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Exosome Engineering and Imaging

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By,

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Abstract

There are many new pharmaceuticals being developed for the treatment of various diseases. One of the major issues these drugs face is the ability to enter a cell or the area of treatment. The current methods for drug delivery are often arduous to complete and do not make it to the diseased area because they are targeted by the immune system. In order to improve the ability for drugs to reach the desired area we propose the use of exosomes. Exosomes are subcellular vesicles that are responsible for the transport of biomaterials or signals within the cell and to other cells. The size of exosomes is small, around 30 nanometers is the average size, which minimizes the potential for immune targeting and also allows them to cross the blood brain barrier. In this project we look at the ability to tag, or add a protein, to the surface of an exosome. The fluorescent protein illustrates the location of the exosome within the cell and its potential to be tagged with other proteins to target various types of cells. Engineering exosomes has the potential to solve the current issues with drug delivery.

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Chapter 1: Introduction

1.1 Background

The treatment of cancer is limited to surgery, chemotherapy, and radiation. The first line of tumor removal is surgery but depending on the progression of the disease, sometimes surgery cannot be performed. Surgery alone does not ensure complete removal of cancerous cells, prompting the use of radiation and chemotherapy. These treatments can help patients achieve remission but it is common for cancer to recur. In 2014 there were nearly 14.5 million cancer diagnoses in the United States and it is expected to rise to almost 19 million by 2024 according to the National Cancer Institute. As cancer rates rise there needs to be alternative, more effective therapy than those currently in practice. The large number of cancer cases has translated to a large field of cancer research. Cellular and protein based therapies are popular potential therapies, however, we are looking to go deeper. Our work is focused on developing the base for a novel, nanoscale system with applications in cancer therapies. Specifically, we are looking to utilize the exosome vesicle for protein attachment.

1.2 Review of the Field

Exosomes have been increasingly studied due to their previously less known role within the cell and potentially useful structure. Exosomes are classified as extracellular vesicles; they are proteolipids with a bilayer, containing lipids, proteins, mRNAs, and microRNAs (Ref. 1). The contents of extracellular vesicles are not random; they maintain specific amounts of the aforementioned products. This is important to the study of extracellular vesicles, specifically exosomes, because their molecular mechanisms such as sorting and biogenesis can be understood in more detail. Intercellular and intracellular communication can be researched further. Exosomes are secreted by “fusion of late endosomes or multivesicular bodies with the

plasma membrane" (Ref. 1). As more components of extracellular vesicles are discovered and studied, the greater the chance for use as biomarkers or immunotherapies.

Cell to cell communication is reliant on extracellular vesicles because they are able to travel to distant cells. Vesicles communicate with cells through transfer of proteins, lipids, and nucleic acids (Ref. 2). They are also able to signal various biological processes. These include antigen presentation and are therefore involved in immunological regulation, transfer transcription factors, oncogenes, and pathogens, in addition, they are able to transfer mRNAs and regulatory RNAs (Ref. 2). Exosomes have a large impact on the various processes conducted in and out of the cell, as well as the introduction of outside products or components, creating the potential for drug delivery, immunological stimulation, and disease biomarking. Exosomes are also present in the brain; they are implicated to be involved with synaptic neurotransmission (Ref. 2). Further, it is has been implicated that exosomes have a role in phenotypic modulation and tissue regeneration post injury. One of the most important implications of exosomes is in disease pathogenesis. One of the more understood exosome related pathologies is tumorigenesis, as exosomes can be involved in the coagulation, proliferation, and immune escape (Ref. 2). Improving the understanding of the role of exosomes in pathogenesis has the possibility of altering the exosomes involvement to prevent the disease from proliferating (Ref. 2-3).

1.3 Literature Review

Cancer research has forced scientists to rethink ideas and discover more intuitive ways of diagnosing and treating patients. Even with current technology many of treatments are unable to accurately assess the potential cancers (Ref. 4). However exosomes are new potential method for overcoming these issues. Considering that cells naturally secrete exosomes, the ability to identify exosomes from different cell types became a field of interest. Recent studies found that, with some degree of accuracy, identify between exosomes produced by lung tumors and exosomes produced by healthy cells (Ref. 4). Current techniques do not necessarily possess a high enough level of accuracy for clinical use. However, expansion on this work could lead to improved diagnostic techniques. The important barrier here is developing a more fundamental

understanding of exosomal surface which are the markers used by scientists to construct diagnostic tests (Ref. 4).

Additionally, research is being conducted regarding the contents of exosomes, as a method to develop a greater understanding of their origin. One group has discovered that oncogene amplifiers and certain DNA fragments linked with tumor growth have been isolated in some exosomes (Ref. 5). This offers an alternative solution to our approach that focuses not on the external focus of exosomes but instead their cargo. While we have chosen to focus on surface proteins, potential of such cargo detection techniques should not be ignored. Our project hopes to open the door to more than diagnostic methods, ultimately the goal is new therapy development.

In order to develop robust drug delivery, a broader understanding exosome function is necessary. Exosomes role in both the development of tumors and potential therapies stems a more broad knowledge of their role in the body (Ref. 6). Specifically their role in the body's immune response is an area of interest and as a result a display system must be established. To accomplish this display system we must turn our focus to membrane proteins and unlock mechanisms by which they can be manipulated.

