Spring 5-2020

Toward Controlling Cardiac Tissue Pacing Using Modified mRNA

Yicheng Zhao
Washington University in St. Louis

Washington University in St. Louis

McKelvey School of Engineering

Department of Biomedical Engineering

Thesis Examination Committee:

Nathaniel Huebsch, Chair

Jonathan Silva

Stacey Rentschler

Toward Controlling Cardiac Tissue Pacing Using Modified mRNA

By

Yicheng Zhao

A thesis presented to the McKelvey School of Engineering of Washington University in St. Louis in partial fulfillment of the requirements for the degree of Master of Science

May 2020

St. Louis, Missouri

Dedication

I want to dedicate this thesis to my parents and grandparents for their life-time guidance, love, and support.

Acknowledgements

I would first like to thank my advisor, Dr. Nathaniel Huebsch, who provided every help I needed guided me through the research process, and also providing the data for virus transfection of this study. I would also like to acknowledge the support from Daniel Simmons, Soore Oguntuyo, and Jingxuan Guo for the iPSC differentiation and iPSC-CM μHM formation.

In addition, I wish to thank Dr. Jon Silva for the support and advices on mmRNA synthesis, Lily Malcolm for the help on the mmRNA synthesis modification, and Kuo-Chan Weng for the support on LED pacing of transduced cells.

Finally, I would also like to acknowledge the committee members, Dr. Silva and Dr. Rentschler, for taking the time to attend the oral defense and reading this thesis.

Yicheng Zhao

Washington University in St. Louis

May 2020

Table of Contents

List of Figures

Abstract

Toward Controlling Cardiac Tissue Pacing Using Modified mRNA

By

Yicheng Zhao

Master of Science in Biomedical Engineering

Washington University in St. Louis, 2020

Research Advisor: Professor Nathaniel Huebsch

Arrhythmia is a common heart disease that happens when the heart is beating too fast, too slow, or irregularly. To study the mechanisms and treatments of this disease, it is important to acutely control the beating rate of the model as it will help distinguish the contribution of different potassium currents and drug-induced action potential in cardiomyocytes. The current method of tissue pacing, electrical pacing, causes contamination and corrosive damage to tissues, thus the tissues fail to be used repeatedly or in future studies. In this study, red-shifted channelrhodopsin (ReaChR) is applied as a non-chemical means to control the beating rate. ReaChR is a light-gated ion channel that opens and allows potassium to enter cardiomyocytes when excited by red lights. To deliver ReaChR into micro tissues, modified mRNA is chosen because of its higher transfection rate comparing to the plasmid, and lower cell toxicity comparing to the virus. Reducible polycation (RPC) is synthesized and used as transfection reagent to acquire a better transfection rate and modifiable structure. The results show successful modified mRNA synthesis and enhanced transfection efficiency with modified mRNA comparing to plasmid in both cells and tissues. The improved transfection efficiency of modified mRNA into iPSC-derived cardiomyocytes and iPSC-derived micro heart muscle using RPC is achieved. The results presented in this thesis demonstrate

the potential of using modified mRNA to control the beating rate of the tissue and eventually control other physiological properties in cells.

Chapter 1: Introduction

Arrhythmia is a common heart disease that happens when the heart is beating too fast, too slow, or irregularly (National Heart Lung and Blood Institute). Over 2.2 million Americans are suffering from arrhythmia, and it may lead to cardiac arrest, stroke, or even sudden death (American Heart Association, 2016, Sep 30b; Health Engine, 2004, Sep 15). Some previous studies have shown that arrhythmia is caused by gene mutations and triggered by the increased mechanical loading of cardiac muscle, which can happen during intense exercise (Herren, Gerber, & Duru, 2009; La Gerche, 2015). Current medications include calcium-channel blocker, beta blocker and anticoagulant (American Heart Association, 2016, Sep 30a). However, some of those drugs have unstable performance in different patients (Krikler, Harris, & Rowland, 1982). Therefore, further study on the mechanisms of arrhythmia and the effects of different medications on the phenotypes is needed.

Acutely controlling the beating rate of cardiac tissue allows the characterization of the conduction velocity, which is a critical factor affecting arrhythmia. It is also a crucial aspect of distinguishing the contribution of different potassium currents and drug-induced action potential in cardiomyocytes (La Gerche et al., 2012). In general, to study the chronic effect of specific drugs on cardiomyocytes and cardiac tissue, and to avoid the interference factors from mechanical environment, adrenergic stimulation, and other physiological signaling, it is essential to have a non-chemical means of controlling the beat rate of cardiac tissue.

Currently, the common method is electrical pacing. Although the electrical pacing can be used to induce the maturation of iPSC-derived cardiomyocytes (iPSC-CM) and to exacerbate the

phenotype of familial cardiomyopathy(Davis et al., 2016; Ronaldson-Bouchard et al., 2018), the corrosive damage and contamination to the tissues impede further incubation after pacing, thus further studies cannot be done on the same sample. Therefore, using an optogenetic approach can allow the cardiac tissue being paced by light and avoid the damages mentioned before.

Channelrhodopsin is a light-gated ion channel on cell membranes. The natural form of it is controlled by blue light, and when the all-trans-retinal in the channelrhodopsin absorbs blue light, a conformational change will happen as all-trans-retinal becomes 13-cis-retinal. This conformational change will lead to a further change happen in the transmembrane protein, which will open up a pore on the membrane and allow an influx of ions. After milliseconds, the 13-cis-retinal will shift back to all-trans-retinal, and the pore will be closed to stop the flow of ions(Nagel et al., 2003). Because the blue light may be toxic to human cells, a red-shift channelrhodopsin (ReaChR) has been used in recent studies(Lin, Knutsen, Muller, Kleinfeld, & Tsien, 2013). ReaChR can be excited by orange or red light, and has a higher membrane trafficking, higher photocurrents, and faster kinetics than channelrhodopsin-2. In addition, the red light will be absorbed less into blood comparing to the blue light(Lin et al., 2013).

It is notably challenging to deliver the ReaChR directly into cardiac tissue because several barriers need to be overcome to reach the nucleus. From the outside, ReaChR needs to go through endothelial barrier, extracellular matrix, and cell membrane before reaching the nucleus [16]. The current method of delivering ReaChR into the human heart or iPSC-derived bioengineered cardiac tissue is by virus infection [14]. Although viral delivery has a higher transfection efficiency and a longer half-life comparing to the non-viral approaches, there is a risk of cytopathic effects, multiple

insertion sites, and mutagenesis [15]. Plasmid delivery has a simple production process, but because DNA needs to go through both cell and nucleus membrane to be expressed, the transfection rate is low and it is less effective in non-proliferating cells, meaning it may not work in tissues(Al-Dosari & Gao, 2009). To find the delivery method that is safe and efficient, modified mRNA is used as the gene delivery vehicle.

Modified mRNA (mmRNA) is a chemically modified messenger RNA that allows the gene to be expressed by cells. Unmodified mRNA will be recognized by the innate immune system in the endosome, and the protein synthesis will be shut down and eventually cause cell death(Karikó, Buckstein, Ni, & Weissman, 2005). The modification is done by substituting uridine with pseudouridine and cytosine with 5-methyl-cytosine during in vitro transcription, and the modification can help avoid the attack from the immune system, and therefore allow the protein to be synthesized and expressed in the host cell(Karikó et al., 2005; Kariko, Muramatsu, Ludwig, & Weissman, 2011).

