Spatiotemporally localized delivery of genes through acoustic droplet vaporization

A research proposal submitted by

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February 2016

Abstract

Gene therapy refers to the insertion of a gene in a patient's cell to prevent or treat diseases, potentially eradicating the need for drugs or surgery [3]. There have been over 1174 gene therapy clinical trials in the United States; however, the Food and Drug Administration has never given approval for a gene therapy product [5]. The primary limitation in gene therapy is the absence of a mechanism that efficiently and safely delivers the gene to the body in very localized areas [4]. The ability to deliver nucleic acids to a localized area without impacting unintended cells/tissues is essential in order to effectively and safely treat diseases. This study aims to demonstrate that ultrasound can non-invasively release plasmid DNA and subsequently transfect cells in a hydrogel scaffold with spatiotemporal precision. Ultrasound will be used in combination with sonosensitive emulsions that contain plasmid DNA. When the emulsions are exposed to ultrasound above a specific amplitude, the outer shell of the emulsion vaporizes into a gas, releasing the encapsulated plasmid DNA. The research will consist of identifying the appropriate relationships between the hydrogel composition, emulsion composition, and acoustic properties to maximize transfection efficiency and cell viability. The objective of the study is to ultimately optimize the ratio of transfected cells in the targeted area to the total number of live cells and minimize the number of dead or dying cells. Application of this proof of concept study will ultimately be applied to deliver genes to treat intractable and refractory diseases, both acquired and genetic.

Introduction

When I was in high school, I read an article by Professor Cathy Davidson from Duke University where she states that 65 percent of students will end up working in fields not yet invented. The article piqued my curiosity. Additionally, statistics and information about several shootings were prevalent in the news at the time. I began researching and discovered that individuals with a history of mental illness instigate 79 percent of mass shootings [2] and that five of the major mental illnesses trace back to the same genetic variation [7]. I imagined a field where technological advances pre-emptively detect and treat visceral behavioral conditions before they lead to senseless violence that leaves a permanent scar on society. Research in the field of genetics and bio delivery opens the doors for pre-emptive treatment of mental illnesses that has the potential to leave a lasting impact on the community. It will be exciting to have the opportunity to gain hands-on experience while exploring the biotechnological aspect of the challenge that truly spans an amalgamation of my interests: engineering, biology, chemistry, psychology, sociology, and ethics. I see this project as an opportunity to apply the knowledge and skills learned in my biotechnology engineering class to a practical solution and to investigate my interests in pursuing a career in research.

I applied to the Undergraduate Research Opportunity Program at the beginning of the school year, as the program, along with the research opportunities on campus, was one of the primary reasons I decided to attend the University of Michigan. Unfortunately, I was waitlisted from the program. In high school, I interned at Caradigm, a healthcare analytics company, for over a year where I worked on cohort-based applications that identified, managed, and treated patients who were at high-risk for various hospital acquired conditions, diabetes, and pressure ulcers. My interest in pursuing research opportunities has increased as I have continued to search for research opportunities throughout the school year and especially after taking the Biotechnology section of Engineering 100. This summer is an optimal time to employ the biotechnology concepts learned and kick-start a research career at UM. Especially since I have not had prior research experience on campus, I plan to start training with Dr. Fabiilli in advance, so I can make ample progress over the ten-week period and I will not be spending the whole summer doing introductory training. I will gain significantly more experience in the summer by working 40 hours a week in comparison to during the school year. I am interested in continuing to do research at the University of Michigan while exploring the option of pursuing a graduate degree and conducting research as a career.

Background Information

The basic paradigm of gene therapy is straightforward: deliver a gene to cells that contain a defective copy of the gene or are lacking the gene entirely. Unlike conventional drug therapy, gene therapy has the potential to permanently cure disease since the delivered gene will be fully incorporated and expressed by the cells. However, the implementation of

gene delivery is far from being straightforward. One of the biggest challenges of gene therapy is finding delivery vehicles for the genes that are both safe and effective. Depending on the gene in question, accidental delivery of genes to unwanted or off-target locations can be very dangerous. Thus, it is crucial that a potential delivery device has the ability to be localized and only target desired (i.e., diseased) regions. Genetic vectors may be administered either vascularly or avascularly at the site to ensure localization [8,11]. However, the approach is not feasible for diffuse or inaccessible diseases. Furthermore, genetic vectors are subject to enzymatic degradation in vivo [6,10], which is another factor that limits their efficacy.

