induction to produce a gfp signal using pPROTetGFP. Again, M9 media was selected due to its low background fluorescence.

2.1 Protein Extraction

The protein extraction protocol was initially carried out with the following in M9 medium: MC4100 cells with ePop and pPROTetGFP, MC4100 cells with only ePop, MC4100 cells with only pPROTetGFP, as well as MC4100 cells with no additional plasmids. Cell samples were inoculated into two 5mL cultures of M9 media and the appropriate antibiotic; one with 1% glucose and the other without. A culture of only M9

and a culture of M9 with 1% glucose with no bacteria inoculations underwent the same protocol to use as a standard or reference. The eight inoculations and two media only samples were incubated in a 37°C shaker for 24 hrs. After inoculation, 200μL samples were taken from the cultures for OD measurements to track the cell growth in the cultures at 3hr intervals. The OD measurements were taken using a 48 well Falcon plate in a Perkin-Elmer Victor3 plate reader with 600 nm light. Every 6hrs, in addition to the OD sampling, 800uL were taken from each culture and put in 1.5ml eppendorf tubes. The samples were centrifuged for 2 minutes at 15,000 rpm to pellet the cells. The supernatant was extracted and stored in a separate microfuge tube at -20°C. The remaining cell pellets were resuspended in 100uL of lysis buffer (0.01M Tris & 4% (w/v) SDS, protease inhibitor) and underwent the following lysis protocol. After adding the lysis buffer, samples were vortexed on high (3000 rpm) for 30 seconds followed by two minutes on ice. This was repeated four times to fully lyse the cells of each sample. The samples were then centrifuged at 4°C and 15,000 rpm for 20 minutes. Lastly, the lysate was pipetted into another eppendorf tube and stored at -20°C until needed for protein quantification.

2.2 24 Hour Culture Growth

The OD 600 readings for the cultures were used to compare with the protein quantification results. The amount of absorbance corresponds with cell density and could be used to normalize the protein amounts based on cell density. Also, the OD

measurements could be used to determine the lysis points of ePop samples and when the greatest protein release into the supernatant could be expected. The 24 hr culture period was long enough to capture the initial lag and growth phases and one lysis event. Since multiple lysis cycles have already been observed as robust and reproducible in cells with ePop, the first lysis cycle was enough to be used as a proof of concept for the use of oscillatory suicidal lysis circuits as a viable protein release mechanism. The first lysis phase appeared to begin around the 18hr time point as observed in the absorbance levels in the plot below.

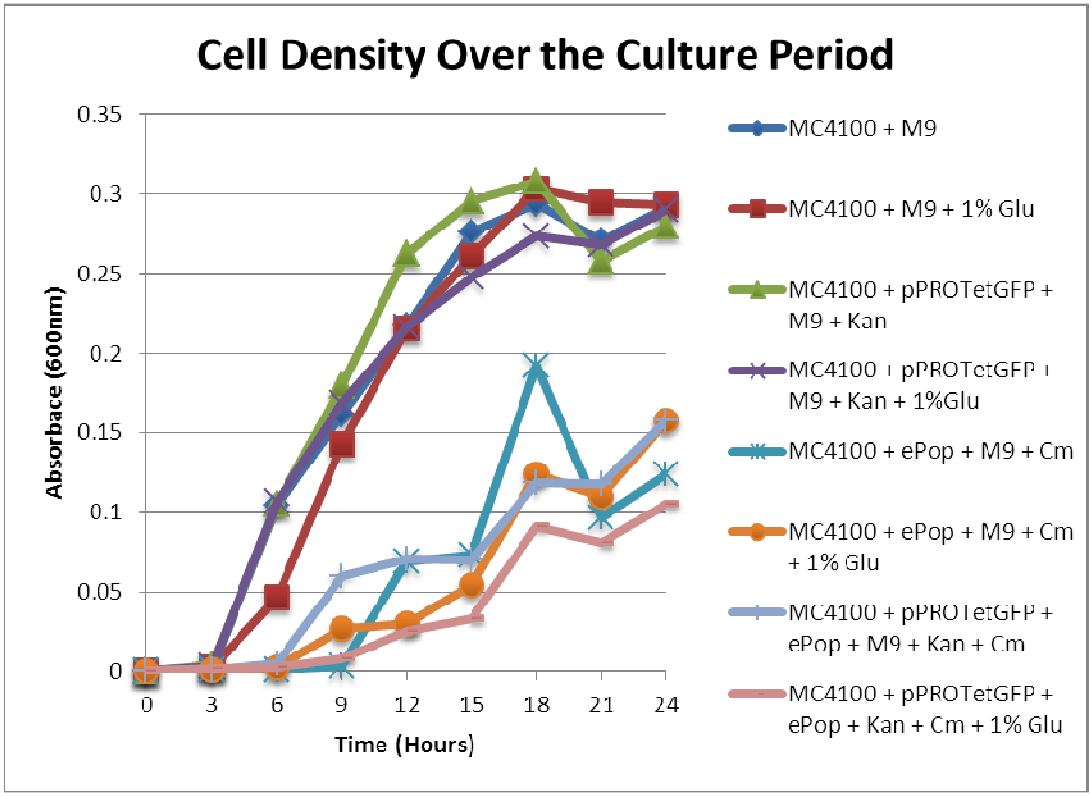

Figure 5 : Time course plot of plate reader absorbance measurements (OD 600nm) of 200mL culture extractions taken every 3 hours after inoculation for a 24 hour time period.