The goal of this study is to develop a safe and efficient method to control the beating of the in vitro cardiac tissue that allows the tissue to be used repeatedly in multiple experiments, and eventually serve to help further understanding of the mechanisms of arrhythmia and finding better solutions using the iPSC-derived micro heart muscle (μHM) previously developed(Huebsch et al., 2016). To improve the transfection result, the reducible polycation (RPC) is used as a transfection reagent of the nucleic acids(Read et al., 2005). The previous study has shown that by coating the nucleic acids with positive charges, the histidine-rich RPC helped improve the transfection efficiency in cells since it can be cleaved by the intracellular reducing environment, which allows the better

release of nucleic acids and lower toxicity(Read et al., 2005). In this study, the RPC is used as the transfection reagent to improve the transfection of ReaChR plasmids and mmRNAs into tissues. This study also shows the possibility to use mmRNA to control other properties of the cell with the gene delivery methods and mmRNA synthesized procedure developed here that help the investigation of other aspects of heart diseases.

Chapter 2: Materials and Methods

In this chapter, the materials and methods used for tissue pacing and gene delivery are described. Three main methods (lentivirus, plasmids, modified mRNA) applied for gene delivery into micro tissues are elaborated. The preparation of plasmids, modified mRNA and the transfection reagent is also included.

Micro Tissue Formation with PDMS Stencils

The dogbone PDMS stencils were first absorbed and disinfected onto a 24-well plate or a 12-well plate with three dogbones per well prior to the tissue formation, and then treated with Pluronics F68 and Fibronectin for better cell adhesion(Huebsch et al., 2016). The iPSC was differentiated into cardiomyocytes by manipulating the Wnt signal following a previously modified protocol(Huebsch et al., 2016; Lian et al., 2013). After differentiation, the iPSC-CMs were singularized using 0.25% trypsin and then combined to a final density of 1×10^8 cells/mL in EB20 media (Dulbecco's Modified Eagle Medium with 20% fetal bovine serum) with 10μM Y27632 and 150μg/mL L-ascorbic acid. The iPSC-CMs were then seeded into a dogbone PDMS stencil with 2μL of the cell/media mixture given a final concentration of 2×10^5 cells per dogbone. The plate was then being centrifuged to get the cells into the dogbone stencils, and then the wells were filled with EB20/Y27632/ascorbic acid media. On the next day, when the cells started beating, they were fed with RPMI/B27/ascorbic acid media, and the cells were fed every other day until the micro tissues were formed.

C2C12 tissues were formed based on a similar protocol. The C2C12 fibroblast was singularized using 0.05% Trypsin, and then they were suspended into Dulbecco's Modified Eagle Medium (DMEM) with 20% fetal bovine serum (FBS) to a density of 1×10^7 cells/mL, and seeded to the

dogbone stencils with 5μL per dogbone, given a final density of 5×10^4 cells per dogbone. The cells were fed with DMEM with 20% FBS. The cells were compacted inside of the dogbone stencils and the tissues were formed around day 4 after cell seeding.

C2C12 Myotube Differentiation

The C2C12 myotubes were differentiated from wild type C2C12 fibroblasts based on a previous protocol(Asano, Ishizuka, Morishima, & Yawo, 2015). On day 0, the C2C12 cells was seeded at a density of 5×10^4 cells/well for 48-well plate in culture media (DMEM with 20% FBS). On day 1, when the cells reached around 80% confluency, transfect the cells with desired plasmid. The transfection result was observed and imaged on the next day, and after confirming the success of transfection, change culture media into differentiation media (DMEM with 5% horse serum), and the media was changed every other day. Observe the morphology of the cells everyday until the C2C12 myotubes formed.

Plasmid Cloning and Modified mRNA synthesis

The ReaChR protein was obtained from pLenti-ReaChR-citrine (Addgene plasmid # 50956 ; http://n2t.net/addgene:50956 ; RRID:Addgene_50956)(Lin et al., 2013). In order to have the plasmids expressed in human cells, the Ef1α promoter was fused into the plasmid using the In-Fusion Cloning kit (Takara #638920) to replace the hSyn promoter. The ReaChR-citrine portion was also fused into GenII plasmid (Cloned by Tim Rand in the lab of Shinya Yamanaka (Warren et al., 2010)) using In-Fusion Cloning, and the T7 promoter and PolyA tail in the plasmid allowed it to be a DNA template for *in vitro* transcription to synthesize modified mRNA (mmRNA). The Citrine is a signaling protein to allow the visualization of the transfection. The LifeAct GFP was also fused into the GenII plasmid to serve as a DNA template for GFP mmRNA.

The mmRNA synthesis process was modified based on a previous protocol using the MEGAscript T7 transcription kit (Thermo Fisher, cat. AM1334), murine RNAse inhibitor (NEB, cat. M0314S), 5-Methylcytidine-5′-triphosphate (Me-CTP; Trilink, cat. no. N1014), Pseudouridine-5′-triphosphate (Pseudo-UTP; Trilink, cat. no. N1019), vaccinia capping system (NEB cat. M2080S), and mRNA Cap 2′-O-Methyltransferase (NEB cat. M0366S)(Mandal & Rossi, 2013). The plasmids were first digested into linear DNA, and the reaction was set up for in vitro transcription as follow:

Amount	Component
To 20 ul	NF H2O
2 ul	GTP Solution
2 ul	ATP Solution
1.5 ul	Me-CTP
1.5 ul	Pseudo-UTP
2 ul	10x Reaction Buffer
0.8 ug	Linearized DNA
2 ul	Enzyme

After incubation for 4 hours at 37 °C, 1µL of TURBO DNase was added and further incubated for 15 minutes at 37 °C to digest the residual DNA template. After the incubation, the phenol-chloroform extraction was done to clean up the synthesized RNA. Before capping the RNA, 10µg uncapped RNA was combined with nucleus free water to a final volume of 13.5 µL, and they were

heated at 65 °C for 5 minutes and then paced on ice for another 5 minutes to denature the uncapped RNA. The reaction was then set up for enzymatic capping as follow:

Amount	Component
13.5 ul	Denatured uncapped RNA
2 ul	10x Capping Buffer
1 ul	GTP (10 mM)
1 ul	SAM (4 mM, dilute 32 mM stock to 4 mM)
0.5 ul	Murine RNase Inhibitor
1 ul	Vaccinia Capping Enzyme (10 U/ul)
1 ul	mRNA Cap 2'-O-Methyltransferase (50 U/ul)

The reaction was done by 60 minutes incubation at 37 °C. Another phenol-chloroform extraction was done to clean up the capped RNA and to obtain our final product of mmRNA. The concentration of the mmRNA was measured by NanoDrop spectrophotometer. As a positive control, CleanCap EGFP mRNA (TriLink, cat. L-7201) was used for transfection.