A recent surge in exosomal research serves to validate the work we are doing and while the methodological approach might change we feel that our system is justified to help us achieve our ultimate goals.

1.4 Project Goal and Objectives

This project is dedicated to modification and imaging of exosomes which have been modified and fluorescently tagged. By developing a deeper understanding of the exosomal surface protein we can begin to explore these nanoparticles unique potential for targeted drug delivery or role as cell messengers.

The first milestone is to develop the protocols necessary for creating and maintaining cell lines that express fluorescently tagged GFP. Using previously established techniques we will be able to create and passage these cells for further study.

Once this has been established we will work extensively on the imaging of these cells. This process will occur in two distinct phases. The first will involve imaging with an optical microscope and the second will require the use of a confocal microscope.

After extensive imaging has conducted we will move forward with further modifications of CD63. We will use existing techniques and protein engineering to insert a new fluorescent tag into our exosomes and prove the robust nature of our established system.

We expect to get a much better image of our modified exosomes by using the confocal lens which is capable of imaging specific layers within the cells. Additionally, modifications with new fluorescent proteins should serve as additional proof that our system is in fact a robust method for the modification of surface exosomal proteins.

1.5 Back-up Plan

The main concern for our research is imaging the tetraspanin protein, CD63 . This M-shaped protein appears to be the ideal scaffold for the attachment of targeting proteins. However, this unique shape could mean that alterations made to the protein could render the exosome unable to link to its target antigen. Due to this fear, an alternative embedded protein on the surface of exosomes has been identified. This alternative protein is a simple linear shape, which could allow for more versatility in terms of the antigen receptors we may target in the future. CD63 is still our ideal target but the identification of a backup was necessary to guarantee the continuation of our research is complications were encountered.

1.6 Significance

Developing a novel system for drug delivery through the use of an exosome could have the potential to treat and/or cure cancer. In addition, it could be applied further to drug delivery in specific tissues or cells. The body naturally creates and secretes exosomes, so they are much less likely to be rejected or cause a reaction in the body if they are used for therapeutics. They are

accepted by cells and release their components within the cell, creating a potential drug delivery system. This could be used to heal specific parts of the body or the entire body. The drugs or therapies could also be highly patient specific, addressing the exact needs. The technology could be simplified to a level where expensive administration techniques could be diminished or eliminated for application to the developing world. Blood-borne pathogens and diseases specific to the developing world could be targeted and eliminated. Exosome based therapies have a great level of potential for curing disease and therapy development as they are readily accepted and exist in every part of the body.

1.7 Team and Management

Senior Design Team:

Joseph Losacco
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Advisor:

Dr. Biao (Bill) Lu

1.8 Project Budget

The most essential of those would be our cell line. Without the HEK 293FT, the basic platform for our research, the entire project would not be possible. The GFP and RFP genes are necessary due to their fluorescent nature. These proteins allow for us to have visual confirmation of the effectiveness of our methods early on in our lab work. The cell culture media is another essential purchase because it provides nutrients for the cells to grow and replicate. Without the media the cells would die off.

The School of Engineering Grant and Roelandts Grant ensured we had no difficulty obtaining the necessary materials required for this project.

1.9 Project Timeline

Our team took the early opportunity to get into the lab to familiarize ourselves with the equipment and refine our protocols to guarantee effective practices. Winter Break was again spent doing literature review. Our team took this time to create a useful reference library that we would be able to emplace in our final thesis. Winter quarter will be mainly spent in the lab executing our experiment. Step one is to manipulate engineer the exosomes to contain GFP on the C terminus of CD63. Once we have established a successful and effective method to execute step one we will begin to further engineer our exosomes. Extensive imaging will follow the success of this method. Spring quarter will be spent finishing up our thesis and executing final tests to confirm our results. Also during this time we will be passing off the project and cell lines to the next group that will further explore the increasing therapeutic possibilities this research can present.

Chapter: 2 Fluorescent Protein Insertion

2.1 Introduction

In order to glean a greater understanding of exosomes and their potential application to drug delivery applications, they must be modified and visualized. One of the most effective ways to visualize subcellular vesicles is attaching a fluorescent protein and viewing them using microscopy. Visualizing the cell after modification can depict the location of exosomes or an incorrect modification.

Viewing specific cell structures, both in and outside of the cell requires luminescence; one of the best and most common ways to accomplish this is utilizing fluorescent proteins. Fluorescent proteins tend to be relatively large in size (~25 kD), especially in comparison to organic fluorophores which are in the ~1 kD range. The applications for fluorescent proteins are wide as they are functional in cellular and animal imaging (Ref 7). The fluorescent proteins, when attached to the proteins of interest, can allow observation of localization, movement, turnover, and time passed from synthesis. In addition, nucleic acids can be used for fluorescent protein attachment (Ref 8). Cells and tissue can be labeled with fluorescent protein in order to image the morphology, location, and movement of cells and tissue as well as the proliferation stages. The most useful application of fluorescent proteins to this project is their ability to tag vesicles and organelles. Fluorescent proteins attached or 'tagged' to vesicles through specific protein attachment, allow visualization of morphology, fusion/fission, and segregation due to cell division or other factors (Ref 8). In order to visualize the tagged structures, the sample must be stimulated with the corresponding frequency of the fluorescent protein in the sample.

