Engineering Light Controllable CAR T-Cells

BENG 187B Final Proposal

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Introduction

Throughout the years, researchers have encountered challenges to create more efficient, accurate, and rapid cancer therapy. The advent of chimeric antigen receptor-T cells (CAR-T cells) has provided a new way to treat cancer. However, CAR-T therapy use has brought concerns over the safety, off-target effects and cytotoxicity of CAR-T due to its potential overexpression. Therefore, it is important to develop CAR-T cells with enhanced safety, which would only have effects on tumors when activated. One of the many possible tools to achieve this goal is through constructing light-inducible genetic circuits that allow the CAR-T cells to specifically target cancer at a certain time and place when needed.

Needs

Level 1: Improve the control of CAR T-cells spatially and temporally to treat solid tumors.

a. Currently, CAR T-cells are only approved by FDA to treat blood cancers but not solid tumors. To ensure the effectiveness and sensitivity of CAR-T therapy, where the CAR-T cells are able to accurately target cancer cells in a short time and not over expressed, there is a need to achieve the control of CAR-T cells' therapeutic protein production[1].
b. Size estimate: Around 50,000 researchers and oncologists according to the Global Survey of Clinical Oncology Workforce[2].

Level 2: Increase the flexibility of CAR T-cell therapy in treating cancer patients

a. Currently, doctors can only administer the drug intravenously but do not have flexibility with where the therapy is targeted. The main stream of cancer treatments like radiation, chemotherapy, and surgery are often invasive.
b. Size estimate: According to the Worldwide Cancer Data, there are around 18 million new patients diagnosed with cancer globally[3]. Consequently, the design will benefit these patients.

Level 3: Minimize the off-target effect and improve the survival rate of CAR T-Cells treatment

a. There is a need to create CAR T-cell therapies that are more specific and less toxic for the patient[4].
b. Size estimate: The global market size in 2019 for CAR T-cell therapy is about $428 million[5]. And CAR T-cell therapy costs about $383,000 per person[6]. The CAR T-cell therapy is estimated to help about 1000 people who are currently using CAR T-cell therapy the past year. However, CAR T-cell therapy is a treatment that is not as accessible to everyone due to constraints such as cost. And consequently, when more widely accessible, CAR T-cell therapy could help the broader population of cancer patients where 18 million patients are diagnosed with cancer a year globally. Currently the estimated growth of CAR T-cell therapy is 51.10% (compound annual growth rate) per year[7].

Who will benefit

At level 1, researchers will use our designed genetic circuit for light-inducible CAR T-cells to do further study on making improved versions of CAR T-cell therapy. At level 2, this design will be beneficial to those patients who have solid tumors and those who could not undergo surgical procedure or other invasive procedure for cancer treatment. In addition, people who are using CAR T-cell therapy are often cancer patients who are not responding to standard of care treatments, and since this design addresses concerns over side effects, at level 3, it will be beneficial to patients who are responding adversely to CAR T-cell treatment.

Problem Statement

CAR T-cell therapy, an alternative to standard cancer treatments, still needs further development to fully control its expression, cut down its costs, expand the number of treatable cancers, and reduce the off-target effects as the public acceptance for the treatment is still low due to these stated reasons.

Previous Versions

1. Current CAR T-cells treatments are not available for solid tumors.
2. Mainstream treatments such as chemotherapy, radiation, and surgery are invasive and can be harmful for the patient.
3. The public acceptance of CAR-T therapy treatment is low due to its high risk of off-target effect.
4. There is a lack of incentives for manufacturers to make personalized CAR-T cells from patients due to the high cost and labor needed to generate such cells.

Topics for Expansion

- Expression Controllability (feedback control)
- Survival Rate/Success Rate
- Treating Solid Tumor
- Non-invasive Cancer Treatment
- Cost
- Target Specificity

Expanded analysis of Significant Functional Sub-problems

Compared to the mainstream treatment, CAR-T therapy shows advantages because it is non invasive. For example, radiation could harm the tissue and organs but CAR T-cells would not harm the organ or tissue because it targets specific cancer cells. However, most CAR-T therapy is still developing in clinical trials, lagging behind its expected widespread release to the public due to concerns over safety and specificity. One major cause for this delay is due to the lack of incentives for researchers to further improve the performance of CAR-T cells by implementing genetic circuits to make the expression of therapeutics more controllable, because

manufacturing such the CAR T-cells have high costs in money and time. Clinical trials have encountered problems with CAR-T cells, including even fatal outcomes. The problems of expression controllability needs to be seriously addressed[5].

Currently, CAR T-cells are approved by FDA to treat blood cancers but not solid tumors due to the lack of spatial and temporal control. Overexpression of CAR T-cells would harm healthy cells, so ideally, a feedback control CAR T-cell expression system would minimize the over expression of CAR-T-cells.

Relevant Background

This section will outline some background technologies applicable to the development process of CAR T-cell engineering, followed by a summary of cancer cell formation, growth and proliferation within human bodies, as well as current cancer treatments that impede these processes. Next, CAR-T therapy will be introduced in detail and discussed in terms of its advantages and drawbacks. Finally, this section will address some current research solutions toward enhanced safety of CAR-T cells.

Scientific and technology background

Applicable technologies

Molecular cloning

Molecular cloning is a technique utilizing polymerase chain reaction (PCR) to amplify DNA fragments and create recombinant DNA through plasmid construction. It enables the engineering of distinct cells (such as CAR-T cells for cancer therapy) to perform specific functions. In particular, fragments of the gene of interest will be amplified through PCR and be inserted into the plasmid vector via ligation to create recombinant plasmids[8]. These plasmids will then be incorporated into E. coli cells and selectively grown in antibiotics media. As only E. coli cells with recombinant plasmids will survive, the plasmids are extracted and used to transform cells through homologous recombination, thus inserting genes into these cells.

Cell culture and in vitro testing

Followed by molecular cloning, cell culture based-in vitro tests can be carried out to investigate their functions. Instead of directly performing studies on live organisms, in vitro tests allow for observation of a specific type of cells in a simulated environment[9]. During cell culture, the cells are grown in a media and treated with a controlled condition. This environment allows these cells to develop in a similar fashion to when they are present inside living organisms. In vitro testing, on the other hand, uses these cultured cells to conduct experiments by administering changes to their surrounding environments (such as drugs, temperature, etc), which is a convenient method that gives insights into how real cells will behave in an experimental condition. Thus, such a technique also facilitates screening for anticancer drugs, including CAR-T antibodies.

Fluorescence microscopy

During in vitro testing, the cells' behavior may not be obvious under naked eyes. Hence, it is sometimes mandatory to implement a microscope that could read fluorescent signals emitted from cells. This tool, called fluorescence microscopy, allows for tracing of specific recombinant fluorescent proteins, or molecules bound by fluorescent tags through a microscope[10]. Combined with molecular cloning and cell culture, it gives qualitative information on the location of molecular interaction, as well as quantitative data on the extent of such interaction.