2.3 Protein Quantification

To quantify protein production, a number of methods were investigated. One method utilized was a version of the Bradford Assay, a standard protocol using the Pierce BCA Protein Assay Kit by Thermo Scientific. Another was sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining, which provided a very reliable relative quantification.

2.3.1 Pierce BCA Protein Assay

The assay exploits the fact that protein in an alkaline medium reduces Cu^{2+} to Cu^{1+}. Then, using bicinchoninic acid's highly sensitive and selective colorimetric detection of cuprous cations, the protein concentration varies the color or darkness of the medium. The absorbance of the medium at 562nm varies based on the protein concentration within it. Creating a regression line using the absorbance values of a range of known albumin concentration standards allowed for the approximation of the protein concentrations of unknown samples. The complete protocol is described in the Pierce BCA Protein Assay Kit – Reducing Agent Compatible by Thermo Scientific. The plots below in Figure (6) display the total protein found in the lysates and supernatants of the samples (only non-glucose samples are shown).

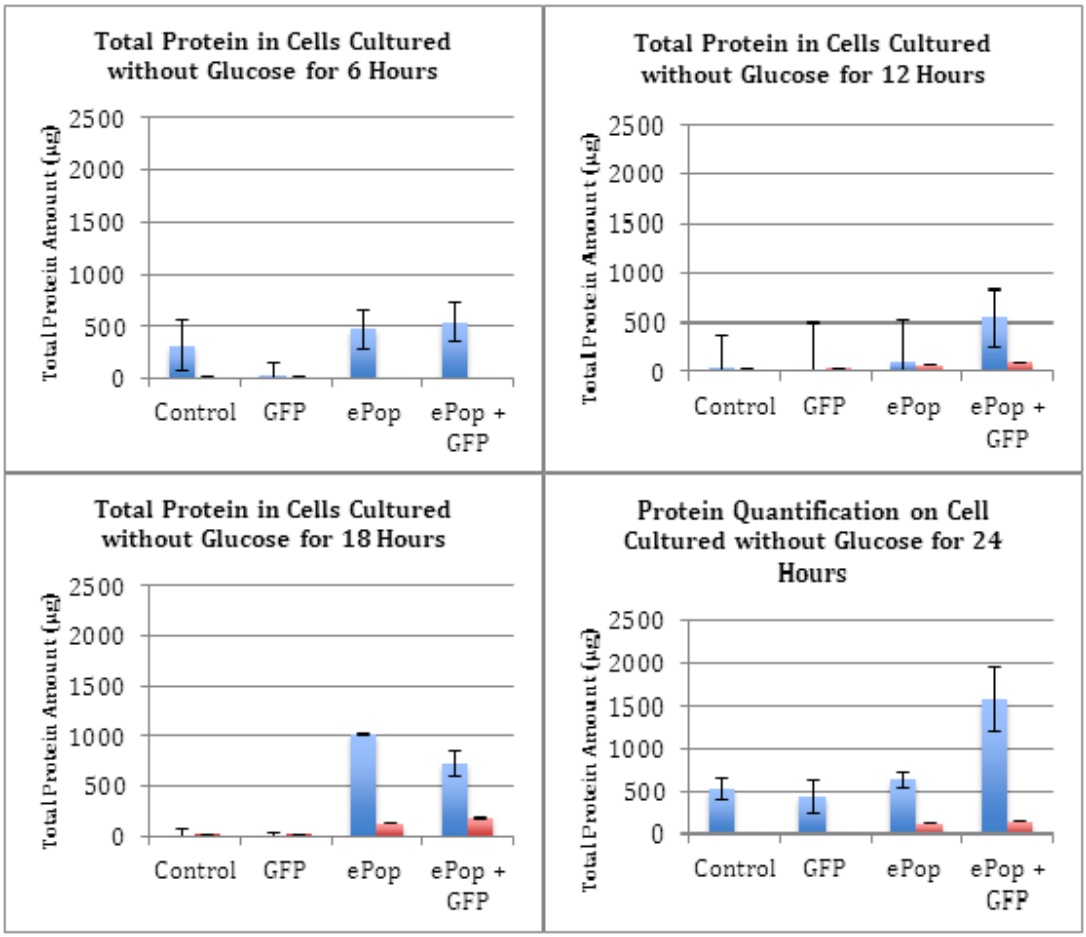

Figure 6 : Bar graphs of total protein amounts in supernatant (blue) and lysate (red) extractions as calculated from the Pierce BCA Protein Assay. It should be noted that these graphs do not represent concentrations but overall quantity of protein. The concentrations estimated by the assay were scaled according to the original sample volumes (100µL for lysate samples, 800µL for supernatant samples). The control and GFP concentrations for the 18hr extractions were calculated as negative values and set to zero.