Exosomes are small vesicles, with a heterogenous size ranging from 30-100 nm and in order to better understand their behavior fluorescent proteins must be utilized (Ref 9). The insertion of a fluorescent protein into the exosome will either be successful or not. In addition, the insertion itself can provide greater information about the exosome activity.

In this experiment, Human Embryonic Kidney (HEK293) cells are utilized, as they are a highly stable mammalian cell line. This specific line has a greater passagability, it does not display mutagenesis in its morphology until the 100 passage time. This allows for easier maintenance and a lower cost for experimentation, as new cell lines were not required as frequently. In addition HEK293 cells have a high growth rate, allowing for quicker results. In addition, HEK293 cells have good transfectability, meaning potential for greater DNA insertion.

Microscopy is the final and key piece of this project, as it will allow for the visualization of the insertion of the fluorescent proteins. The microscopes utilized were the Leica DMI3000 20x Inverted Microscope, 40x Zeiss Axioscope Fluorescence Microscope, and a 63x Confocal Microscope. The Leica microscope is the least powerful of the microscopes but it allows for quick visualization of cells within the lab. The Leica microscope allows for live cell visualization and fluorescence. The 40x Zeiss Axioscope Fluorescence Microscope is more powerful than the Leica and is utilized for its fluorescence. Finally, the Confocal Microscope is the most powerful imaging technology available for use. Its greatest advantages are its ‘z-slicing’ and high magnification properties. The ‘z-slicing’ feature allows the user to indicate a specific location on the z-axis in which to image the cells. This is highly advantageous to work with subcellular vesicles because they are much smaller in size.

2.2 Key Constraints

The key constraints of this experiment were the cell line used, the fluorescent proteins: GFP and RFP, and the microscopes used for imaging.

2.3 Design Approach

The four basic tenants of our experimental design are modifiability, human compatibility, ability to visualize, and maintenance of functionality. The design must be modifiable so that it can be altered if the original fluorescent proteins do not work initially or be applicable to different tags

and serve as a platform for different proteins or cells. Human compatibility is integral as the overarching goal for this research is targeted drug delivery in humans. There is limited research on the use of engineered exosome systems in mammalian cells but for the system to be successful it must work in humans. Using a mammalian cell line is the starting point for later use in humans. The system must allow for visualization in order to determine the location of the exosomes. Finally, the system must maintain its functionality. The platform or design should not breakdown over time, it should be able to maintain its integrity.

In order to create this system, the CD63 tetraspanin is chosen as the target for modification. The CD63 protein crosses the exosome membrane four times, putting it in the tetraspanin class of proteins. This protein is one of the most abundant protein on the exosome surface and is oftentimes considered a key component of exosomes (Ref 10). In addition, CD63 has the potential for use as a scaffold.

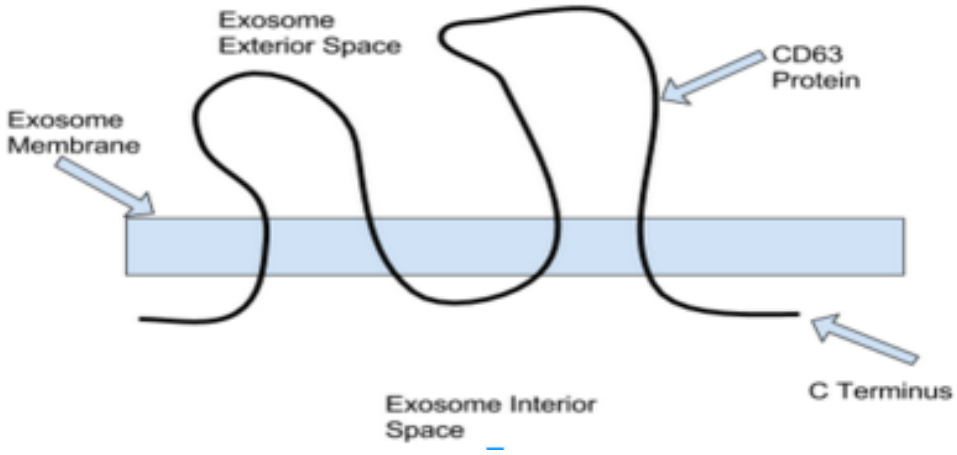


Figure 1: Schematic of the CD63 Tetraspanin.

Figure 1 depicts the CD63 tetraspanin, a key part of this schematic is the C Terminus. For this design, GFP is inserted onto the C Terminus and nothing in the loops as indicated in Figure 2.