Expression thresholds

After gathering data, analysis may be carried out with the help of computational tools. Integral feedback, feedforward, and transcritical bifurcation motifs can generate thresholds.

Computational pathways of these motifs will help assess the risk in vitro cellular assays[11]. Bifurcation behavior occurs when a small smooth change is made to the parameter values. Genetic circuit is an assembly of biological parts encoding RNA or protein that enables individual cells to respond and interact with each other to perform some logical functions. To design a gene that creates bifurcation, gene editing tools like Cre-Lox, Crispr, Zinc fingers, TALENS from addGene can be used Genome editing.

Cancer cell physiology

The causes of cancer cell formation are of great importance in learning how to fight against it. Generally, cancer results from environmental factors that cause damage to DNA repair mechanisms, which allows mutations in oncogenes and tumor suppressors (such as p53), leading to the onset of cancer cells[12]. Researchers have found out that carcinogens could promote more mutations by acting as mutagens for certain genes critical for cell cycle maintenance. With such understanding in carcinogenesis, CAR-T therapy can be more precisely targeted to cancer cells.

Besides the onset of tumor formation, the development of cancer is also crucial for it to be harmful to human cells. Researchers have found out a list of different proteins that are factors in such development[13]. These include proteins involved in signal transduction pathways, cell metabolism, as well as apoptosis and hormonal signaling. Having an understanding of these mechanisms can also help with developing targets for CAR-T therapy. The development of cancer cells has strong implications on the potential damage of cancer cells that can be done to the human body[14]. Its symptoms comprise different stages, and it can ultimately result in change in blood vessels, organ failure, and even death. Thus having understanding of the symptoms of patients also pertains to best success of cancer therapy.

Current cancer therapies

Tumor removal surgery

In effort to counter the malignancy of cancer through therapy, surgery has been used as one of the first treatment methods for cancer. Through surgery, removing tumor directly from the patient proves to be a convenient way to immediately reduce the number of cancer cells within the patient's body, thus attempting to eliminate cancer[15]. However, research has also demonstrated that such surgery may put the residual tumor at risk of metastasis. While surgery removes most part of the tumors, chances are there will be residual cancer cells stuck with healthy tissues or traveling within blood vessels that will lead to potential resurgence of cancer.

Cytotoxic chemotherapy

To address surgery's inability to completely wipe out cancer, chemotherapy has been widely used to complement surgery. This most common first-line therapeutics works by injecting drugs into a patient's blood and letting them kill the cancer cells[16]. It has lower risks of cancer recurrence than direct surgery but may have more side effects. One of the most common side effects is leukopenia, a symptom characterized by a patient's abnormally low white blood cell count. This observation is accounted for by the fact that chemotherapeutics are also toxic toward healthy tissues. These drugs could impede the bone marrow's ability to regenerate white

blood cells; and lower white blood cells count could compromise patients' immune system, making them more susceptible to infection.

Radiotherapy
In an effort to resolve side effects that arise from chemotherapy, this method called radiotherapy was developed that allows for curing of cancer in local tumorigenic sites[17]. Through the use of ionizing radiations, cancer cells can be rapidly eliminated. However, besides the high cost, the dosage needs to be carefully considered for effective treatment while avoiding potential harmful effects on nearby normal tissue cells.

State of the art
Monoclonal antibodies as therapeutic agents
CAR-T therapy works by the action of monoclonal antibodies (mAb) that can be produced at the surface of its membrane[18]. It is shown that mAb is composed of Fc (constant) and Fv (variable) domains. While the protein sequence of Fc stays the same throughout all human T cells, the highly distinct Fv determines the antigen which it can bind to. Therefore, the Fv domain of CAR-T cells can be engineered via molecular cloning so that the antibodies can be massively produced and specifically target certain types of cancer cells, making personalized treatments easier. Also, it is of great importance to study how CAR-T cells migrate within the patient's body to target cancer cells, as well as how they adapt to various different microenvironments[19]. By recognition of cancer cell membrane proteins, CAR-T cells are able to perform their functions by killing cancer cells.

How CAR T-cell therapy works
Foreign substances can be recognized by the immune system because of their antigens on the surface of those cells[20]. The receptors on the T cells can attach to foreign antigens that then trigger other parts of the immune system to destroy the foreign substance. Each foreign antigen has a unique immune receptor that it is able to bind to. Cancer cells have antigens which can only bind to unique immune receptors. With the right receptor, immune cells can attach to the antigens and help destroy the cancer cells. T cells used in CAR T-cell therapies are reengineered with a chimeric antigen receptor. This helps them better identify specific cancer cell antigens. Since different cancers have different antigens, each CAR is made for a specific cancer's antigen.

CAR-T cell therapy advantages
CAR-T cell therapy is a major advancement in personalized cancer treatment. One of the advantages of CAR-T cell treatment is that the patient will have less active treatment days than undergoing traditional therapy[21]. The goal of treatment is to help get a patient into complete remission for many years[22]. In addition, infused T-cells can persist in the patient's body, therefore enabling a long-term effect in cancer treatment[23]. As a result, CAR T cell therapy shows dramatic clinical responses and high rates of complete remission.

Side effects of CAR-T therapy

While the novel CAR T cell therapy has its appealing advantages over conventional ones, there are still drawbacks. There are many side effects observed in clinical study. One of the side effects of CAR-T cell immunotherapy is that it can lead to cytokine release syndrome and excessive inflammation[24]. Cytokine release syndrome is a common side effect of cytokine release syndrome. Cytokine release syndrome refers to the process of immune cells and tumor cells to release a large number of cytokines, which trigger cell chain reaction. The most common is excessive inflammation, in which patients develop flu-like symptoms, such as high fever and cold, and sometimes a coagulation cascade. CAR-T cell immunotherapy can also be neurotoxic, causing neurological problems and possibly organ failure. Neurotoxicity can cause neurological problems. Symptoms are shown as confusion or even coma. CAR-T cell immunotherapy may also cause hypotension and dyspnea, and in severe cases can lead to multiple organ dysfunction and failure, which can be life-threatening. Therefore ongoing research effort in developing controllable CAR-T cells is necessary for their safety during application.

Light-induced translocation and dimerization

Recent advances in developing controllable CAR-T cells have been focused on building genetic circuits where external stimuli can be used as a switch which triggers certain biochemical interactions. This light-induced translocation and dimerization (LINTAD) system works by using blue light stimulus[25]. When this light is administered into CAR-T cells, it causes conformational change of a recombinant nuclear localization signal-transcription factor, allowing for such protein to be imported into the nucleus. Followed by this process, this recombinant protein dimerizes with nuclear transcription factors, and induces transcription of reporter genes. These reporter genes can be potentially antibody-producing, which lead to enhanced inducibility of CAR-T therapeutic effects.