2.3.2 SDS-PAGE Coomassi Staining

The SDS-PAGE Coomassie protocol begins by adding Laemmli buffer to samples and vortexed. The β-mercaptoethanol, BME, in the buffer breaks down the disulfide bonds and bridges of the proteins. Next the samples are boiled for 10 minutes to break

down the hydrogen bonds, further linearizing the proteins. The samples are then loaded into the wells of the gel. Creating a voltage gradient across the gel causes the proteins to migrate through the gel based on charge and size. After allowing the proteins to migrate and separate, the gels are immersed in destain buffer for 20 mins to fix the proteins in their place in the gel. Then, the destain buffer was removed and replaced with Coomassi Berillium Blue for 30 minutes to stain the gel and proteins. Next, the Coomassi Berillium Blue is discarded and the gel is re-immersed in destain buffer and placed on a table top shaker at a low rpm setting to slowly remove the stain of the gel and reveal the remaining stained protein bands.

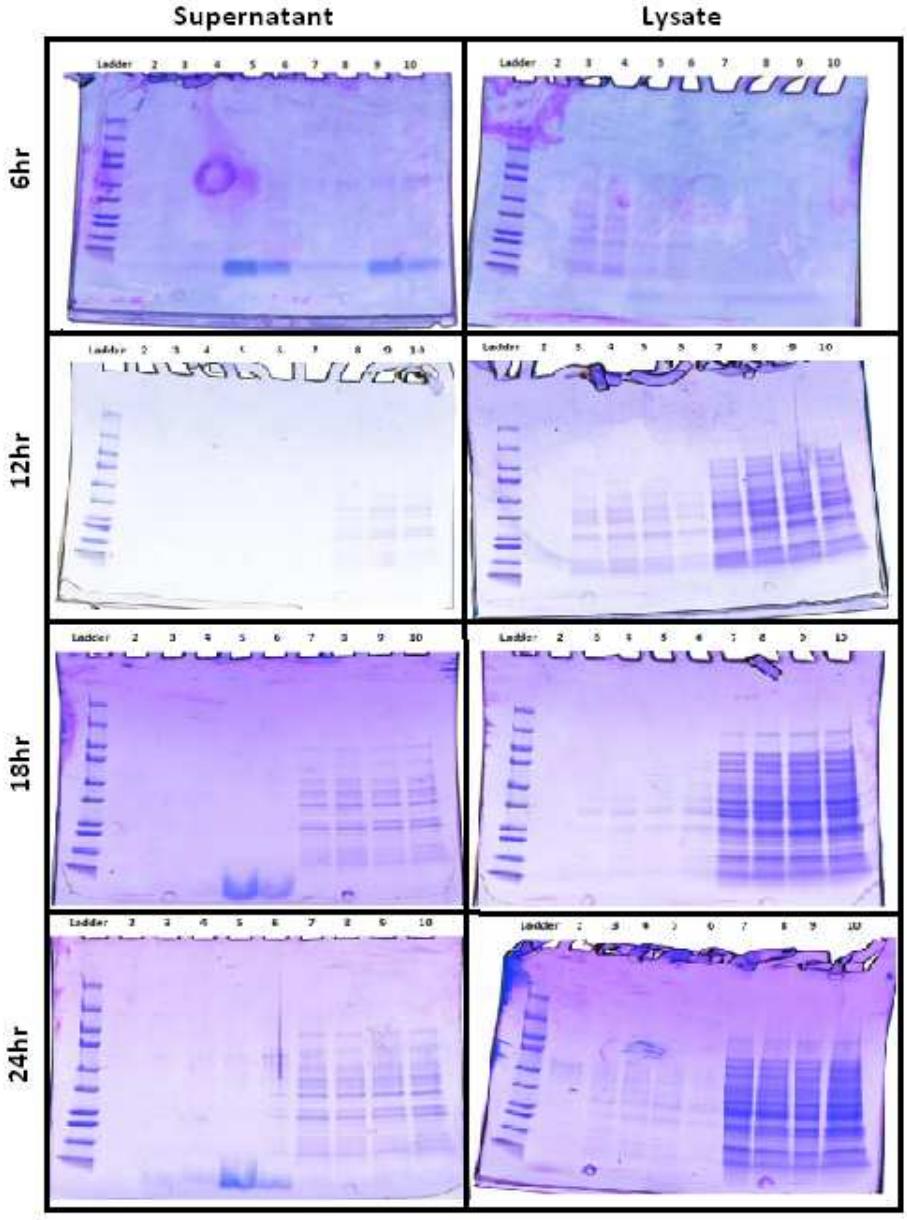

Figure 7 : SDS-PAGE results of supernatant and lysate samples taken from culture extractions at 6, 12, 18, and 24 hours of growth. Gel lanes contain the following samples: 1- Ladder, 2-M9 media only, 3-MC4100 cells in M9, 4-MC4100 cells in M9+ 1%Glu, 5-MC4100 cells with pPROTetGFP in M9, 6-MC4100 cells with pPROTetGFP in M9+1%Glu, 7-MC4100 cells with ePop in M9, 8-MC4100 cells with ePop in M9+1%Glu, 9-MC4100 cells with ePop + pPROTetGFP in M9, 10-MC4100 cells with ePop + pPROTetGFP in M9+1%Glu.