One method to localize gene delivery is via the use of ultrasound, which is non-invasive. Ultrasound, which consists of acoustic waves typically in the megahertz frequency range, is used in the clinical setting for both diagnosis and therapy. Dr. Fabiilli's laboratory has developed a targeted drug delivery platform utilizing "sonosensitive" emulsions in conjunction with ultrasound. The emulsion contains the delivery payload. When the emulsion is exposed to high amplitude ultrasound, the payload is released from the emulsion in a process known as acoustic droplet vaporization (ADV). Since ADV uses focused ultrasound, the release is confined to a small region (i.e., submillimeter). A recent study in the Fabiilli Laboratory has shown that bioactive plasmid DNA, complexed in a lipoplex, can be encapsulated and released from a sonosensitive emulsion using ADV (see Figure 1). The next step is to demonstrate that the release of plasmid DNA can generate highly localized gene expression (i.e., transfection) through spatial localization.

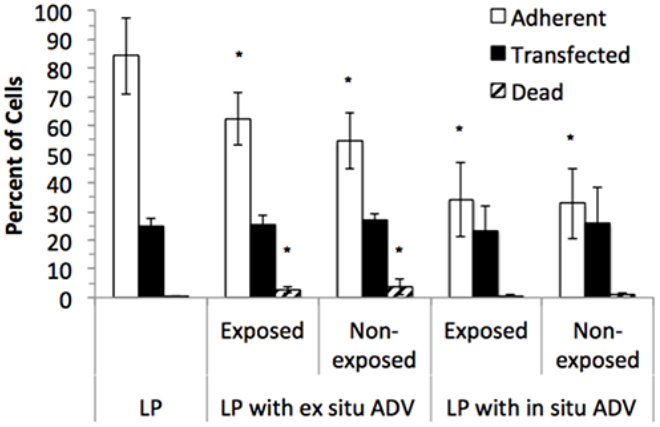

Figure 1: Cell adherence, percent transfected, and percent dead for five different lipoplex preparation methods from a recent study in the Fabiilli Laboratory.

Objectives

The overall objective of the study is to demonstrate that ultrasound, specifically ADV, can non-invasively release plasmid DNA and subsequently generate gene expression (transfection) with spatiotemporal precision. The below variables will be interrogated and optimized in order to maximize the ratio of transfected cells to the total number of live cells (within a region of interest) while minimizing the number of dead or dying cells.

- Cell presentation: 2D or 3D culture
- Hydrogel: fibrin density (1-10 mg/mL)
- Emulsion composition: perfluoropentane (C_5F_{12}) or perfluorohexane (C_6F_{14}) core
- Acoustic parameters: Frequency (2.5 or 7.5 MHz); mechanical index (1.8-2.5); duty cycle (0.01-0.1%)

Methodology

Sonosensitive emulsion droplets will be prepared based off a previously published method [1]. These particles have greater in vivo stability and longer circulation lifespans than the previously used sonosensitive microbubbles [12] and will release lipoplex-containing plasmid DNA with an enhanced green fluorescent protein reporter through ADV (Figure 2, Step 1 and 2). All experiments will utilize cultured cells of the mouse multipotent line C3H/10T1/2 (clone8) contained within a fibrin hydrogel scaffold along with the droplets. Hydrogel scaffolds are commonly used with the field of tissue regeneration as an adhesive substrate for cells. Immobilizing the cells and droplets within the scaffold enables the study of spatial location. Furthermore, regeneration applications (ex., blood vessel or bone growth) can use this cell-droplet composite scaffold. Ultrasound waves will be focused on a small subset of cells (indicated by the orange rectangle in Figure 2, Step 2). When an emulsion droplet is exposed to ultrasound above a threshold acoustic amplitude, the perfluorocarbon phase within the droplet vaporizes into a gas and releases its payload into the environment (Figure 2, Step 3) [9]. Once the plasmid DNA

enters the cells, the number of transfected cells can be determined by counting the number of cells that express green fluorescent protein through the microscope (Figure 2, Step 4).