Reducible Polycations Preparation

The Reducible Polycation (RPC) was prepared based on a previous study by Read et al(Read et al., 2005). The polycondensation reaction was done with 2 mg $CH_6K_3H_6C$ monomer into 150 µL phosphate-buffered saline (PBS) containing 30 vol% DMSO. The reaction was performed at room temperature for 48 hours with the solution was continuously being gently shaken on a vortex mixer. After the reaction was done, the RPCs were purified from DMSO and cyclic by-products using centrifugal ultra-filters with molecular weight cut-off 10,000. The concentration of RPCs was measured by BCA assay using the BCA Protein Assay kit (Thermo Fisher, cat. 23227). Finally,

the RPCs were filter sterilized (0.2μm) and stored at 4 °C. The gel-shift assay was done to exam the functionality of RPC. The RPCs were combined with Plasmid DNA and incubated for 30 minutes, and then the polyplexes were run on the gel at 135 volts for 15 minutes.

Cell and Tissue Transfection

For cell transfection, the C2C12 cells were seeded to a 48-well plate at 2.5×10^4 cells per well a day before the transfection. Right before transfection, the plasmid or mmRNA was diluted into Opti-MEM (Thermo Fisher, cat. 31985088) to a final concentration of 50 μg/mL, and then the RPC was added with desired weight ratios of RPC/nucleic acid and the mixture was mixed well by gently pipetting the solution up and down. The polyplexes were formed by incubation at room temperature of the solution for 15-30 minutes. After incubation, the polyplexes were added directly into the culture media drop by drop, and the plate was being rocked gently to enable even distribution of nucleic acids. The amount of nucleic acids added were 0.5 μg plasmids per well and 0.25 μg mmRNA per well, and each well was seeded with 2.5×10^4 cells. As a positive control, the commercial reagent, Lipofectamine 3000 (Thermo Fisher, cat. L3000001), was used for plasmid transfection, and TransIt-mRNA (Mirus, cat. MIR2225) was used for mmRNA transfection. For tissue transfection, both C2C12 tissues and iPSC-CM micro tissues have three tissues per well, and the ratio of the amount of nucleic acid delivered over the numbers of cells seeded was tested with the same ratio of cell transfection and double the ratio. Around 18 hours after mmRNA transfection and around 26 hours after plasmid transfection, the cells and tissues were imaged to get the transfection result using Nikon microscope. The transfection efficiency was quantified using Matlab by finding the number of cells expressing the signaling protein and the overall number of cells.

Chapter 3: Results of Modified mRNA Synthesis and Gene Delivery

This chapter provides the results of modified mRNA synthesis and gene delivery into tissues. The transfection efficiency in cells and tissues using plasmids and modified mRNA are analyzed and compared. The gene delivery results of C2C12 tissues and iPSC-derived micro heart muscles with the use of reducible polycation are also compared.

Expression of Synthesized ReaChR mmRNA in C2C12 Cells

The synthesized ReaChR mmRNA and LifeAct GFP mmRNA were transfected into C2C12 cells, and the transfection result is shown (**Figure 1**). The signal proteins are showing green under GFP for EGFP mRNA, LifeAct GFP mmRNA, and ReaChR mmRNA, and they are showing the shape of the C2C12 cells (**Figure 1A-C**). Comparing to the images showing the nucleus of the cells (**Figure 1D-F**), most of the cells are expressing the delivered proteins, indicating that the synthesized mmRNA has a high transfection efficiency, and the toxicity to cells is limited.

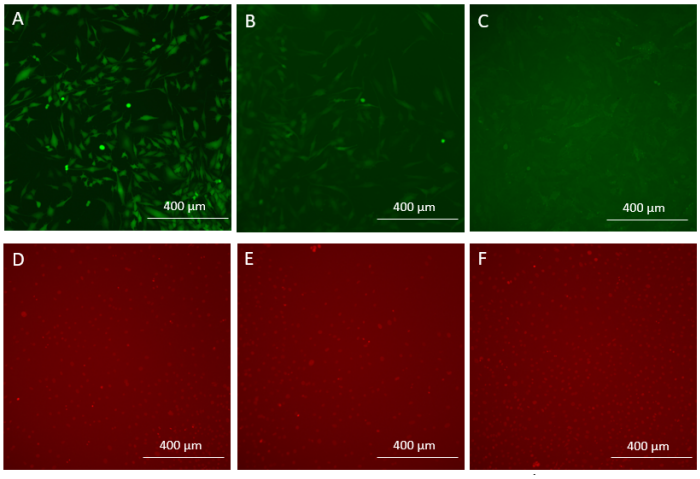


Figure 1. The result of mmRNA shows the successful synthesis of mmRNA, the high transfection efficiency of mmRNA, and the limited toxicity to cells. (A-C) Images showing the transfection result of EGFP mRNA, GFP mmRNA, and ReaChR mmRNA respectively, and green is the expressing protein, proves the cell is transfected. (D-F) Red is the nucleus of C2C12 cells, showing the total amounts of cells of each condition in the same order.

Application of RPC on Plasmid Delivery and mmRNA Delivery

RPC was proved to be functioning by gel-shift assay (**Figure 2)**. The assay was done with different weight ratios of RPC over Plasmid DNA, and as the ratio increases, less free Plasmid DNAs are released in the gel, proving that the RPC has capsulated the plasmids. The transfection of Plasmid DNA into C2C12 cells using RPC showed a higher transfection rate comparing to the commercial reagent **(Figure 3)**. The imaging results showed the C2C12 cells are transfected successfully with GFP plasmids using both Lipofectamine 300 and RPC (**Figure 3B-I**), where RPC greatly improves the transfection efficiency. The images are showing the transfection results of Lipofectamine 3000 (**Figure 3B, F**), RPC/DNA w/w 100 (**Figure 3C, G**), RPC/DNA w/w 200 (**Figure 3D, H**), and RPC/DNA w/w 300 (**Figure 3E, I**). The best efficiency happened at the RPC/DNA weight ratio of 300, and the transfection rate is around 20% while the transfection rate with Lipofectamine 3000 is around 8% (**Figure 3A**). Therefore, we can conclude that RPC doubles the transfection efficiency of Plasmid DNA delivery into cells comparing to using Lipofectamine 3000.

However, RPC failed to improve the transfection of mmRNA delivery into C2C12 cells comparing to the commercial reagent, TransIt (**Figure 4**). The transfection rate was quantified using Matlab and calculated by GFP expressing cells (**Figure 4B-E**) over overall cell numbers (**Figure 4F-I**). Comparing the transfection rate of EGFP mRNA, it is clear that RPC has a lower transfection efficiency given that the transfection rate of TranIt is around 60%, and the RPC is around 30% (**Figure 4A**). The images are showing the transfection results of TransIt (**Figure 4B, F**), RPC/RNA w/w 40 (**Figure 4C, G**), RPC/RNA w/w 80 (**Figure 4D, H**), and RPC/RNA w/w 160 (**Figure 4E, I**). Comparing the results of Plasmid DNA delivery and mmRNA delivery, mmRNA has a significantly higher transfection rate than plasmids. As for iPSC-CMs, the RPC with

RPC/RNA w/w ratio of 40 seems to improve the transfection efficiency of EGFP mRNA delivery comparing to TransIt, but the difference is not significant (**Figure 5**).