3. Observations

The supernatant samples show the expected trends in both the Pierce BCA Assay and the SDS-PAGE. Looking at Figure (7) the supernatant gel results (left), samples not containing ePop (lanes 2-6) did not exhibit significant banding while samples containing ePop (lanes 7-10) show significant protein release into the supernatant. The protein bands became more pronounced in the 18 and 24hr samples. This observation is consistent with the timing of the lysis event at ~18 hrs of culture incubation. The banding exhibited in the supernatant gels supports the idea that population level lysis oscillator circuits could be integrated into protein production systems for improving the protein release process. The protein quantities measured using the Pierce BCA Assay should be discussed. The milligram quantities of protein in only hundreds of microliters of supernatant and lysate is a high amount and may not be an accurate measurement. However, one could still use the assay as a sufficient method for relative amounts between samples. Another observation that was seen across both methods of quantification was the apparent increased general protein production in cells containing ePop. The OD readings at the extraction time points show that the samples with ePop had a much lower cell density or concentration per volume as compared to samples without the ePop plasmid (roughly 1:10). This distortion in cell density makes the greater overall protein presence in samples with ePop even more impressive when normalized with cell density. Carrying out the experiment repeatedly produced similar

results, indicating that the ePop circuit augmented protein production. However, the likelihood of the ePop circuit causing increased protein production was very low and other explanations were sought. One such explanation was an insufficient lysis protocol.

3.1 Insufficient Lysis

Since the E gene induces lysis essentially through weakening the cell wall via inhibiting the function of MraY, it is logical to assume that even before lysis, the cell walls of bacteria possessing the ePop plasmid will become increasingly fragile. With this thought in mind, it was proposed that the observed increase in protein production was not an increase in protein production at all. Instead, if the lysis protocol was not sufficient for the complete lysis of healthy cells, the results would simply be due to the fact that ePop cells are the only samples with effective lysis due to the weakened state of their cell walls. To test this hypothesis, another lysis protocol was deployed.

3.2 New Lysis Protocol

After the cells had been pelleted and the supernatant removed during the extraction, instead of resuspending the cells in the lysis buffer, cells were resuspended in 100μL of Laemmli buffer. The sample eppendorf tubes were then placed in water and boiled for 15 minutes. Boiling the samples breaks down the cell walls and causes the cells to lyse. By resuspending in Laemmli buffer, the samples can be directly loaded into the SDS-PAGE gel without having to introduce the Laemmli buffer later. Next the cell

debris was removed by centrifuging at 16,000 x g at 4°C for 20 minutes and separating the lysate by pipetting.

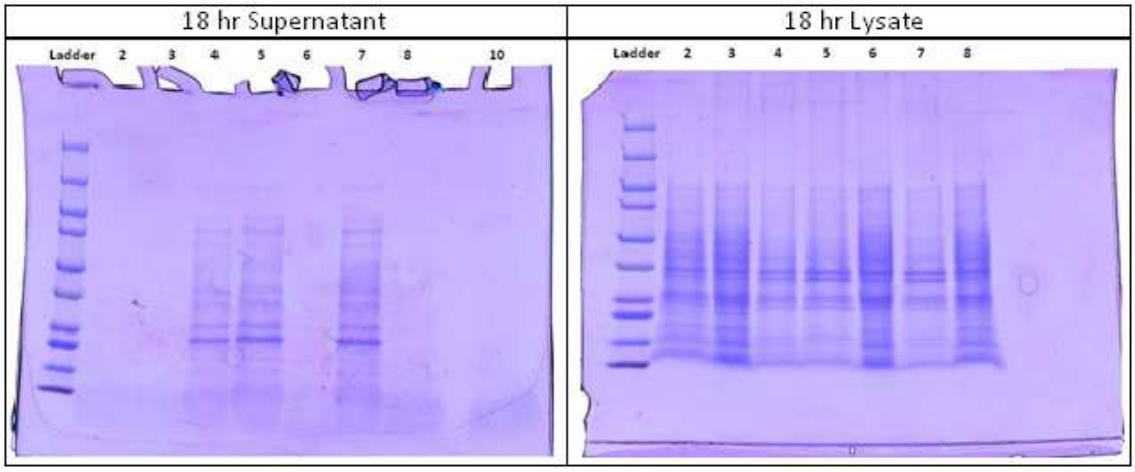

Figure 8 : SDS-PAGE results of time course supernatant and lysate culture samples after 18hrs of growth. The lysate samples underwent the new lysis protocol with boiling. Lanes contain the following samples: 1- Ladder, 2-MC4100 cells in M9, 3-MC4100 cells with pPROLarA in M9, 4-MC4100 cells with ePop-mini in M9, 5-MC4100 cells with ePop + pPROTetGFP in M9, 6-MC4100 cells with pPROTetGFP in M9, 7-MC4100 cells with ePop in M9, 8-MC4100 cells with pZS31GFPk in M9, Supernatant gel lane 10-M9 media only.

The results of the SDS-PAGE, shown in Figure (8), strongly support the hypothesis that the initial lysis protocol was insufficient for the substantial lysis of healthy non-ePop cells. Again, only samples with ePop plasmids exhibited protein banding in the supernatant (lanes 4, 5, 7). In accordance with the initial expectations, and contrasting with the results of the previous lysis protocol, the non-ePop samples displayed noticeably more protein banding (lanes 2, 3, 6, 8). These results make sense because the cell density in the ePop samples is much less due to the lysis of a percentage of the population. If the ePop circuit had been shown to augment protein production by

some hidden mechanism, it would have been a substantial unanticipated benefit of ePop; improving not only the release stage but also the overall synthesis capability. This was an unlikely scenario and turned out not to be the case.