Tetracycline-Inducible System (Tet)

This system can also be incorporated into the controllable circuit of CAR-T cells in addition to LINTAD. Within this system, TetR works as a transcriptional repressor by binding to TetO, which can be deactivated if bound by tetracycline[26]. Since overdose of protein products of reporter genes may lead to cytotoxicity, the Tet system proposed here could act as a part of negative feedback where over-induced reporter gene transcription can be sequestered to avoid potential harmful effects.

miRNA-mediated circuit

This alternative design also aims to increase efficacy of CAR-T therapy and reduce its cytotoxicity[27]. It works similarly to Tet where blue light induces proteins that promote miRNA transcription and ultimately blocks the expression of reporter genes. This miRNA acts by base-pairing with reporter genes, which blocks the RNA polymerase from travelling toward the reporter genes, thereby disabling their transcription.

Design Goals and Constraints

The goal of our project is to engineer a genetic circuit inside CAR T-cells that will increase the controllability and limits of CAR T-cells with blue light. Therefore, the functional goals of our experiment are to increase the sensitivity, on-target specificity, and consistency of CAR T-cells.

Functional Goals

Goal 1 (40%): Increased sensitivity of CAR T-cell therapy using a blue light-inducible positive feedback loop

Our goal is to make the protein expression of CAR-T cells have increased sensitivity to blue light induction.The importance of this goal stems the concern over the controllability of the current CAR-T cells as expression of CAR can be difficult to express. One way to evaluate the outcome is by having a mCherry placed where the CAR gene would have been. Upon blue-light activation, the fluorescence from mCherry with respect to time and fold-activity can be recorded to examine the genetic construct and see whether our design has the desired response. Another way to evaluate the controllability of this design is by placing two tumors close by in a mouse model and shining blue light only on one tumor. If only the tumor that was shined with light is reduced, then we can say with greater confidence that we have more spatial and temporal control over our therapy. We weighted this goal 40% because controllability is the main goal of engineering these CAR T-cells to more safely and accurately use CAR T-cell therapy.

Goal 2 (30%): Decreased off-target effects of CAR T-cell therapy while also maintaining the effectiveness of the therapy.

Our second goal is to minimize the off target effect which leads to harm to the patients. Safety is a major concern regarding treatment. The current public acceptance of CAR-T cell treatment is low because the potential risks for the treatment are high. One way to measure the off-target effects of CAR T-cell therapy is to test the CAR T-cell therapy in mice models. You could compare cytokine levels in mice as this is one off-target effect induced by CAR T-cell therapy. Another way to measure the off-target effects indirectly is to measure the lifespan of mice and compare it to other mice who have undergone CAR T-cell therapy. If there mice are major discrepancies, off-target effects are a likely reason behind this. The goal is weighted at 30% as it is an important factor for a project aimed at clinical treatment but as in the earlier stage of the research, safety is not as large of a concern compared to making sure the genetic circuit works first.

Goal 3 (30%): Ensure that the genetic circuit in the CAR T-cell therapy is robust and consistent with results.

Consistency is an important factor for a project aimed at clinical treatment. The repeatability of the genetic circuits function is important because there are often many different ways proteins can interact and consequently can change the outcome of the genetic protein. Ways to evaluate or measure the reliability of the CAR T-cell therapy is to evaluate the variability of the

experiment of luminescence. By placing mCherry, a red fluorescent protein, as a reporter gene, we can measure the luminescence from the cells to see if the gene expression behavior is consistently displaying the same behavior over time. You could measure the variability and use statistical analysis to compare different experiments. A second way to measure the reliability is to test the CAR T-cell therapy in mice and see if mice are consistently seeing reduced tumor sizes between experiments.We weighted this goal at 30% because it is important that the genetic circuit is working reliably and performing its intended function in order to use the genetic circuit in the future, both in research and the hospital.

Other Areas for Consideration

1) Ease of operation
2) Method development
3) Efficacy
4) Cost

Significant constraints

Constraint 1: Time

All of the data needed for the project will need to be done by Fall and Winter quarter. And due to COVID-19, we are not able to go into the lab to run experiments. There will likely be issues running the experiments at first as we need to do the cloning, transfect the cells, and image the cells. Each set of experiments if everything goes smoothly will take 2 weeks. However, if problems arise with running experiments, one round of experiments could potentially take 1-1.5 months. Consequently, time is the largest constraint.

Constraint 2: Cost

Money is a major constraint as we are given a $500 budget for our senior design project. If extra is needed, the budget can go up to $1000 if deemed acceptable for lab funding. Some major costs would include ordering plasmids and primers needed. Additionally, the budget will need to account for paying the cost for Gibson Assembly reagents and PCR purification

Constraint 3: Components

Although there may be components that we find that could be promising for our design. Ultimately, there are many components that we may find that we do not have access to. Therefore, most of the components that we use will mainly be found through Addgene and possibly other sources. This constraint limits which components we assemble together for our design.

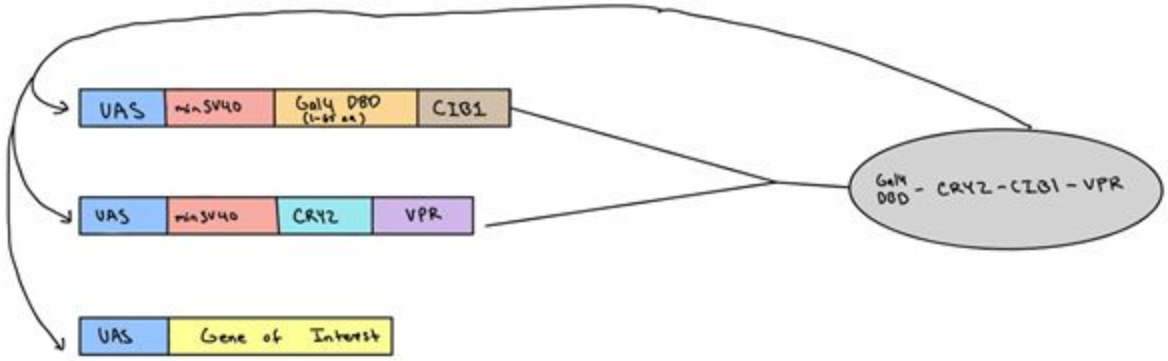
Constraint 4: Safety

Safety is also a constraint as we must be careful with handling biohazards. The experiment must also be conducted with sterile techniques to avoid contamination for both the experiments and personnel handling the experiment. Also safety must be considered in COVID as working in the laboratory for too long may increase the risk of receiving COVID.