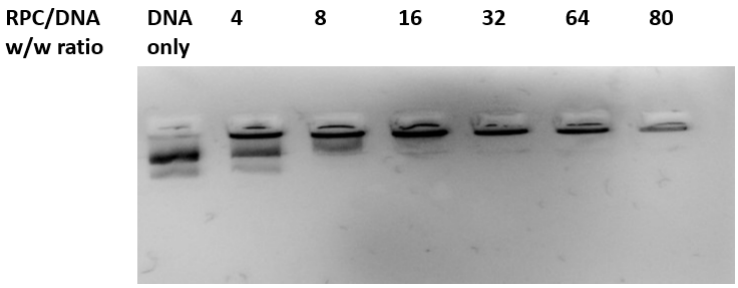


Figure 2. The gel-shift assay showed that the Plasmid DNA is encapsulated inside the RPC as less free plasmids are released as the RPC/DNA weight ratio increased.

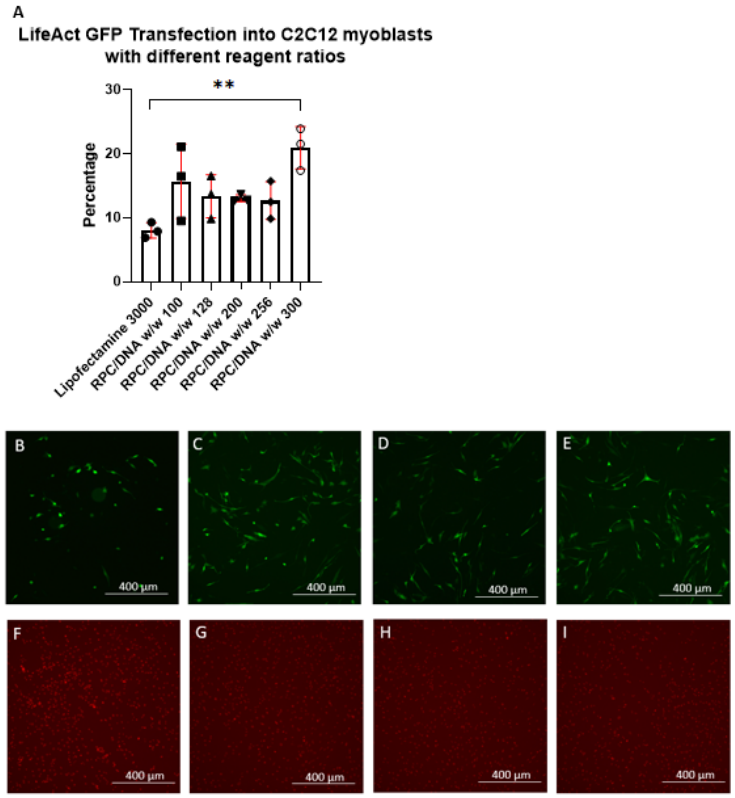


Figure 3. The result of Plasmid DNAs transfection shows RPC as transfection reagent improves the transfection efficiency. (A) Comparing the percentage of transfection, RPC has a better transfection rate than Lipofectamine 3000, and the best result is at RPC/DNA w/w ration of 300. (B-E) The green indicating transfected cells expressing GFP, and the imaging results of Lipofectamine 3000 and RPC/DNA w/w ratio of 100, 200, 300 respectively show the C2C12 cells expressing GFP, and (F-I) red shows the nucleus of the cells. (** indicated $P < 0.005$)

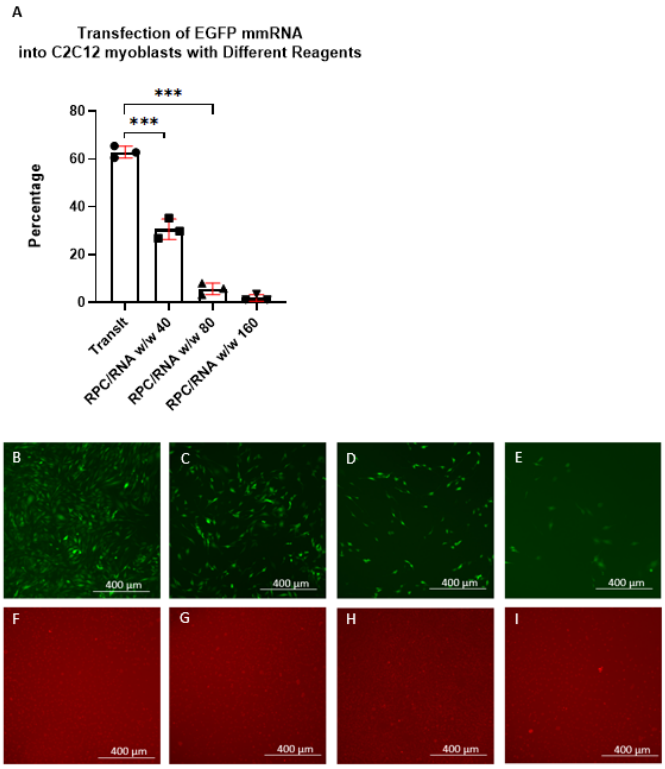


Figure 4. The result of EGFP mRNA transfection into C2C12 cells shows RPC as transfection reagent gives lower transfection efficiency comparing to TransIt (A) Comparing the percentage of transfection, RPC has a lower transfection rate than TransIt, which is 60% and the best result of RPC is at RPC/RNA w/w ration of 40, which is around 30% (B-E) The imaging results of TransIt and RPC/RNA w/w ratio of 40, 80, 160 respectively show the C2C12 cells expressing GFP, which is the green, and (F-I) red is the nucleus of the cells, showing the total number of the cells. (*** means $P < 0.001$)

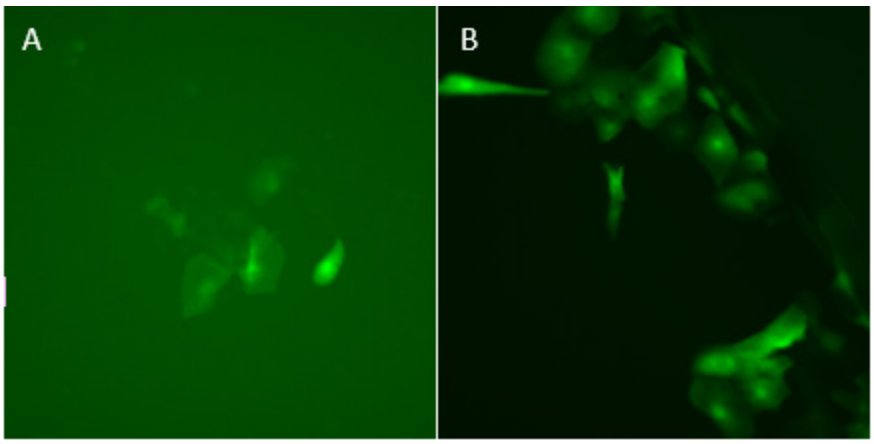


Figure 5. The result of EGFP mRNA transfection into iPSC-CM shows RPC as transfection reagent gives slightly higher transfection efficiency compared to TransIt. (A) 0.5 µg mRNA was transfected into one well of cardiomyocytes using TransIt reagent. Green is indicating the cells that are transduced. (B) The same amount of mRNA was delivered into one well of

cardiomyocytes using RPC with the RPC/RNA w/w ratio of 40. The difference in transfection rate was not significant.