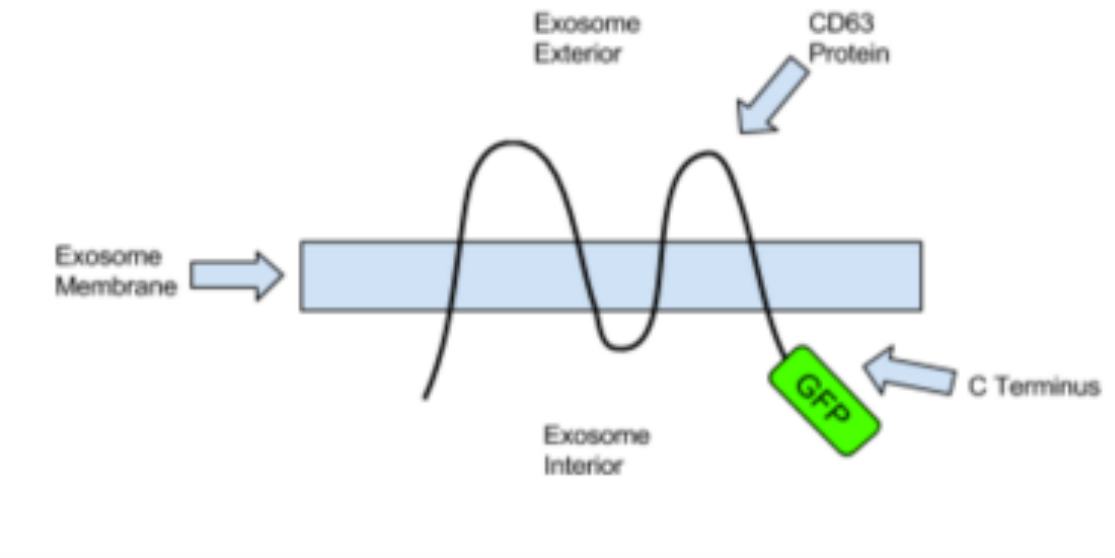


Figure 2: Schematic of the inserted GFP onto the C Terminus of CD63

The GFP is attached to the C Terminus through the system indicated in Figure 3. The vector is constructed using constitutive cytomegalovirus (CMV) promoter, the CD63 virus, GFP, and a polyadenylation signal. EF1α with a puromycin resistance gene is incorporated.

Figure 3: C Terminus vector for mammalian cell insertion.

This step allows for exosome fluorescence, in order to analyze their location a reference is necessary. Green fluorescence will also be inserted into the cytosol for imaging comparison.

RFP is inserted into the nucleuses via transient transfection reagent. The cell line should express the GFP and RFP when imaged. These components should allow visualization of the location of exosomes relative the nucleus. The cells are imaged initially using the Leica DMI3000 20x microscope to ensure proper insertion of the fluorescence and analyze the location. After initial analysis, some cells are fixed onto glass coverslips in order for imaging by the40x Zeiss Axioscope Fluorescence Microscope and 63x Confocal Microscope. Fixation on glass coverslips is necessary for proper imaging.

2.4 Supporting Analyses

Exosome modification and imaging is a newer movement in current research. However, modification and imaging of other subcellular vesicles and bodies is not nearly as new. In order to image subcellular vesicles or bodies, plasmid based transfection is often utilized (Ref. 11). The liposome is a larger vesicle analogous to the structure of the exosome and more visualization has been conducted using exosomes. Fujii, et al. analyzed membrane protein visualization by inserting fluorescent proteins (Ref. 12). These authors were able to develop a system for surface display and then analyzed the varying fluorescence of the liposomes. The surface display system is applicable to other proteins in lipid-bilayer membranes (Ref. 13).

However, the use of most plasmid-based transfection does not usually have a high rate of fluorescence. There are other ways to visualize these subcellular vesicles, one of these is the use of Cyto-Tracers (Ref. 11). Cyto-Tracer constructs encode fusion proteins; these proteins contain a targeting peptide and a fluorescent protein sequence. The targeting peptide should then guide the fluorescent protein to the correct location. The vector design presented in Figure 3, contains a Cyto-Tracer, indicated as CMV. Fong, et al. have shown success with Cyto-Tracers in mitochondria, peroxisomes, microtubules, and other intracellular structures. The work of these authors indicates that our experimental set up should yield proper fluorescence of the exosome.

2.5 Expected Results

For the correct insertion of GFP and RFP, the exosomes should appear surrounding the nucleus. Microscopy should depict a red nucleus with green exosomes or endosome areas outside of the nucleus area. As exosomes are prepared for secretion, they remain in the endosomes, until secretion (Ref. 14). However, it is not expected that all nucleuses will fluoresce, as the insertion of RFP is the result of a transient transfection. Depending on the passage the nucleuses will fluoresce red but the percentage may vary.

2.6 Backup Plan

If this system does not work, the first step is to return to the traditional form of plasmid based fluorescent insertion for surface display. If reverting back to the traditional system, the fluorescent proteins will be reevaluated and replaced with different proteins or different forms of the initial fluorescent proteins to identify the problem.

2.7 Full Protocol

2.7.1 Cell Culture Basic Protocol:

Protocol adapted from ThermoFisher

Cell Culture Protocol

Preparing Medium

Prepare the complete D-MEM medium containing 10% FBS supplemented with 0.1 mM MEM Non-Essential Amino Acids, 1 mM sodium pyruvate and 2 mM Lglutamine

1. Remove 1/10 of D-MEM and replace amount with 50 mL FBS.

2. Also add:
    - L-Glutamine
    - Non-Essential Amino Acids (mix)
    - Sodium Pyruvate
3. Sterilize the medium
4. Store at 4°C

Thawing

1. Remove the vial of frozen cells from liquid nitrogen and thaw quickly in a 37°C water bath.
2. Decontaminate with ethanol solution and transfer to sterile tube with PBS. Then centrifuge and resuspend.
3. Transfer cells to flask containing complete medium.
4. Incubate flask.
5. The next day aspirate off medium and replace with fresh medium.
6. Incubate cells and check daily for 80-90% cell adherence.