Design Solution

Design Solution: UAS-Gal4 CRY2-CIB1 Positive Feedback Loop

The design solution would use the UAS-Gal4 Cry2-CIB1 system[28]. In this system UAS would be attached to the CRY2-Gal4 and CIB1-VPR transcription activator. The reporter gene would also have UAS. Consequently this would create a positive feedback loop as all three of the genetic constructs have UAS as shown in the figure below. Cry2-Gal4 and CIB1-VPR would bind together in the presence of blue light. And then they would either bind to one of the three UAS sequences. Creating more of these constructs creates even more of these two proteins. And these proteins would dimerize together to bind to the UAS sequence of the genetic construct. In order to make this genetic construct, we would have to make perhaps three separate plasmids for each of these constructs. A minimal weak core promoter, minSV40, would be used between UAS and the gene of expression in order to express the basal levels needed to start the positive feedback loop. Implementing this genetic circuit could add ultrasensitivity to blue-light inducible CAR T-cells and increase the level of CAR expression.



Subprojects**:** Logical breakdown of the solution/design into major components/subprojects

a. Plasmid/Primer Design: Plasmid and primer design for molecular cloning procedures. Use Benchling tools to simulate Gibson assembly to get recombinant plasmid maps and obtain optimal primer sequences for PCR reactions.
b. Wet Lab: Molecular cloning for recombinant plasmids for cellular transfection. Designing genetic circuits that will be light inducible and have the desired gene expression kinetics. Afterwards, we will execute the wet lab experiments which includes cell culture, cloning, transfection, and staining.
c. MATLAB/ Imaging: Microscopic analysis for fluorescent protein expression in response to blue light induction. Use ImageJ to count expressed T-cells automatically and quantify fluorescence response.
d. Statiscal Analysis: Using Statistical applications like t-test and One-way Anova to validate the significance of the design. Comparing and contrasting between control groups like UAS construct and mCherry construct to our designs. Using MATLAB/Excel to observe the expression level whether it's negative or positive feedback or linear.

Leaders and Contributors to Each Subproject

a. Plasmid/Primer Design: Fan Xu
    i. Contributors: Lyhour Lay
b. Wet Lab: Jeffrey Chen
    i. Contributors: Fan Xu
    ii. Contributors: Lyhour Lay
    iii. Contributors: Yifan Xiang
c. MATLAB/ Imaging: Yifan Xiang
    i. Contributors: Lyhour Lay
    ii. Contributors: Fan Xu
d. Statiscal Analysis: Lyhour Lay
    i. Contributors: Jeffrey Chen

Risk Mitigation

Significant Risk 1: HEK-293T cells have low transfection efficiency

It is well known that HEK-293T cell lines have a high rate of failure to transfect properly. One way to decrease this risk is to take good care of HEK-293T cells while maintaining cells and running the transfection protocol in order to increase transfection efficiency. This could include practicing good PPE while handling HEK cells, not leaving the cells out of the incubator for too long, and splitting/feeding cells at appropriate time intervals and confluency percentage. And another way to reduce this risk is to repeat transfection multiple times to make sure we get the desired transfection outcome, with each time tweaking incubation conditions.

Significant Risk 2: Genetic circuit does not work properly

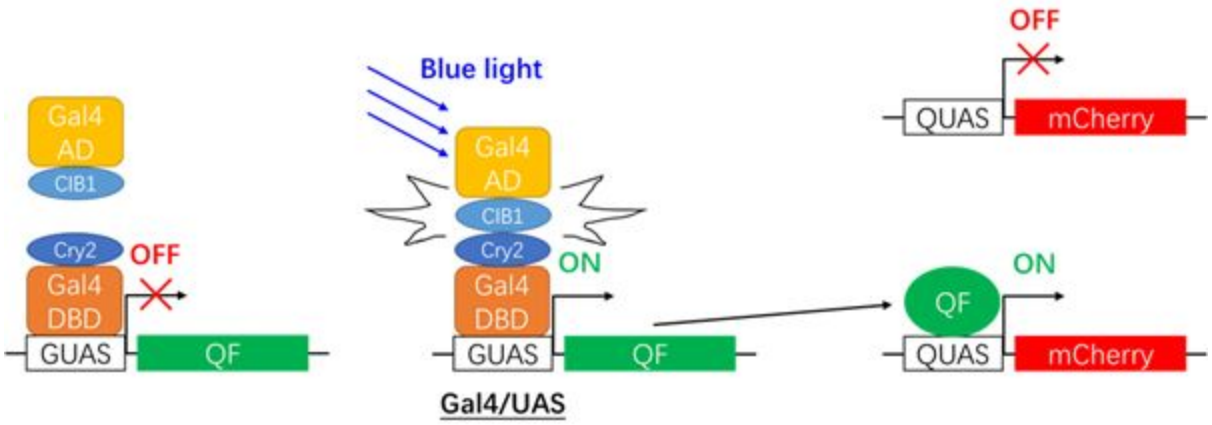
In order to help reduce this risk, the design implemented in the HEK293T cells needs to be well thought out and consider multiple ways the design can fail. This would involve conducting a comprehensive literature search to see what has worked for other people and what has not. Reducing this risk also involves using trusted components and making sure the design is not over-engineered and too complicated. We would mitigate this risk by starting with a design that is not too complicated and then adding more parts as necessary. Finally, another way to reduce this risk is by creating multiple designs. It is possible that the fundamental nature of our initial design is flawed. And consequently, planning multiple plausible designs and ordering primers and plasmids in advance would help to reduce the risk of wasting time, the largest bottleneck of this project.

Design Alternatives and Analysis

Current Design: UAS-Gal4 System with Positive Feedback Loop

Our current design includes the implementation of using the UAS-Gal4 Cry2-CIB1 system. In this system UAS would be attached to the CRY2-Gal4 and CIB1-VPR transcription activator. The reporter gene would also have UAS. Consequently, this would create a positive feedback loop as all three of the genetic constructs have UAS. Cry2-Gal4 and CIB1-VPR would bind together in the presence of blue light. And then they would either bind to one of the three UAS sequences. Creating more of these constructs creates even more of these two proteins. And these proteins would dimerize together to bind to the UAS sequence of the genetic construct. In order to make this genetic construct, we would have to make perhaps three separate plasmids for each of these constructs. A minimal weak core promoter, minSV40 would be used between UAS and the gene of expression in order to express the basal levels needed to start the positive feedback loop. Implementing this genetic circuit could add ultra-sensitivity to blue-light inducible CAR T-cells and increase the level of CAR expression [28].