mmRNA Delivery into C2C12 Tissues and iPSC-CM μHM

After confirming mmRNA and Plasmid DNA can be transfected in cells, they are used to transfected into tissues directly. In order to find an efficient delivery method, the transfections are done in C2C12 tissues first. With three tissues per well for a 12-well plate, and each dogbone seeded with 5×10^4 cells per dogbone, given 1.5×10^5 cells per well. The amount of LifeAct same DNA/cells ratio in the protocol of Lipofectamine 3000. Similarly, the amount of GFP mRNA was decided to be 1 μg per well. The transfections were done on day 4 or day 5 after cell seeding when the tissues were fully formed with both conditions of RPC and commercial reagents. The RPC/DNA w/w ratio was 300 since it has the best transfection rate in cell transfection, and similarly, RPC/RNA w/w ratio was 40. From the imaging results, RPC has a higher transfection efficiency in tissues for LifeAct GFP plasmid (**Figure 6A-B**), but has a lower transfection efficiency for GFP mRNA (**Figure 6C-D**). Also, the GFP mRNA has a significantly higher transfection rate than GFP plasmid, especially the more condensed parts of the tissue indicating by higher intensity of the red fluorescence from nuclei (**Figure 6).** It is shown in the images that at the most condensed part of tissues, the LifeAct GFP plasmid hardly has any expression, but on the other hand, the EGFP mRNA has a similar level of expression throughout the tissue. This result indicate that it is hard for Plasmid DNAs to be transfected into intact tissues. Another transfection doubling the amount of the nucleic acid was done with both Plasmid DNA and mmRNA, but shows no noticeable increase in transfection rate (data not shown).

The iPSC-CM μHM was transfected with EGFP mRNA and LifeAct GFP Plasmid DNA, but the expression GFP plasmid can hardly be seen when imaging inside the μHM (data not shown). The

EGFP mRNA transfection was done with four different conditions, including using TransIt as transfection reagent with normal ratio of mRNA/cells (**Figure 7A**), TransIt with double ratio of mRNA/cells (**Figure 7B**), RPC with normal ratio of mRNA/cells (**Figure 7C**), and finally RPC with double ratio of mRNA/cells (**Figure 7D**). The results are showing that with doubling the amount of mRNA transfected, the transfection rate increased, and the RPC has a higher transfection rate comparing to TransIt. Overall, the transfection using RPC with double ratio of RPC/RNA has the best outcome regarding of transfection efficiency. This result is inconsistent with the results from C2C12 tissue transfection, leading to the possibility that cardiomyocytes have different barriers for Plasmid DNA and mmRNA delivery.

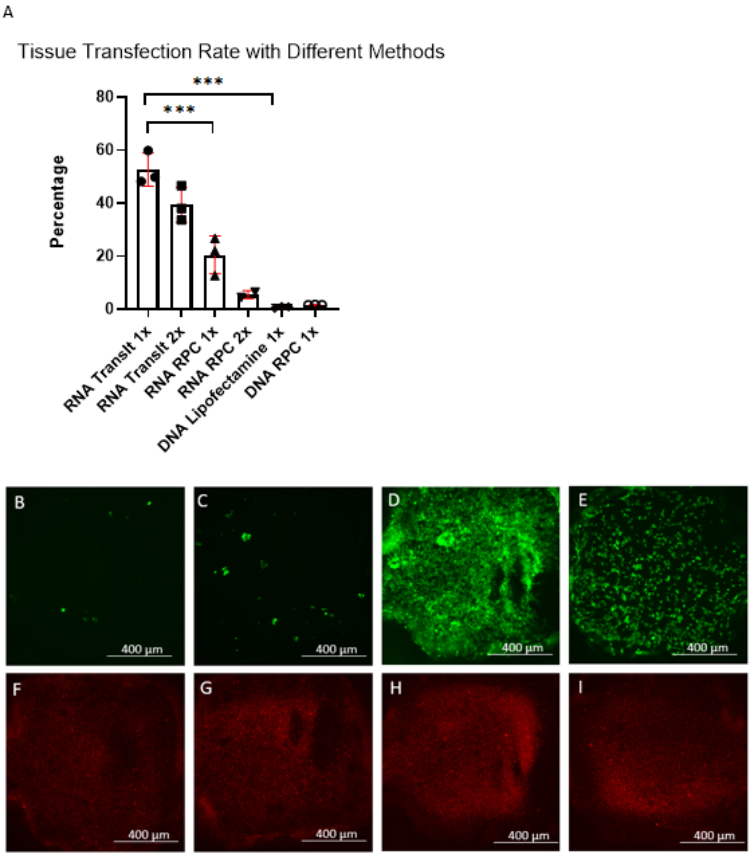


Figure 6. The result of LifeAct GFP plasmid DNA and EGFP mRNA transfection into C2C12 tissues shows mmRNA has a much higher transfection efficiency in tissues than DNA, especially for the most condensed part. The green indicating the expressing GFP protein, and the red indicating the cell nucleus. The higher intensity of the red means more condensed of the part of

the tissue. (A) The quantification result shows that mmRNA has a significantly higher transfection rate using TransIt comparing to RPC and plasmid transfection, but doubling the amount of mmRNA has limited effect. (B, F) The GFP plasmids can hardly be expressed in C2C12 tissues with Lipofectamine 3000. (C, G) The transfection efficiency was slightly improved by RPC as reagent with RPC/DNA w/w 300, but the plasmid DNA was still hard to get into the condensed part. (D, H) EGFP mRNA transfection using TransIt has a significantly higher transfection rate comparing to plasmid DNA, and even the condensed parts have a high level of cells being transfected. (E, I) mmRNA transfection using RPC with RPC/RNA w/w 40 has a lower transfection rate than using TransIt, but is still much higher than plasmid DNA. (*** means $P < 0.001$)

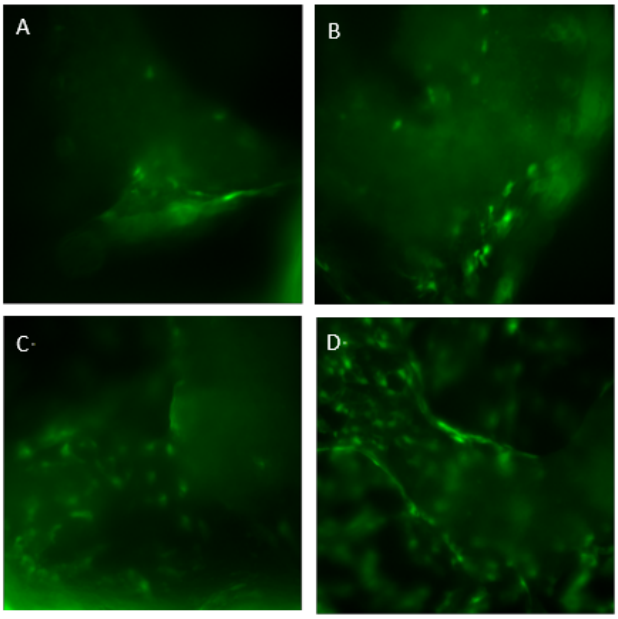


Figure 7. The result of EGFP mRNA transfection into iPSC-CM μHM shows RPC as transfection reagent and with double amount of mRNA delivered gives the best transfection efficiency. (A) Normal ratio of mRNA/cells, 1 μg per well for 24-well plate and three tissues per well, were transfected using TransIt reagent. Scattered green dots indicating the cells that are transduced, and the blurry green background is the GCamp indicating calcium activities. (B) Double ratio of mRNA/cells, 2 μg per well, were transfected using TransIt reagent. (C) Normal Ratio of mRNA/cells were transfected using RPC with RPC/RNA w/w 40. (D) Double ratio of mRNA/cells were transfected using RPC.