Freezing

1. Freeze cells at 3 x 106 viable cells/mL density
2. Prepare medium with 10% DMSO. Should be done immediately prior to use.
3. Culture desired quantity to 70-90% adherence.
4. Remove cells from tissue culture flask(s).
5. Centrifuge the cells suspension, remove medium and resuspend cells in the pre-determined volume of chilled freezing medium.
6. Dispense aliquots of suspension into cryovials.
7. Freeze cells in apparatus with temperature decrease of 1°C per minute.
8. Transfer vials to liquid nitrogen storage.

2.7.2 Transfection into Mammalian Cells

Protocol Adapted from ThermoFisher

Mammalian Cell Transfection

Check the confluency of your cells. They should be ~75-90% when transfecting

Preparing your materials:

1. Pre-heat cell media (dMEM+10%FBS)
2. After checking cell confluency, wipe down the under surface of your cell plate with

70% EtOH on a kimwipe and place in hood

3. Turn on the aspirator and spray EtOH on the tubing that will be inside the hood
4. Obtain proper pipettemans and pipette tips. Sterilize with EtOH
5. Obtain three 1.5mL centrifuge tubes from your instructor
6. Sterilize hands with EtOH before putting in hood.

Transfections

1. Label your 1.5mL tubes for the transfection reactions you calculated
2. Add media to the tubes and then add the calculated amount of DNA. Mix thoroughly by pipetting
3. Add the proper amount of Fugene6 DIRECTLY to the reaction without touching the sides of the tubes.
4. Incubate for 10 minutes at RT inside the hood
5. Aspirate off old media from the cells and replace with 2mL of new media. Add media to the side of the plate while tipping to prevent detachment of the cells
6. Add the transfection reaction mixture dropwise to each well
7. Tilt the plate back and forth to distribute the mixture evenly
8. Put the plate in the 37C and check back at 24, 48, 72 hours to take fluorescent pictures

2.7.3 Cell Fixing

Protocol Adapted from R&D Systems

Cell Fixation on Glass Slips

1. Culture cells by adding 500 µL of culture media containing approximately 5000 cells to the wells of a cell culture plate containing gelatin-coated coverslips.
2. When cells have reached the desired density/age, remove the culture media from each well and wash twice with PBS.
3. Add 300-400 µL of 2-4% Paraformaldehyde Fixative Solution to each well, and incubate for 20 minutes at room temperature.
   Note: Some cell types can be damaged by the change in surface tension that occurs when the culture medium is entirely removed and replaced with wash buffer. If this is the case, pre-fix the cells by adding 500 µL of 4% Formaldehyde Fixative Solution directly into the culture medium. After 2 minutes, replace the pre-fixation culture medium with 300-400 µL of 2% Formaldehyde Fixative Solution and incubate, for 20 minutes at room temperature.
4. Wash the wells twice with PBS and cover with 400 µL of wash buffer. The coverslips can be stored at 2-8 °C for up to 3 months or they may be stained immediately.
   Note: Fixation can result in hydrophobic cross-linking of tissue proteins. The time, temperature, pH, and fixative used will determine the degree of cross-linking. Once the fixation protocol has been optimized, the same procedure should be used consistently.

2.8 Results

2.8.1 Brightfield Microscopy

The Leica microscope is the least powerful of the microscopes utilized. Figure 4 depicts the GFP fluorescence in cells and those cells in a brightefield setting to illustrate the cell shape and location of exosomes. This pair of images indicates the exosomes are at the edge of the cell in the endosome space, indicating that the GFP vector targeted the exosomes.

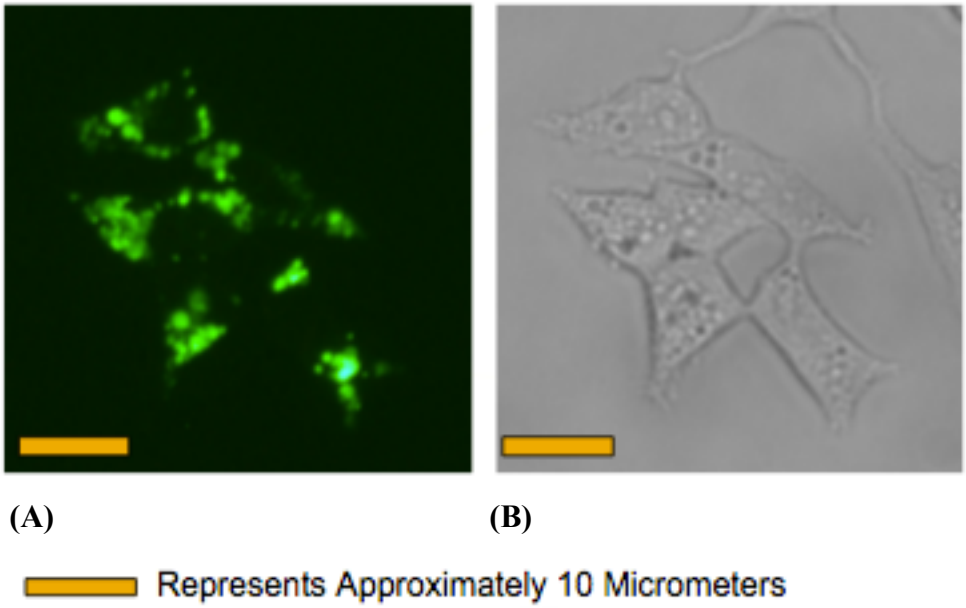


Figure 4: Exosomal GFP Tagged CD63. (A) Under Argon laser (B) Under white light.