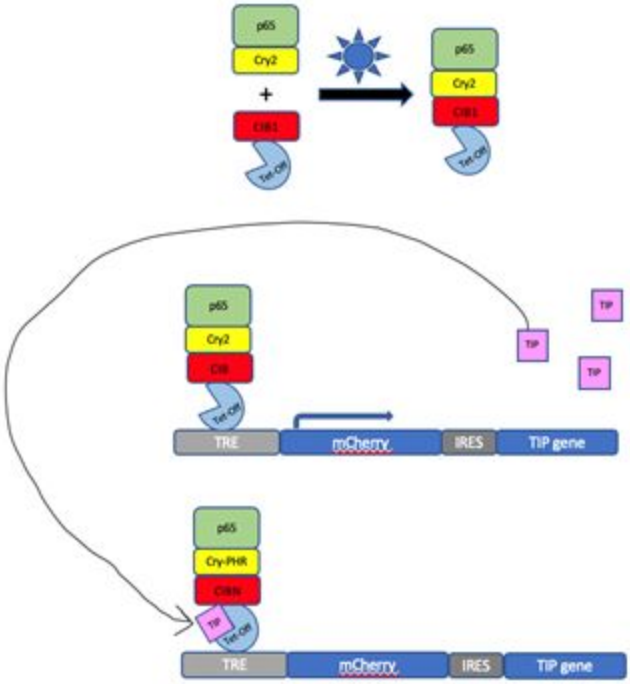
Design Alternative 1: Q system



Currently, our design intends to implement the Gal4/UAS system in conjunction of blue light-inducible Cry2-CIB1 optogenetic circuit to allow the induction of CAR expression. Although this system is well studied and can be confidently implemented in our preliminary design, this system can only achieve positive feedback where upon CAR-T recognition of tumor cells, we can make the CAR-T cells to mass produce a lot of antibodies by shining blue light. To enable more comprehensive spatial and temporal control of gene expression in CAR-T cells, which allows us to shut down CAR expression if necessary, we can apply the Q system into the CAR-T cells. Q system encompasses two genetic cassettes plus one reporter gene of interest. The two cassettes are transcription regulator genes called QF and QS that can be upregulated by certain promoters (designated as “P1” and “P2”). While QF is an activator that, upon binding to the QUAS (Q-upstream activation sequence), the reporter X will be actively transcribed and expressed, QS acts as the repressor where, if bound to QF, will deactivate the function of QF and blocks the reporter gene X from transcribing. The result is a binary repressible behavior that

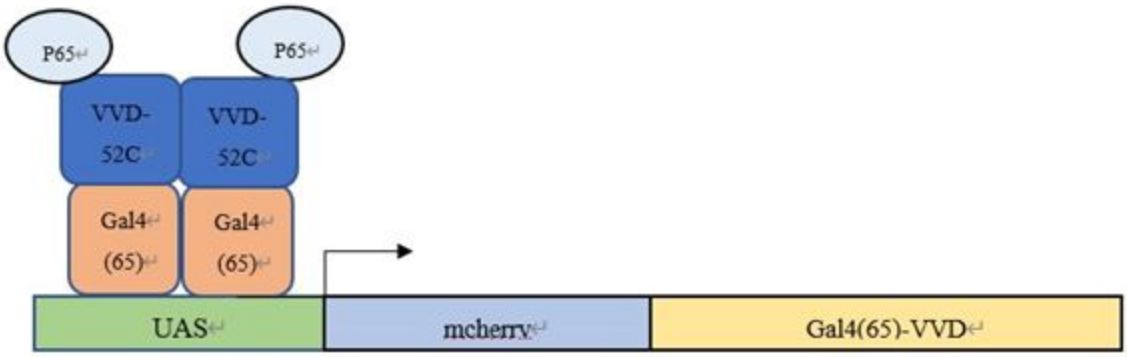
is easy to control and characterize. Major advantages of this system are that there is a low basal level of gene X expression without QF binding, and the expression level between QF and QF-QS complex binding has significant differences that can have distinct physiological effects on the CAR-T cells targeting. Moreover, we can also use this Q system in conjunction with Cry2-CIB1 or TetR system to fine tune the expression of QF and QS[29].

Design Alternative 2: TetR-TIP-Cry2-Cib1



It would be ideal to create a genetic circuit that provides an autoregulation so that the system can use positive feedback behavior to control the expression of Car-T cells. To do so, we can add additional sequences when expressing the Car-T cells in which additional proteins/enzymes are expressed to repress the transcription. So, a TetR system can be implemented in the system to regulate the expression of the Car-T which is mCherry for our first experiment before we are moving forward into actual T cells. TIP is a transcription inducer protein that is fused with Trx-A protein to induce/repress the transcription [30]. The limitation of this design, we have yet to find the sequence. However, we have found papers that prove that TIP has been used as an artificial tetracycline.

Design Alternative 3: VVD-Gal4 UAS system



In this design we use Vivid rather than use the CRY2-CIB1 system as chemical inducers of dimerization. Vivid is a photoreceptor that can form a rapidly exchanging dimer with blue-light activation. Under blue light, the chimeric protein consisting of Vivid and Gal4 dimerizes and becomes a transcriptional activator. In absence of blue light, the active dimer disassociates [31]. Although the Vivid system has a limitation in its low dimer-forming efficiency. There is a mutagenesis optimization of vivid genes that can reduce the background expression to a minimal level and significantly increase the dimer forming efficiency[32]. Since the VVD system works as a homodimerization system, it is easier to form a positive feedback loop. The weakness of this system is that there is probability that the system will be activated without blue light. The system is also not well tested in mammalian cells therefore is not promising as the CRY2-CIB1 system.

Evaluation of the design alternatives

Table

Goals	Weight (%)	Design 1 (UAS-Gal4 Positive Feedback Loop)	Design 2 (Q system)	Design 3(TetR-TIP-Cry 2-Cib1)	Design 4 (VVD)
Successfully inducing CAR expression	40	80	70	50	50
Having expected protein expression level	20	90	80	50	60
Ability to produce feedback loop	30	90	90	60	60
High throughput (Can be done rapidly in parallel)	10	90	70	50	50
Total	100	86	78	53	55

Assessment

According to the matrix evaluation, the GAL4-UAS positive feedback loop design, which is our current design received the highest scores, followed by the Q system design. Comparing the GAL4-UAS positive feedback loop design and the Q system design. The GAL4-UAS system is more simple than the Q system and has much fewer components. Simple system is more desirable since it takes less time when performing the cloning process and it is more likely to react as we expected. The GAL4-UAS system is also a well tested system that is demonstrated to work with CRY2 and CIB1 in HEK cells. Therefore it seems reasonable for us to lean on the GAL4 system which is much more promising.

Parts, Resources, and Costs

Team Budget

Product	Part number	Cost	Size	Company
Phusion High-Fidelity DNA Polymerase (https://www.neb.com/products/m0530-phusion-high-fidelity-dna-polymerase#Product%20Information)	M0530S	$111.00	100 units/ 50uL	NEB
Plasmid vectors (http://www.addgene.org/28244/, http://www.addgene.org/28246/)	28244, 28246	$150.00	~ 10ng/ 50uL	AddGene
Primers for PCR reactions	N/A	$50-200 depending on the numbers	25nmol/ ~ 100uL each vial	Eton Bioscience/ genewiz
Gibson Assembly Master Mix (https://www.neb.com/products/e2611-gibson-assembly-master-mix#Product%20Information)	E2611S	$164.00	100uL	NEB
QIAquick PCR Purification Kit (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-pcr-purification-kit/?clear=true#orderinginformation)	28104	$123.00	50 purifications	Qiagen
Lipofectamine LTX Reagent	TM500	$0(provided by the lab)	500 mL	BrainBits

DMEM + L-Glutamine	10013CV	$0(provided by the lab)	500 mL +4.5g/L	Thermo Fisher
Fetal Bovine Serum	FBS002	$0(provided by the lab)	500 mL	Thermo Fisher
Confocal microscope (for fluorescent imaging)	N/A	$0(provided by the lab)	N/A	Bioengineering dept.
Various other equipment for cell culture and reactions	N/A	$0(provided by the lab)	N/A	Bioengineering dept.
Total		$648.00 - $798.00		

Some of the parts and resources needed in our project are provided in Wang's lab as shown in the table. Taking this into consideration, our project's budget is approximated between 600 to 800 dollars. The total budget is slightly over 500 dollars but is still acceptable for PI's funding to invest (less than $1000).