4. Presence of Protein of Interest (GFP)

The results of the polyacrylamide gels discussed previously are effective in divulging relative total protein production. However, in order to test the viability of the oscillatory lysis circuit for the production of a specific protein of interest, a different method was needed. A western blot protocol using antibodies specific to the protein of interest, POI, (green fluorescent protein) was carried out in order to prove that the protein was being effectively released into the supernatant.

4.1 Western Blotting

The western blot protocol begins the same way that the SDS-PAGE protocol does. The proteins in the sample were linearized by adding Laemmli buffer which contains β-mercaptoethanol, to sever the disulfide bridges, and then boiled for 15 minutes to break down the hydrogen bonds of the proteins. From there, the samples were loaded in the wells of the gels, and the gel box was filled with running buffer. A 150V was applied across the gels for 1.5 hrs to allow the proteins to migrate and separate according to size. Sponge pads and filter paper were immersed in transfer buffer (20% methanol, 14.4g glycine, 3.14g TRIS buffered to 8.3pH) for 5 minutes. During this time, polyvinylidene fluoride (PVFD) transfer membrane was immersed in methanol for 5 minutes for activation. The gels were then placed in a clamp box between the pads, transfer membrane, and filter paper. A 30V gradient was applied across the clamp box overnight to drive the proteins across the filter paper and onto the membrane. Blotto

(5% blotto -> 1g skim milk in 20 mL TBST -> 3.14g TRIS Base, 8g sodium chloride, 1 tween20, buffered to 7.6pH) was used to block the PFVD membrane for one hour. This was followed by another hour blocking with a 1:1000 dilution of primary antibody (GFP) in blotto. The membrane was washed three times in TBST for 15 minutes each on a table shaker. After washing, the membrane was covered by substrate reagents, wrapped in saran wrap, and fixed to the inside of an exposure cassette. Films were exposed to the membranes in the cassette for 1, 2, 5, and 10 minutes.

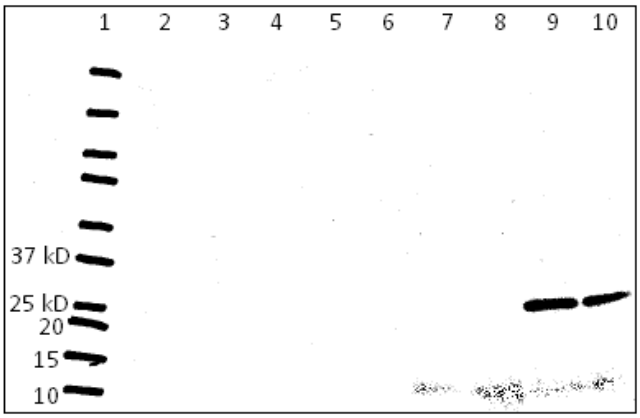

Figure 9 : Western Blot image of film exposed to PVFD membrane of 18hr supernatant samples for 10 minutes. Samples in each lane are as follows: 1- Ladder, 2- M9 media + 1% Glucose, 3- MC4100 cells in M9, 4- MC4100 cells in M9+1%Glu, 5- MC4100 cells with pPROTetGFP in M9, 6- MC4100 cells with pPROTetGFP in M9+1%Glu, 7- MC4100 cells with ePop in M9, 8- MC4100 cells with ePop in M9+1%Glu, 9- MC4100 cells with ePop and pPROTetGFP in M9, 10- MC4100 cells with ePop and pPROTetGFP in M9+1%Glu.

Figure (9) shows a western blot film produced by supernatant samples extracted after 18 hours of culture. This exhibits the desired results. Lanes 3 and 4 contain supernatant samples from MC4100 cells with no additional plasmids, 5 and 6 contain supernatant from MC4100 cells with the pPROTetGFP plasmid, and lanes 7 and 8 have

supernatant from MC4100 cells with ePop. However, only lanes 9 and 10, the supernatant from ePop + pPROTetGFP samples, exhibit protein banding. The other samples either did not release their proteins to the supernatant or did not synthesize the protein of interest. Note that even though the samples in lanes 5 and 6 produced GFP, without a lysis mechanism, no GFP was detected in the sample's supernatant. These results indicated the need for samples to both produce the desired protein and undergo some degree of lysis to release the protein of interest, in this case GFP.

4.2 Functionality of POI

In industrial scale recombinant protein production processes, while a great deal of focus is directed at quantity, the quality of the end product is equally important. For each specific protein, different culturing processes and conditions are required to produce properly folded, fully functional, and stable proteins in the most efficient manner possible[3][6][9-11]. Often, if environmental conditions are not sufficient, proteins will become degraded or fold incorrectly, thereby limiting and possibly extinguishing their functionality. This would become a concern especially when changing the environment from the cytoplasm within the cell to the surrounding media, as would be the case with lysis. While no considerations were taken into account regarding the optimal conditions for producing fully functional green fluorescent proteins, the functionality of the released proteins was maintained. The western blot shown in Figure (9) suggests that the degradation of the protein in the supernatant is minimal. The bands

produced in lanes 9 and 10 of the film suggest that the desired protein was approximately 27kD. According to the National Center for Biotechnology Information's website, the green fluorescent protein is comprised of 238 amino acids and is 26.84 kD. The lack of degradation increased the likelihood that the protein was functional but did not confirm it. The GFP signal displayed in Figure (4) also supports the notion that the GFP remains functional even after being released into the supernatant. Rather than exhibiting oscillations, adhering to the dynamics of the cell density as would be expected if function became extinct upon lysis, the GFP showed a continued growth, suggesting that the released GFP are still contributing to the overall fluorescence signal. Lastly, through the observation of cells containing ePop and pPROTetGFP within micro-encapsulations, discussed in the Application section later, the functionality of GFP in the surrounding media is confirmed. Figure (10) shows the comparison of the distinct and discrete fluorescence of GFP contained within cells along with the dispersed or blended fluorescence throughout the capsule after cellular lysis and release of the GFP.