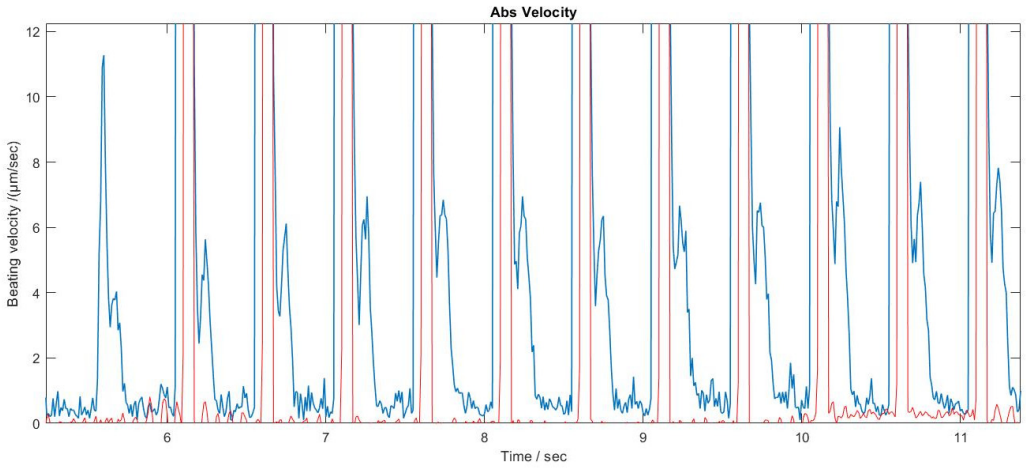


Figure 8. The red line is the shining of LED light, and the blue line is the beating of iPSC-CM. The first blue peak is the spontaneous beating of the cardiomyocytes with the first larger peak being the contraction, and the second lower peak being relaxation. The peaks and frequency of LED light and cell beating matches, indicating that the cardiomyocytes have a spontaneous beating, but it follows the frequency of the LED light when the light started to shine.

ReaChR Plasmid Transduced C2C12 Myotubes Pacing

To prove that the ReaChR can be used in cells for optogenetic pacing, the C2C12 fibroblasts were differentiated into myotubes and reverse transfected with EF1α ReaChR Plasmid DNA using Lipofectamine 3000. The myotubes started to form starting from day 5 after differentiation, and the expressing GFP is showing the shape of the myotubes. On day 9, the transduced myotubes were imaged and paced under TRITC (532nm). As the light shining, the transduced cell twitched when the light is on (Supplementary video 1). The twitching indicated that the ReaChR is being expressed in the cell, and can be excited by the light. The myotube diameter was measure using ImageJ, and the shortest length was when the cell twitched and longest length was when the cell in relaxation. The contracted length was 12.5 pixels, proving that the cells are twitching and changing the diameters under the light.

The ReaChR was also delivered into iPSC-CM using virus transfection. On the next day of the transfection, the transduced cells were paced by red LED light, and the cells were beating at the same frequency of the light shining (Supplementary video 2). The frequency of the cardiomyocytes under the shining LED light was recorded, and the results showed that the frequency of cardiomyocytes changed from spontaneous beating to match the frequency of LED light (Figure 8) Overall, the pacing results from transduced C2C12 myotubes and virus infected iPSC-CM indicate that ReaChR can be excited by light and leads to the beating of the cells.

Chapter 4: Discussion and Conclusion

In this study, we compared the transfection efficiency under different condition using different transfection reagents. Overall, the mmRNA has a significant higher transfection efficiency comparing to Plasmid DNAs for both C2C12 cells and tissues. Considering unlike plasmid DNA, mRNA does not require entering cell nucleus and is effective in non-dividing cells, this result is reasonable(Leonhardt et al., 2014). Reducible polycations (RPCs) is a lipopolymer that combine with nucleic acids to give them positive charges, therefore allow them to enter the cell membrane and to be delivered into the cells. For C2C12 fibroblasts, the RPC improved the cell transfection efficiency for Plasmid DNAs comparing to the commercial transfection reagent, and the transfection rate was doubled at the highest when the RPC/DNA w/w ratio was 300. The improvement in transfection efficiency is consistent with previous study(Read et al., 2005). On the other hand, RPC did not work well for mmRNA transfection comparing to the commercial reagent, TransIt, as the transfection rate decreased from 60% to 30% with the RPC/RNA w/w ratio of 40. Similar results were showed in C2C12 tissues. The RPC helped to improve the transfection efficiency for Plasmid DNA, but for mmRNA transfection, TransIt has a higher transfection rate. In addition, the transfection rate was not improved by doubling the ratio of nucleic acids over cells. The reason of RPC did not work for mmRNA as well as with plasmid DNA may be the difference in the mechanisms of mRNA transfection and DNA transfection, and may also be the different sizes of the nuclei acids. In order to find out the exact reason and to make modification on the transfection process for mmRNA, further researches may be needed.

The transfection of iPSC-CM and iPSC-CM μHM showed slightly different results from C2C12 cells and tissues. Consistently, the plasmid DNA had very limited delivery into both the

cardiomyocytes and the μHM, and mmRNA has a significantly higher transfection efficiency. However, using RPC as transfection did improve the transfection efficiency of mmRNA into μHM, as well as doubling the amount of mmRNA being delivered. The best transfection result of mmRNA into μHM was using RPC as transfection reagent with RPC/RNA w/w ratio of 40, and double the ratio of mmRNA/cells. Transfection into iPSC-CM has proven to be harder than undifferentiated cells as more integral membrane proteins are on the apical membrane, delimiting the interaction between membrane and transfection reagent, therefore decreasing the transfection efficiency(Rybakovsky et al., 2019). With this, it is reasonable that the results are not identical between iPSC-CM and C2C12s.

With the virus transfection into iPSC-CM and the C2C12 myotubes transfection, the results showed that ReaChR could be expressed and excited by light, leading to optogenetic pacing using LED light. However, the virus infected cardiomyocytes did not look as healthy as non-transduced cells, indicating that virus transfection gives a relatively high toxicity to cells. Therefore, it is important to find a safer, at the same time efficient method for transfection. Comparing to the virus, mmRNA has lower toxicity to cells, and from the results above, it is relatively efficient compared to plasmid DNA. The ReaChR mmRNA was synthesized by a two-step process: in vitro transcription followed by the capping of mRNA. The cell transfection results showed that the ReaChR mmRNA can be transfected into cells and expressing the protein. The ReaChR mmRNA was also transduced into iPSC-CM and expressing the signal fluorescence protein, but in order to pace the transduced cardiomyocytes, further studies are needed.