Planning and Scheduling

Gantt Chart:

Bottlenecks and Planning:

Dependencies: Plasmids and primers must be ordered before cloning. Therefore, timing and a well planned schedule is required. Due to limited access to resources like lab equipment and materials, we need to check and ensure that everything is accessible prior to the experiment day. Another dependency is that we must begin culturing cells in early January so that they are ready for transfection. Since each experiment could take up to one month. We need to allocate time to analyze and repeat the experiment due to contamination and undesired results. Lastly, the lab must be available to use by January in order for us to have a buffer time to repeat the experiment. With the uncertainty of lab access, we need to start the experiment process as soon as possible after approval from Dr. Peter Wang.

Key Steps: First, the recombinant plasmid constructs must work as expected in initial models. The transition from HEK cells to Jurkat cells must maintain the same genetic circuit behavior. There is a high probability that the transition between cell models will not necessarily work, which would take more time tuning of the genetic construct. In addition, another key bottleneck step to consider is the low transfection efficiency of HEK and T-cells resulting in low gene expression in the cell model. The cultured HEK and T-cells need to be ready for testing during their exponential growth phase.

Resource-related Setbacks:The largest bottleneck in the project is time as we are unsure how much time there is to execute all of the experiments given the conditions with COVID. It will be difficult to make sure that there is ample time to run all of the necessary experiments. This is perhaps the biggest constraint as time is one of the most important things needed in case the testing of HEK cells does not go well and the genetic construct needs to be redesigned. Also while transitioning from HEK cells to Jurkat cells there is a high chance the model does not work while transitioning in which case the team may need to modify the genetic circuit. There may

also be issues such as transfection efficiency as HEK cells are known to have low transfection efficiency. Also, the design of the plasmid needs to be correct. If there are any errors with the placement of the promoter or how the genetic components are spaced, there will be issues with the experiment and consequently experiments will have to be redone. The team is limited by the number of rounds of experiments that can be conducted to validate the genetic construct. And so any problems with these steps will lead to issues with time. Our budget is limited to $500 which would mostly be spent on plasmids, primers, cell culture maintenance, sequencing orders. Such restrictions should be well considered before spending all of the money allocated for our budget. Although we will be using protocols tested in literature, we could still encounter situations where cells do not respond as expected. To prevent the setback, we may do multiple cloning at same time to make sure at least some of them work. We also need to book access to the confocal microscopes ahead of time to be able to test the fluorescence responses when cells are ready for the test.

Risks

Summary: There are different types of risks throughout the project. Some of the technical risks associated with the project is the disability to get desired recombinant plasmids because of random mutations and potentially failed Gibson assembly(R), the transfection of HEK293T cells may exhibit low efficiency(A), the final results of reporter gene expression may differ from initial expectations(A), and the risk of not being able to transition from HEK293T to mammalian T-cells(R). A resource risk can be that the desired reagents may not be available(P). A safety risk could be the exposure of toxic reagents while handling and performing experiments without proper training and proper equipment(P). A potential environmental risk can be that biohazards may be improperly disposed that lead to contamination(P). A social risk can be caused by the constant experimental failure that may lead to loss of confidence. Lastly, additional catastrophic events like family emergencies or Covid-19 infection need to be considered during this pandemic time.

Significant Risk: One of the biggest risks that needs to be addressed is that HEK293T cells have low transfection efficiency. It is well known that these cell lines are very likely to have failed transfection. The only thing we can do is to repeat transfection multiple times to make sure we get the desired transfection outcome, with each time tweaking incubation conditions. Therefore, we need to take good care of HEK293T cells while maintaining cells and running the transfection protocol in order to increase transfection efficiency. This could include practicing good PPE while handling HEK cells, not leaving the cells out of the incubator for too long, and splitting/feeding cells at appropriate time intervals and confluency %. Another significant risk is that the final results of reporter gene expression differ from initial expectations. Therefore, we need to do more research and try to find multiple papers that validate the functions of the design components. We also need to plan for multiple alternative designs as back up for the experiments. Another significant risk that we need to consider is the transition from HEK cells into the mammalian T-cell. HEK cells are different from mammalian T cells, therefore there are a lot of considerations when upscaling the experiments. Since they have different cell functions which means that their genes are different, we need to ensure that T cells have the ability to express the construct that we have made in HEK cells.

Preliminary Assessment

Strength:

Cell Growth and Report: When we get the desired transfected cells, they should be easy to grow and analyze. The plasmid design and cloning workflow is pretty similar throughout different cell strains, but with small changes among different plasmids. Our design can be set up to just simulate cellular response to blue light induction through analyzing mCherry reporter gene expression. Robust system that expresses genes well

Resources: The equipment for experiments as well as basic molecular biology reagents are already provided by Wang Lab. Since the lab has used the same Gal system before, they have all the components ready for the experiment. We just need to perform experiments with our design but the same as the biological system.

Innovation: This project is new in that its goal is to optimize CAR-T controllability through using feedback-loop genetic circuits. Although similar attempts have been made to implement circuits for other purposes, they are rarely done in cancer therapy.

Skills/Knowledges: We are very passionate about immunotherapy research and would like to devote as much time as possible. We have some preliminary knowledge in experimental techniques necessary for that purpose, too. With BENG 160 and 162, we have general knowledge and understanding of these wet lab techniques.

Market: Because CAR-T therapy has been shown to be promising to cure cancer, we are confident that it will have a very prominent market once it is widely commercialized. We believe that is going to be the future to treat cancer because it is powerful and less invasive compared to other cancer treatments.

Likelihood to Reach Desired Result: Research has shown that there are pretty much bifurcation behaviors. Since we have adapted our design based on the previous published paper. We are confident that the design will be able to produce significant results as expected. The replication of well known experiments allows us to be able to perform the experiments without any major concerns and uncertainties of the procedures.

Treatment Time: One of the major advantages of CAR-T cell treatment is that the patient will have less active treatment days than undergoing traditional therapy. The goal of treatment is to help get a patient into complete remission for many years. Infused T-cells can persist in the patient's body, therefore perform a long-term effect in cancer treatment.