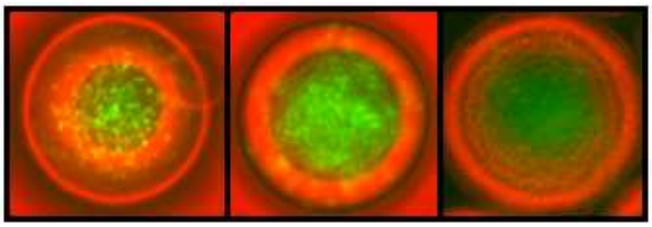

Figure 10 : Microscope images of encapsulated MC4100 cells with ePop + pPROTetGFP. Initially the GFP signal seems to be discrete and contained within cells (left) but as time goes on and lysis occurs the GFP signal begins to leak out and become less discretized (middle, right). Encapsulation and observation images were taken by Ying Zhang of the Kam Leong lab.

5. Application

There are a number of applications where protein production and release via controlled or oscillatory cellular lysis could be taken advantage of. Two such applications are currently being investigated in ongoing research projects. One is simply a continuation on the initial viability study discussed here. Now that the proof of concept has been shown, the design, testing, and optimization of a continuous protein production system involving the oscillatory lysis circuit can be investigated. The initial design involves a filtered flow system. Another application being currently pursued is the use of such a bacterial circuit as a platform technology for controlled and sustained drug delivery.

5.1 Protein Production System

Once the concept of protein production and release via oscillatory lysis circuits was shown to be viable or possible, the project began to head in the direction of designing a system that would make it possible for continuous and sustained protein production. Initial designs involve a flow system that continuously feeds the cell culture with fresh media to prevent the need for the repeated culturing and regrowth of the bacteria stock. Implementing a filtering scheme that would allow proteins to diffuse past a filter wall while preventing the passage of cells, could serve as a mechanism for streamlining the protein isolation process. Filters with various sub-micrometer pore sizes, such as those being developed and produced by Corning Incorporated Life

Sciences, can be inserted into the wells of plate reader culture plates to test the feasibility of the design.

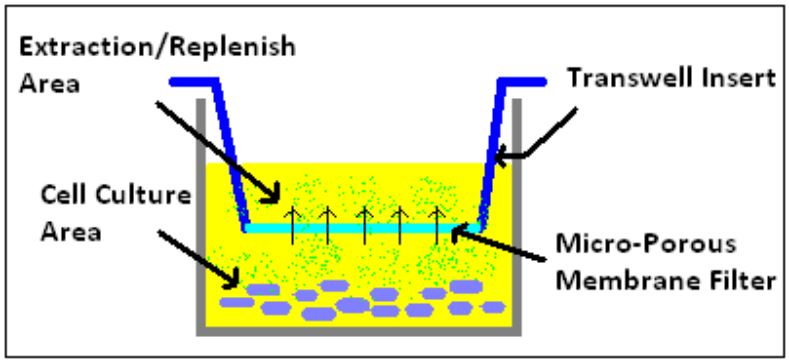

Figure 11 : Diagram of Transwell technology. The insert fits into the well of a plate reader culture plate separating the extraction area from the cell culture area. The micro-porous membrane allows proteins to diffuse through while preventing the passage of cells.

A time course experiment will be carried out, taking advantage of the system displayed in Figure (11). By extracting the volume of media above the filter for protein quantification at discrete time points followed by replenishing the well with fresh media of the same volume, continued growth and protein release could be sustained. An approximate protein production rate could be extrapolated from the protein quantifications at discrete time points.

Beyond the filter, an isolation method could be used to extract the protein of interest specifically. Such a system could be employed at the laboratory level for research requiring sufficient amounts of a certain protein. After a pilot/laboratory scale of the system has been created, an approximate volumetric protein production rate can

be calculated. This rate could then be compared with current commercial production capabilities.

5.2 Controlled and Sustained Drug Delivery

Another project involves investigating the ability of the gene circuit to be used for controlled and sustained drug and therapeutic protein delivery. The goal of the research is to develop a platform technology in which bacterial populations could be contained or encapsulated stably, while still being able to interact with the surrounding microenvironment. Environmental inputs experienced by the contained population could initiate population oscillations and subsequent protein/drug release. The project has been a collaborative effort with the lab of Dr. Kam Leong, Duke University Biomedical Engineering Department, whose focus has been primarily on optimizing the encapsulation process. The current method of encapsulation is a double emulsion technique with bacteria media in oil in fresh media. The oil micro-beads are created using two microfluidic chips in parallel as depicted in Figure (12.A).