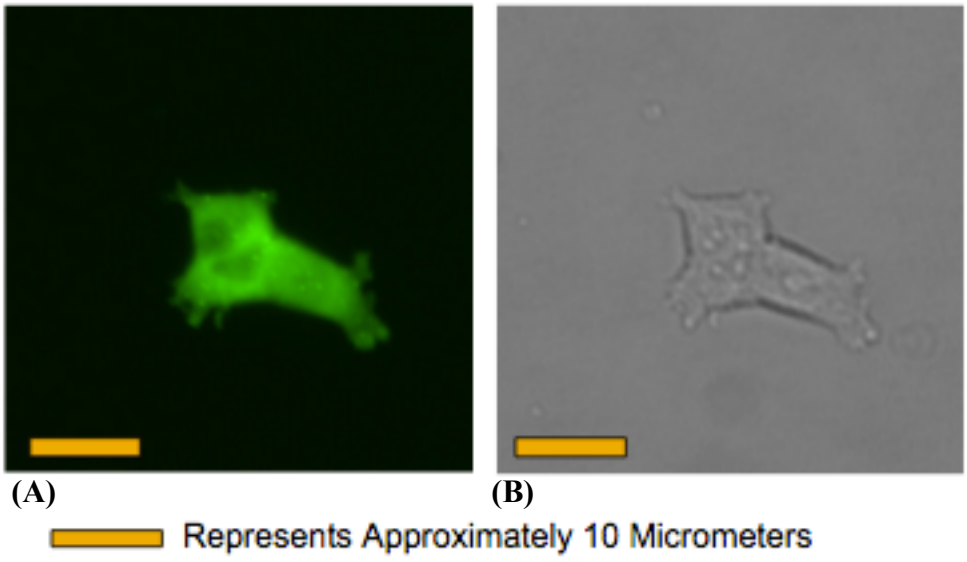


Figure 5: Cytosolic GFP. (A) Cytosol under Argon laser (B) Cytosol under white light.

In addition to the exosome fluorescence, the cytosol was targeted with GFP to depict the capacity of the GFP. The pair of images in Figure 5 are images of the same cell under the argon and brightfield conditions to indicate the shape.

2.8.2 Zeiss Microscopy

These images were obtained through the use of the Ziess Microscope. The images taken are of the same cell and surrounding area. The yellow arrow indicates the nucleus location in each image.