Less Invasive: Car-T cell helps patients to be treated in a less invasive method. All 3 common cancer treatments are very invasive. For example, a surgery method could damage tissue and organs while Car-T is based on delivering cells into the body and letting the cells fight off cancer.

Weakness:

Lab Access: Due to Covid-19, we are still unsure when we are able to use the lab and start our experiment. Since our experiment is heavily dependent on wet lab experiments, the lab access is a major concern for all of us. We have finished the training prior using the lab. Now we are waiting for the approval from Dr. Peter Wang to let us start working.

Cloning: Cloning experiment has high failure rate and the transfected cells may not respond as we expected. Although attempts can be made to produce desired cells, we may need to think about alternative ways to address potential failures

Level of Expression: Our design might not produce a significant change in term of expression due to the background noise that interferes in the genetic circuit. Though we have used the literature paper to back up our design proposal.

Safety: Clinical trials have pointed out some problems including off-target effects that may be detrimental to the patients. Solving these problems is necessary and may take up some time before the CAR-T can be really used safely. This means that much more research is needed before putting the Car-T Cell therapy in public for a common way to treat cancer.

Blue Light Technology: Blue light has weak penetration, so blue light inducible CAR T-cells would treat skin cancers or there would need to be creative ways to reach other parts of the body such as laparoscopically shining blue light.

Cost: Cost may be an issue as CAR T-cell therapy currently is already expensive. Since each Car-T cell therapy is very personalized, it requires time and resources to develop the treatment for patients. These factors prevent the big biotechnology and pharmaceutical companies to mass produce the treatment.

Limited Validation Experiments: Since this design heavily modifies how cells would function. There are lack of validation methods to characterize the cells. We would be able to validate the cells producing the desired reporter proteins but we are not able to validate whether introducing the new DNA information will negatively affect other basic cell functions.

Remote Work: There is not much work that can be done remotely. The project is heavily on wet lab experiments. So we are able to do anything in the moment besides doing more literature search to propose more alternative designs in case our design fails. Most of the remote work like statistical analysis and microscopic evaluation can be done after wet lab experiments.

Availability of Plasmid Constructs: Though addgene has our current design components, addgene does not contain all the components for alternative designs that we have come up with. For example, we are able to find the plasmid of TIP protein construct. If our design failed, we are limited to design constructs that produce a significant result.

Appendix

Interview Summary

Interview #1: (Dr. Peter Wang) - Wang Lab of UCSD Department of Bioengineering
Interviewers: Jeffrey Chen, Lyhour Lay, Fan Xu, Yifan Xiang

Questions asked:

1. What makes you interested in CAR-T Cells?
2. What are potential problems that need to be addressed about CAR-T cells?
3. What is the most challenging part of your research and engineering CAR T-cells?
4. Saw an article involving your paper on using Tamoxifen and blue-light inducible CAR T-cells to control when CAR T-cells attack, and you mentioned how blue light-inducible CAR T-cells would most likely be used in head and neck skin cancer. Is there a reason in particular why you said head & neck skin cancer and not other types of skin cancer?
5. Also would the CAR T-cells be injected at the site of the skin tumor?
6. Also mentioned collaborating with clinicians to test the CAR-T therapy, are you currently or looking to collaborate with clinicians right now? And if so what are you doing with these clinicians?
7. Next week we are meeting up with a head & neck oncologist at UCSD who is part of the Precision Clinic of Immunotherapy at UCSD. We were wondering if you had any particular questions for him regarding blue-light inducible CAR T-cells that maybe you had and we could ask him/pass on the response to you.
8. What is your prospect for applying CAR-T therapy with enhanced safety in the future?
9. Do you have any advice for our design project?

Dr. Peter Wang and his team have done a lot of biochemical and biomolecular engineering. They have been working on how to characterize how cells are signaling and thinking. Now they want to understand the nature of the cells and he hopes to change the nature of cells and molecules. Car-T is one of the engineerable cells that is practical to work on and can potentially be useful. Car-T cells would be a good experimental group because it currently has the drawbacks. The drawback of the car-t is that it goes everywhere, they can amplify the signal, it is powerful enough to kill the tumor but meanwhile it also kills healthy cells, therefore the current Car-T cells application also harm the healthy cells/tissues/organs. So this drawback motivates Dr. Peter Wang and his team to design genetic circuits that allow them to control the expression temporally and spatially.

There are many genetic circuits in biochemical and biomolecular engineering applications. The key part is to determine which one is sufficient and what is important. The desirable goal for the design of the genetic circuit for Car-T cells is an elegant system that is powerful and sufficient. Designing a working gene circuit is important but the ultimate goal to produce a design that can cure/treat solid tumors. The most challenging is safety and efficiency. So there are challenges when upscaling in vivo into clinical trials because there are a lot of considerations to take into account when designing them for humans.

In general skin cancers are a good experimental group. But Dr. Peter Wang and his team wants to expand to neck and head cancers because he wants to include various types of

cancer being treated with Car-T cells. Other than skin cancer, Colon cancer can also be a potential target of the light inducible car-t treatment since now we can directly shine blue light into the Colon. If everything goes well, 5-6 years. that the controllable Car-T cells will be available to treat cancers. His team has finished experimenting on animals. Now they are preparing for the clinical trials.

Now, his team is working with clinicians. They currently work on injecting laparoscopically to the liver. Today, Dr. Peter Wang just had a meeting with the clinicians who specialize in chemotherapy. The collaboration between them will be huge because they will be able to connect the delivery method and Car-T cells treatment to target the tumor site precisely and accurately. A collaboration with Dr. Erza Cohen would be also great because he would connect the gap between animal and human. For example, Dr. Cohen would be able to provide the expectations from the data that he has with human's cancer. This will help Dr. Peter Wang and his lab to ensure that his design is working in clinical trials.

Solid tumors are usually from their own cells. Therefore, the traditional Car-T is hard to differentiate the healthy cells vs the tumor cells. Luckily, his team is able to activate the Car-T cells at a specific tumor site and not other untarget tumors. Dr.Peter Wang's team have done a comparison experiment with a target tumor and a distant tumor, the normal car-t cell kills both tumors while controllable car-t treatment is only activated at target tumor site using ultrasound and it only kills the target tumor. The result of the experiment proves the safety of the design.

Lastly, he advised us to be wild and ambitious about designs. He wants us to present ideas as much as possible. He and Phuong Ho, our graduate student mentor will discuss and give feedback on what is practical.