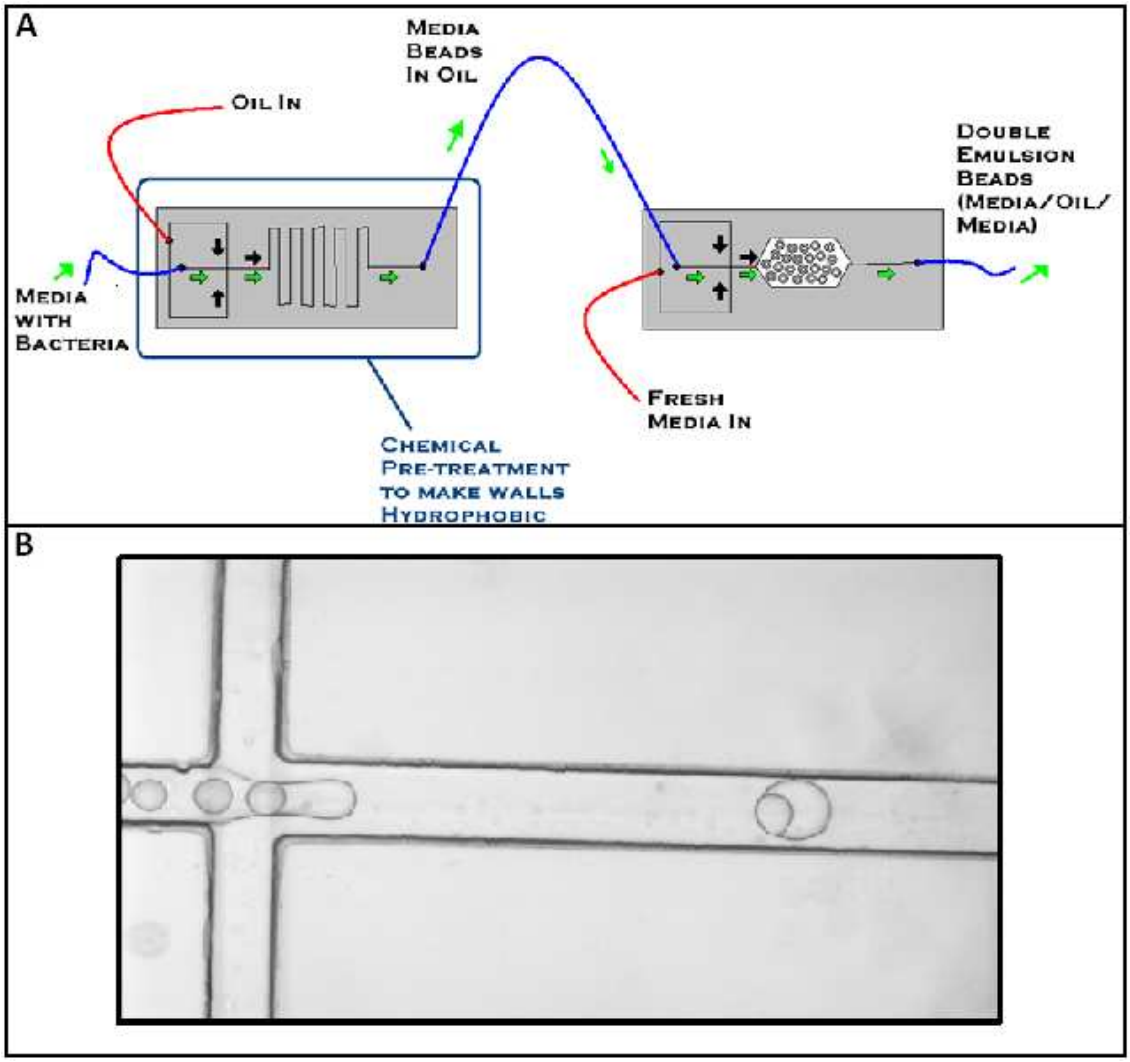

Figure 12 : A. Schematic of the two microfluidic chip setups used by the Kam Leong lab to accomplish the double emulsion encapsulation. The first chip encapsulates bacteria media droplets in oil. The second chip immerses the media in oil beads in media again. B. A microscope image of the junction of the second chip taken by Ying Zhang. Bacterial media droplets in oil (coming in from the left) are met at the intersection with media (coming from the top and bottom) creating a bacterial media droplet, encased in oil, immersed in media again.

The first chip has been chemically treated to make the walls hydrophobic and thus repellant to the bacteria media to ensure that the media beads up and becomes immersed in oil. The bacteria media beads in oil then flow into the second chip where media surrounds the oil again at the junction as shown in Figure (12.B).