Overall, the study supports that ReaChR can be used for optogenetic pacing of cardiac tissues, and demonstrates a transfection process with high transfection efficiency using mmRNA and RPC as transfection reagent. It also shows a method of tissue transfection with mmRNA. With the support from this study, it is possible to control the beating and even other properties of the cells using mmRNA.

Chapter 5: Limitations and Future Work

From this study, we have demonstrated the possibility using ReaChR mmRNA to control the cell beating. However, there are still some limitation we need to overcome to reach the goal. Although the experiments results showed the success transfection of mmRNA into cells and tissues, those ReaChR mmRNA transduced cells have not yet been paced with light successfully. One possibility we are considering is that although mRNA transfection gives a high transfection rate, the expression of the protein may not be strong enough because mRNA degrades fast in the cells comparing to plasmid DNA and virus, therefore the amounts of protein being produced may not be enough to drive the beating of the cardiomyocytes. Another limitation is that the signal protein of the ReaChR mmRNA is citrine, which is a yellow fluorescence protein, and because of the GCamp in our iPSC-CM, the citrine is hard to be imaged, thus provides obstacle in transfection efficiency quantification. Therefore, another fluorescence protein may be used to replace Citrine in the future as necessary. There is also a concern regarding to the light source for pacing. The current LED light is not concentrated enough to apply only on one well at a time, and the spread of the light may decrease the intensity of the light that are delivered into the cells or tissues. To resolve this problem, a modification on the light source may be needed.

References

Al-Dosari, M. S., & Gao, X. (2009). Nonviral gene delivery: principle, limitations, and recent progress. *The AAPS journal, 11*(4), 671.

American Heart Association. (2016, Sep 30a). Medication for arrhythmia. Retrieved from https://www.heart.org/en/health-topics/arrhythmia/prevention--treatment-of-arrhythmia/medications-for-arrhythmia

American Heart Association. (2016, Sep 30b). Why arrhythmia matters. Retrieved from https://www.heart.org/en/health-topics/arrhythmia/why-arrhythmia-matters

Asano, T., Ishizuka, T., Morishima, K., & Yawo, H. (2015). Optogenetic induction of contractile ability in immature C2C12 myotubes. *Scientific reports, 5*, 8317.

Davis, J., Davis, L. C., Correll, R. N., Makarewich, C. A., Schwanekamp, J. A., Moussavi-Harami, F., . . . Houser, S. R. (2016). A tension-based model distinguishes hypertrophic versus dilated cardiomyopathy. *Cell, 165*(5), 1147-1159.

Health Engine. (2004, Sep 15). Arrhythmia. Retrieved from https://healthengine.com.au/info/arrhythmia

Herren, T., Gerber, P. A., & Duru, F. (2009). Arrhythmogenic right ventricular cardiomyopathy/dysplasia: a not so rare "disease of the desmosome" with multiple clinical presentations. *Clinical Research in Cardiology, 98*(3), 141-158.

Huebsch, N., Loskill, P., Deveshwar, N., Spencer, C. I., Judge, L. M., Mandegar, M. A., . . . Mathur, A. (2016). Miniaturized iPS-cell-derived cardiac muscles for physiologically relevant drug response analyses. *Scientific reports, 6*, 24726.

Karikó, K., Buckstein, M., Ni, H., & Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity, 23*(2), 165-175.

Kariko, K., Muramatsu, H., Ludwig, J., & Weissman, D. (2011). Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic acids research, 39*(21), e142-e142.

Krikler, D. M., Harris, L., & Rowland, E. (1982). Calcium-channel blockers and beta blockers: advantages and disadvantages of combination therapy in chronic stable angina pectoris. *American heart journal, 104*(3), 702-708.

La Gerche, A. (2015). Defining the interaction between exercise and arrhythmogenic right ventricular cardiomyopathy. *European journal of heart failure, 17*(2), 128-131.

La Gerche, A., Burns, A. T., Mooney, D. J., Inder, W. J., Taylor, A. J., Bogaert, J., . . . Prior, D. L. (2012). Exercise-induced right ventricular dysfunction and structural remodelling in endurance athletes. *European heart journal, 33*(8), 998-1006.

Leonhardt, C., Schwake, G., Stögbauer, T. R., Rappl, S., Kuhr, J.-T., Ligon, T. S., & Rädler, J. O. (2014). Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers. *Nanomedicine: Nanotechnology, Biology and Medicine, 10*(4), 679-688.

Lian, X., Zhang, J., Azarin, S. M., Zhu, K., Hazeltine, L. B., Bao, X., . . . Palecek, S. P. (2013). Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nature protocols, 8*(1), 162.

Lin, J. Y., Knutsen, P. M., Muller, A., Kleinfeld, D., & Tsien, R. Y. (2013). ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. *Nature neuroscience, 16*(10), 1499.

Mandal, P. K., & Rossi, D. J. (2013). Reprogramming human fibroblasts to pluripotency using modified mRNA. *Nature protocols, 8*(3), 568.

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., . . . Bamberg, E. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proceedings of the National Academy of Sciences, 100*(24), 13940-13945.

National Heart Lung and Blood Institute. Arrhythmia. Retrieved from https://www.nhlbi.nih.gov/health-topics/arrhythmia

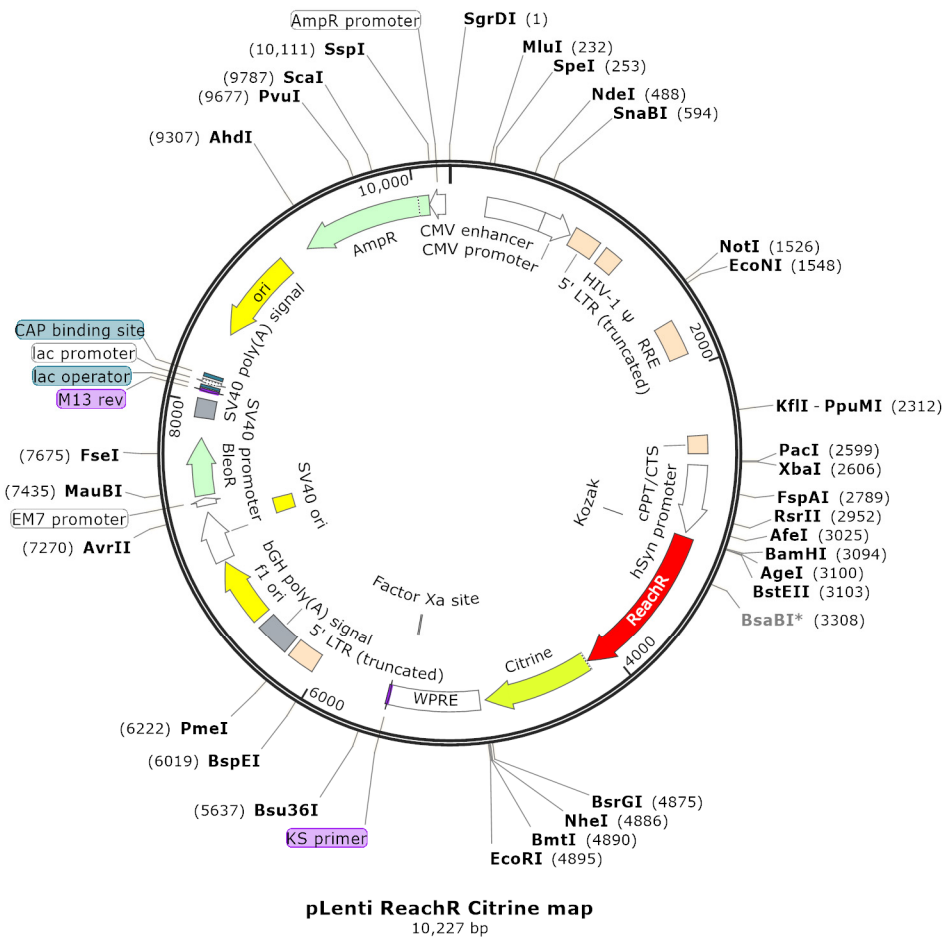
Read, M. L., Singh, S., Ahmed, Z., Stevenson, M., Briggs, S. S., Oupicky, D., . . . Berry, M. (2005). A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. *Nucleic acids research, 33*(9), e86-e86.