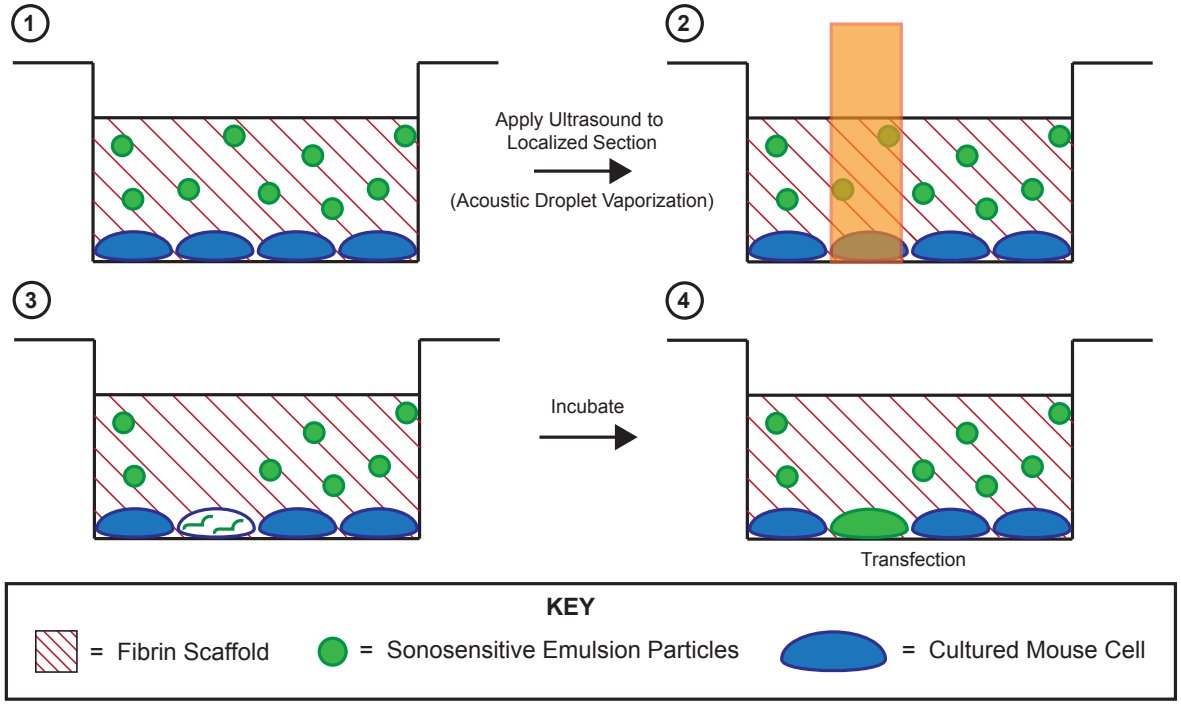

Figure 2: Schematic illustration of lipoplex release using acoustic droplet vaporization with localized ultrasound.

In addition to the number of transfected cells, the number of total cells and dead cells will be obtained via staining with Hoechst and propidium iodide, respectively. The variables listed in the 'Objectives' section will be investigated to determine how each impacts how the cells and DNA move within the gel, how the ultrasound reacts with the emulsion particles, the transfection of surrounding, non-targeted cells, and the vitality of all the cultured cells in the environment.

In order to account for different scenarios in which non-targeted cells may be exposed to the genes, the study will examine two separate cell culture approaches: a 2D and 3D layout (Figure 3). In the 2D layout, there will be a monolayer of cells plated on either the top or bottom of the gel. In the 3D layout, the cells will be placed at various levels within a hydrogel, adding a depth of field aspect.

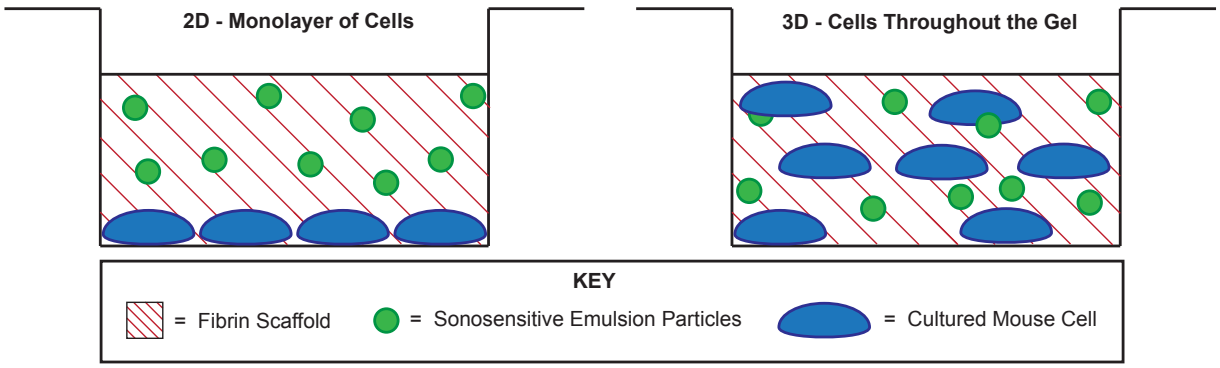

Figure 3: Schematic comparison of the two environments in which the study with be completed