Interview #2: (Dr. Nate Yoon) - Wang Lab of UCSD Department of Bioengineering
Interviewers: Jeffrey Chen, Lyhour Lay, Fan Xu, Yifan Xiang

Questions asked:

1. What is your background? What are your specialties?
2. What is your current research? Previous published papers/ abstracts?
3. Why are you interested in the mechanogenetics and ultrasound CAR T-cell research?
4. What is your vision for Car-T Cell and Ultrasound research and how do you think it will be implemented as a medicine in the future?
5. What do you find is the most interesting thing about what other people are doing in ultrasound CAR-T research?
6. What would you say are important considerations for the design of the genetic circuit?
7. What is the most challenging part of your research and engineering CAR T-cells?
8. What are your thoughts on different genetic circuits (such as ultrasound, blue light-inducible Cry2-CIB1, TetR system, and others you heard about)?
9. Our project currently involves engineering light-inducible CAR T-cells. Do you have any advice for us on designing and executing our design project?

Our group met with postdoctoral researcher Dr. Yoon on November 16. He is a member of professor Yingxiao Wang's lab and is currently designing ultrasound-inducible mechanogenetic circuits to direct calcium signaling in CAR-T cells that specifically target

prostate cancer. He informed us that (1) genetic circuits are useful tools for inducing protein expression with a big fold change, (2) different genetic circuits can be implemented for different purposes, and (3) there are challenges when designing circuits due to possible interference by internal cellular pathways.

He firstly introduced his academic background in ultrasound mechanics. Graduated with a PhD from an electrical engineering lab, he Had worked with ultrasound stimulation with different cells to observe cellular behaviors. Having tackled molecular engineering, he went to Wang Lab to pursue his interest in designing inducible CAR-T cells. His specific goals are to establish ultrasound inducible genetic circuits for CAR-T cells but also expand the utility of such circuits into other types of cells as well.

He stated that ultrasound can create a lot of intriguing bioeffects. For example, continued ultrasound can induce heating around targeted tissue, which could be a useful tradeoff for inducing heat-related pathways. There exists a variety of neuronal modulation using ultrasound stimulation, and ultrasound has very deep penetration into the body, compared to blue light which can only reach the surface level of an organism. He mentioned that research about inducible systems was promising because stem cells have certain differentiation patterns that can be shown to be directed by such means. Especially popular topic within the field is optogenetics, which has a lot of preliminary data available.

Dr. Yoon liked the idea of us using the Cry2-CIB1 system because it has a very low basal level of expression, so we would expect better fold changes when activated. As for Dr. Yoon, he was trying to induce CAR-T expression using calcium signaling. He stated that it was easier for him to design this system because calcium signaling has been well-studied. However, he suggested some potential challenges we still have to face.

When designing his ultrasound inducible circuit to direct calcium signaling, he has to consider how to suppress the effect of non-specific calcium-dependent pathways so that he could achieve an on-and-off effect using ultrasound stimulation. There are a lot of ways for cells to decode calcium signals, but he wants to direct calcium signals to only induce the response he wants. Besides, mechanical sensors in the cells are not robust because they are also affected by cell morphology. If these sensors can be more specific to ultrasound stimulus, then he could get a finer control. Another important thing we might consider is that the stimulation time needs to have shorter exposure time due to limited time in clinical trials, so that the expected transient translation of protein products can be achieved.

As advice for our design project, he commented that we can combine different genetic components together to achieve independent protein expression through different stimuli. However, we have to make them compatible with each other and make sure they do not interfere. There is no good system for everything, and it takes time and multiple testing to get a well-designed product that can be ready for application in clinical situations.

Interview #3: (Phuong Ho) - Wang Lab of UCSD Department of Bioengineering
Interviewers: Jeffrey Chen, Lyhour Lay, Fan Xu, Yifan Xiang

Questions asked:

1. What is your background? What are your specialties?
2. What is your current research? Previous published papers/ abstracts?

3. Why are you interested in this Optogenetics ?
4. What is your vision for CAR-T cell and Optogenetics research?
5. What finds the most interesting what other people are doing in optogenetic CAR-T research?
6. What would you say are important considerations for the design of the genetic circuit?
7. What is the most challenging part of the project?

Phuong Ho is our design mentor and his main research is focused onto genetics and cell signaling, mainly to regulate gene expression. Moreover, his secondary interests also include ODE modeling, which is modeling gene dynamics from quantitative data from fluorescence imaging. His current research topic is blue light inducible protein to control how cells can respond to different signals. He planned to apply the techniques he learned in undergraduate school which gave him a published paper in virology to conduct his research.

He stated the reason for his interest in optogenetics is because it is easy to do studies in in-vitro work and study the cell processes. Other inducible systems such as drug inducible are harder to carry out experimentation. His vision for optogenetics and CAR-T cell research is that we need a staging ground for CAR-T and test a whole bunch of ideas.

Actually, applying optogenetics is kind of tough, because the stimulus is not going to penetrate deep into patients. Optogenetics is like a sandbox where we can try to apply different ideas into different concepts. However the response may not be as expected, so many tests have to be carried out to get the inducible systems that we can get desired results. However, through some alternative designs such as ultrasound or heat inducible genetic circuits, we can test these tools when we apply them into clinical situations. For example, red light can have better tissue penetration. But for optogenetics, blue light is further in development than red light. Also, people started to work on red light-inducible nanoparticle conversion which allows better tissue penetration of the light signal, for deep level cancers.

He addressed that important considerations for design of genetic circuits is that we need reliable parts to verify that all the protein components are as intended. For many instances we assume they work, but when we actually use it it isn't as good as they advertise it in the paper. Thus, one of the most challenging aspects for our project is translating our work in HEK293T cells into real human T cell types. Also, having all the components work as intended is also challenging and opportunistic. Be prepared to see it's not working.

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Work Breakdown

All of our team members have contributed to the literature review, planning, brainstorming, and writing of team assignments for the senior design project.

Completed Design Phases

1. Conduct literature search to find relevant background, concepts, and experiments that can be replicated
2. Brainstorm and evaluate design alternatives: so far, we decide to start with implementing Gal4/UAS system in HEK293T cells
3. Construct and obtain plasmids and primers for transfection

Leaders and Lieutenants for Completed Design Phases

Design Phase	Leader	Lieutenant
1	Yifan Xiang	Lyhour Lay
2	Jeffrey Chen	Lyhour Lay
3	Fan Xu	Lyhour Lay

Team Assignment Leaders

Team Assignment	Principal Author	Principal Editor
Literature Review	Lyhour Lay	Fan Xu
Needs Assessment	Jeffrey Chen	Fan Xu
Problem Statement	Yifan Xiang	Lyhour Lay
Team Goals	Jeffrey Chen	Fan Xu
Interview Target List	Jeffrey Chen	Fan Xu
Team Constraints	Jeffrey Chen	Fan Xu
Project Exchange Summary	Jeffrey Chen	Fan Xu
Design Review	Lyhour Lay	Yifan Xiang
Planning and Bottlenecks	Yifan Xiang	Lyhour Lay
Design Alternatives	Jeffrey Chen	Fan Xu
Risks Assessment	Lyhour Lay	Yifan Xiang
Team Budget	Jeffrey Chen	Fan Xu
Team Progress	Lyhour Lay	Yifan Xiang