Ronaldson-Bouchard, K., Ma, S. P., Yeager, K., Chen, T., Song, L., Sirabella, D., . . . Vunjak-Novakovic, G. (2018). Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature, 556*(7700), 239-243.

Rybakovsky, E., Valenzano, M. C., DiGuilio, K. M., Buleza, N. B., Moskalenko, D. V., Harty, R. N., & Mullin, J. M. (2019). Improving Transient Transfection Efficiency in a Differentiated, Polar Epithelial Cell Layer. *Journal of biomolecular techniques: JBT, 30*(2), 19.

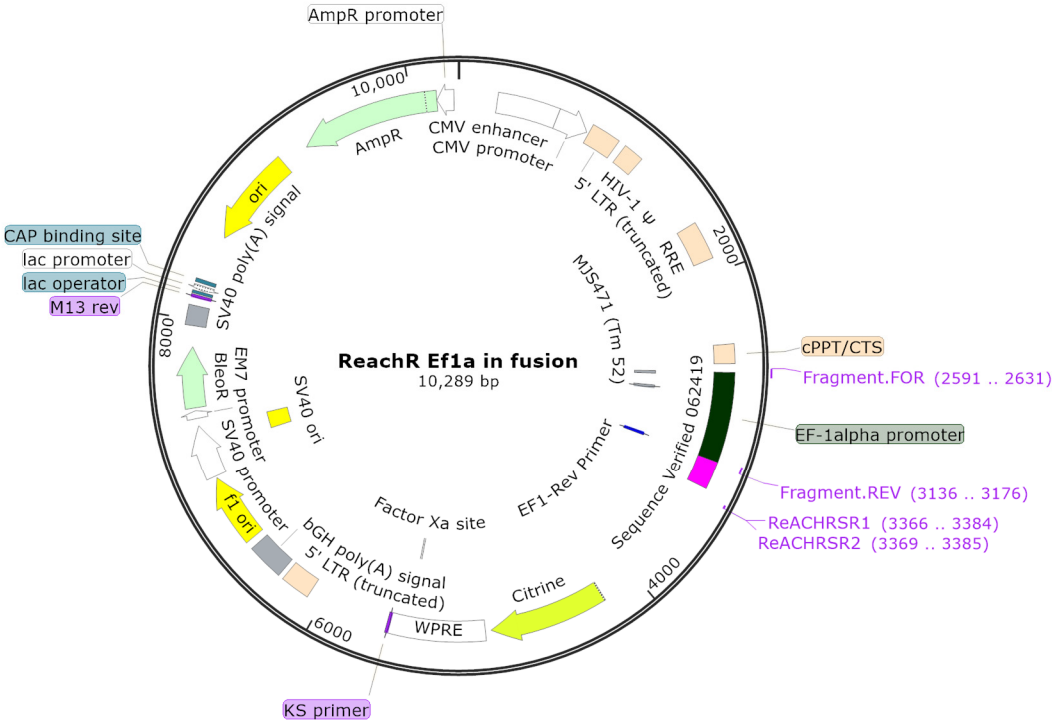
Warren, L., Manos, P. D., Ahfeldt, T., Loh, Y.-H., Li, H., Lau, F., . . . Meissner, A. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell stem cell, 7*(5), 618-630.

Appendix A: Plasmid Map of pLenti ReaChR Citrine

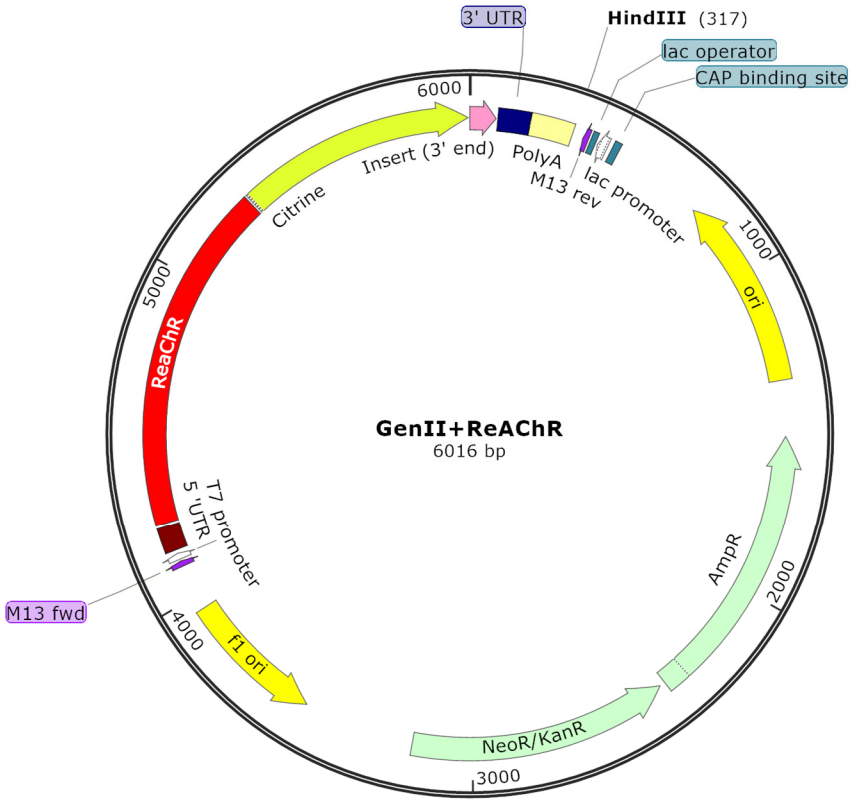


pLenti ReachR Citrine map
10,227 bp

Appendix B: Plasmid Map of Ef1α ReaChR



Appendix C: Plasmid Map of GenII ReaChR



Appendix D: Plasmid Map of GenII LifeAct GFP