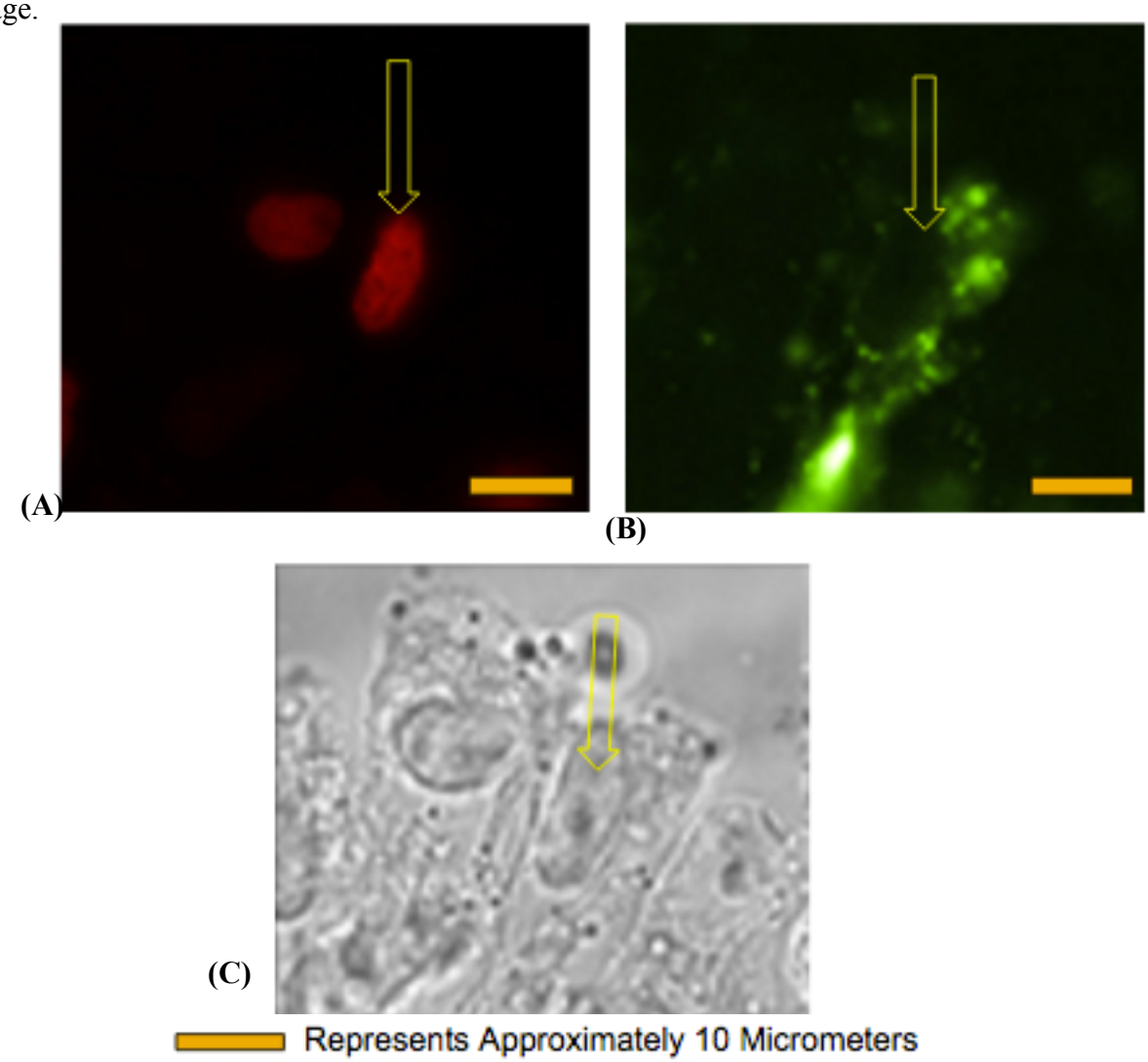
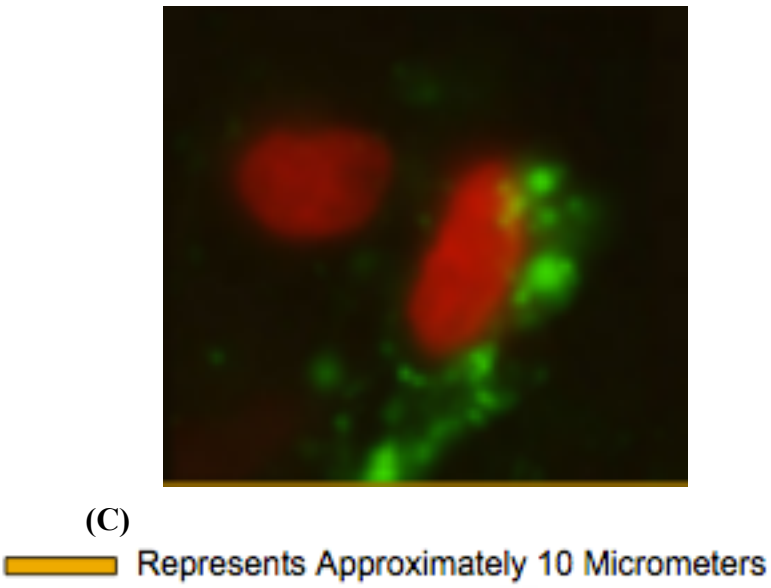


Figure 6: Fluorescent Tagging. (A) Nuclear RFP insertion (B) Exosome GFP insertion (C) White light image.

Zeiss microscopy allows for a greater magnification as compared to the Leica microscope. Figure 6 depicts the same area with RFP, GFP, and no fluorescence. The exosomes appear outside of the nucleus. These images further confirm the location of the exosomes and vector success.

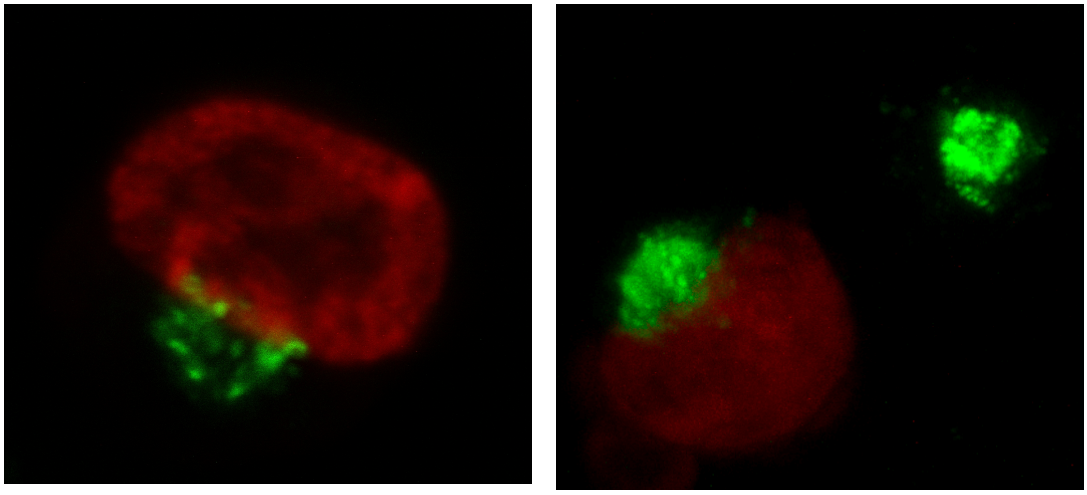


The images are then overlaid to more clearly show the location of the exosomes. The overlay depicts some overlap of the nucleus and exosomes but this is due to the three dimensional structure of the cell. The exosomes are not in the nucleus but they appear to based on imaging constraints.