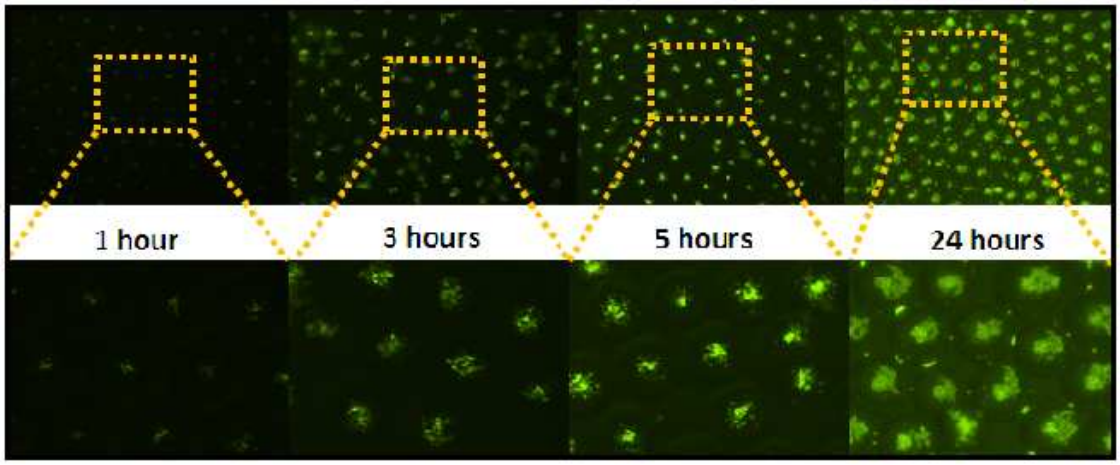

Figure 13 : Time course culture of MC4100Z1 cells with pPROTetGFP within oil encapsulations in M9 media carried out by Ying Zhang of the Kam Leong lab. There is no aTc in the initial bacteria media. aTc was only added to the media outside of the encapsulations. Microscope images were taken at 1, 3, 5, and 24 hours after encapsulation and immersion of the encapsulations in the M9+atc media.

Once inside the encapsulations, absorbance measurements are no longer effective for characterizing population growth dynamics. Thus, green fluorescence has been used for the characterization of cell population growth dynamics within the encapsulations. Fluorescence was shown to be sufficient for characterization using constitutive GFP expression via pPROTetGFP plasmids in MC4100 cells. However, when investigating the ability of small molecules to diffuse through the oil encapsulation, MC4100Z1 cells with pPROTetGFP were used. The MC4100Z1 cells express a tet repressor preventing

synthesis of GFP unless induced by aTc. By adding aTc to the media outside of the encapsulations the diffusive ability of the molecule could be shown through the eventual occurrence or absence of a fluorescent signal from the cells within. Fluorescence was produced within an hour depending on the thickness of the oil layer so the coefficient of diffusion for aTc through the oil layer appears to be fairly high, though more characterization is required. Also, the ability of the cells to release drugs and proteins through the encapsulation and into the environment has yet to be explored.

While the oil encapsulations have been able to successfully encapsulate the bacteria, their stability remains suspect. Notable percentages (typically ~25%) of the encapsulations break down within 24 hours. This is most likely due to two factors: the cell growth within causing increasing pressure and evaporation of the oil. This instability occurs without any significant motion or movement of the encapsulations, which would be necessary for sustained delivery scenarios. For these reasons, methods, such as solid polymer encapsulation, are being pursued to increase stability. If stable encapsulation can be achieved and the oscillatory growth dynamics maintained, it could prove to be a very useful and effective technology.

In the future, the release of a tumor necrosis factor related apoptosis inducing ligand, or TRAIL, will be tested. The TRAIL protein has been shown to be very effective in the specific targeting and killing of tumor cells and therefore would give the proposed platform technology a specific and significant application. For this instance, the

established dynamics of ePop happens to serve as a relevant control mechanism. Since the lysis and oscillations exhibited by ePop have been shown to be influenced by the environmental glucose concentrations, the circuit can be modulated by the local levels of glucose experienced by the cells. Since tumors are formed by the uncontrolled growth of a population of cells, the increased metabolic activities consume higher amounts of glucose leading to a lower relative glucose concentration in the micro-environment surrounding the tumor. In this way, the encapsulated bacteria could deliver therapy via lysis when needed, based on environmental cues such as glucose levels.

6. Conclusions

The ePop circuit investigated in this study serves as a proof of concept for controllable oscillatory lysis circuits to be used for various applications. In order to be effective and applicable, the dynamics would need to be sustained for extended periods of time. The oscillatory constructs transformed as a plasmid are likely to become mutated and not maintained as needed. For the purpose of longterm protein production and release, integration of the oscillatory/lysis constructs into the host genome would be required. Due to its current oscillation mechanism of plasmid copy number fluctuations, ePop in its current form would not be ideal. Other well-defined oscillation methods such as the initial ePop design, exploiting the luxR/luxI quorum sensing system, would be better suited. The ability of controlled and sustained lysis to effectively release proteins into the media could prove to be an extremely advantageous mechanism when integrated into recombinant protein production processes. Proteins could be continuously synthesized and released without requiring the complete annihilation and regrowth of the stock culture. Optimization of circuit dynamics could lead to the lysis/death rate equaling the growth rate of cells in the exponential growth phase, creating a truly continuous release of proteins.

For the drug delivery or protein therapy application, the encapsulation technology must first be perfected. The public will not be comfortable with implanting bacteria into the body until it has been proven that the bacteria can be successfully

contained. However, oscillating bacterial populations would be able to provide sustained delivery of a drug when implanted near an area of need. The ability of an oscillatory circuit to respond to environmental inputs opens up many other possible applications for such controlled or programmable protein release. For example, instead of a patient taking medication at the onset of pain or insulin after checking blood sugar levels, bacteria could be programmed to sense the desired changes in the environment and initiate oscillations and subsequent protein or drug release accordingly.

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