While analyzing the results of the monolayer of cells, an epifluorescent microscope is used, whereas the 3D layout requires a confocal florescence microscope to add the depth of field dimension. Additionally, the variable factors (i.e. mechanical index, scaffold density, etc.) may differ for each of the cases. However, the overall methodology of the study will remain the same for both scenarios.

Timeline

Timeframe	Deliverables
Weeks 1-2	• Learn how to make the necessary components: fibrin scaffold, emulsion droplets, and lipoplex. • Learn how to operate the necessary laboratory equipment: ultrasound exposure setup, epifluorescent microscope, and confocal fluorescence microscope. • Learn how to perform the necessary laboratory procedures: in vitro setup, microscopy, cell staining, and image analysis. • Practice techniques and procedures beforehand to ensure precision and accuracy
Weeks 3-5	• Attend and participate in seminars co-sponsored by the Medical School • Alter fibrin scaffold concentration for optimal density for the cells and emulsions • Identify and test emulsions for optimal vaporization properties • Identify and test for the optimal mechanical index and amplitude of the ultrasound waves
Week 6	• Prepare and present midterm report and presentation to full research team
Weeks 7-9	• Repeat deliverables for Weeks 3-5 with the 3D hydrogel environment
Week 10	• Finalize last minute modifications to research report • Prepare research poster and presentation

Expected Results

This study aims to demonstrate that ultrasound can be used to non-invasively release plasmid DNA with spatiotemporal precision, without affecting the surrounding cells. It is expected that the application of focused ultrasound will 1) initiate transfection in the mouse cells within the targeted area as shown by the orange square in Figure 4 and 2) minimize the number of surrounding cells that are transfected and/or killed by the gene delivery process.

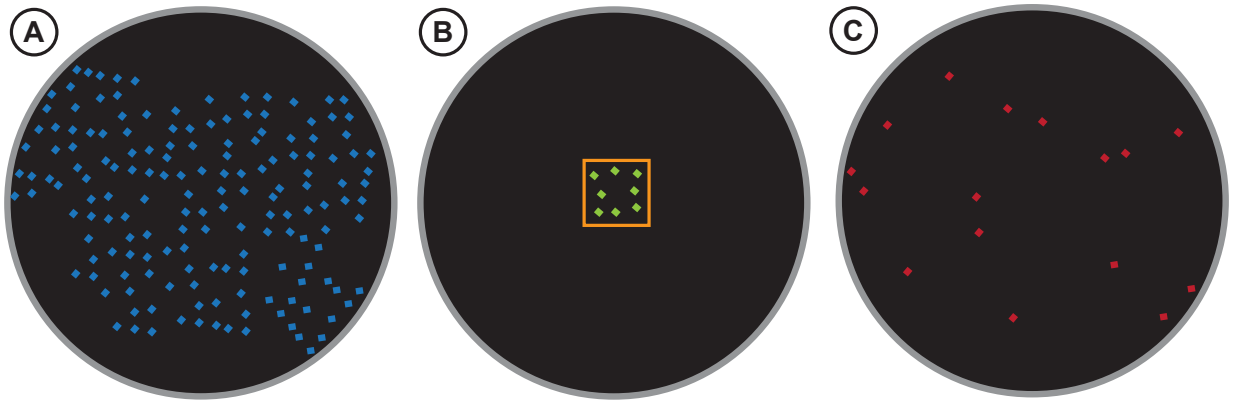

Figure 4: Schematic illustration of the expected results with three different filters showing total (A), transfected (B), and dead/dying cells (C).

The application of different filters on the image from the fluorescent microscope reveal a unique subset of cells. Figure 4, Part A shows all the cells, which were previously stained with Hoechst. Figure 4, Part B shows all the transfected cells, which now express the green fluorescent protein. A majority, if not all, of the transfected cells is expected to lay in the intended area where the ultrasound was focused. The membrane of all the dead and dying cells will have a hole, thus allowing the propidium iodide to stain. Figure 4, Part C shows these cells.

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