2.8.3 Confocal Microscopy

Images taken using confocal microscopy. These images are at a significantly higher magnification than the prior images. The confocal microscope allowed for 0.5 micron cuts along the z-axis plane within the sample. The difficulty with the confocal imaging and Zeiss is the

bleaching that occurred. Bleaching means the loss or minimization of fluorescence and it occurs when the cells are fixed on the coverslips and undergo imaging. The fixation of cells is necessary to image the sample but the bleaching minimizes the amount of time and areas available for imaging.



(A) **(B)**

Figure 8: Confocal Images of RFP inserted nucleus and GFP inserted exosomes

The images in Figure 8 depict the much higher microscopic power of the confocal microscope. Image (A) depicts the individual cell nucleus with RFP fluorescence overlaid with the GFP fluorescent exosomes. The exosomes appear below and over the nucleus however, cells and their components are three-dimensional and the location along the z-axis can cause the appearance of overlap.

2.9 Discussion

The insertion of fluorescence into exosomes was successful. The use of GFP and RFP in HEK293 proved to be a working combination when imaged with each microscope. The limitation we faced was using only fixed cells for imaging with the Zeiss and Confocal. If it was possible to image the live cells, we could have gleaned more information about the exosomes

and their movement within the cell and between cells as the Leica could not provide that level of detail.

The Zeiss and Confocal microscope gave greater insight into the location of exosomes, particularly the confocal because of its ability to stack the images to form three-dimensional images in video form. We were able to use the software with the images we obtained to have a greater visualization of the nucleus and exosome locations. The Zeiss and Confocal microscopes are in the Biology Department, which created some difficulty for use of both devices as extensive scheduling and training was necessary for their use. We were very fortunate to access the machines at all but it would have been preferable to spend more time imaging the cells to analyze the insertion further.

The exact location of the modification on or in the exosome cannot be known solely through imaging itself. Microscopes are not strong enough to view the surface proteins of exosomes. In order to evaluate the exact location of modification, other methods must be used such as antibody beads, which would link to the area of modification and provide greater validation of accuracy. If we had a greater amount of time with this project we could employ various forms of validation methods to identify the location of the modification.

Chapter 3: Conclusions

3.1 Summary and Conclusion

Exosomal engineering has not been studied extensively but is quickly gaining more attention due to its potential in drug delivery, particularly its potential to target diseased cells such as cancer. The current options for drug therapeutics overwhelmingly face issues with delivery to the correct place with in the body and entrance into the cell. The pharmaceutical or drug that is being delivered may have a high potential to kill toxic cells or target a disease but due to the lack of deliverability the pharmaceutical is useless. Exosomes have the potential to overcome issues with deliverability as they are natural vesicles that travel throughout the body.

To better gauge the potential for exosomes they must be modifiable. The exosome must be able to express or maintain a modification because modifications or engineering is required for their use in therapeutics, prior to carrying a pharmaceutical. In this project we used fluorescent proteins to gauge initial modifiability. The GFP insertion proved successful throughout the images we took on all microscopes. The areas outside of the nucleus fluoresced green which indicated that the correct vesicles were targeted. The exosomes tend to clump and because they are small in size the images could not depict each exosome individually but the groups. This project indicated that surface level expression modification does work.

The next requirement of our design is that the exosomes must be human compatible. Exosomes are naturally forming and cell derived vesicles, by nature they should be human compatible. Much of the research conducted on exosomes is done using bacteria, not creating the standard of potential viability in humans. Mammalian cells are particularly different from bacterial cells and can be much more complex. For this reason, research conducted with exosomes should begin with human cell lines if the goal is to use it in humans later on. The research that we conducted used HEK293 cells or Human Embryonic Kidney cells throughout the project so that we focused solely on human exosomes within human cells.

In order to evaluate the success of modification within human cells, the modifications must be visualized. The best way for us to evaluate the modification and visualize it was to utilize fluorescent proteins. The success or failure of the modification would be visible using fluorescent microscopy. Each form of microscopy confirmed that the modification did occur and in the correct area. Incorrect modification would have produced fluorescence in the wrong areas or there would be an absence of fluorescence. Using three different types of microscopes with different magnifications allowed for further confirmation of modifiability. The Leica microscope served as the starting point for analyzing the cells. If there was no fluorescence with the Leica, we would have revisited the materials and protocol. The initial success prompted cell fixation for use of the Zeiss and Confocal microscopes. Both of these microscopes depicted the success of the modification as the nucleus and exosomal areas were in the proper areas.

The exosomes must be able to maintain their modifications and maintain their functionality. Engineering exosomes should not result in a loss of capacity or ability to move between cells and the body as a whole. One of the first indicators of loss of function would be death and the exosomes would not fluoresce but with the scope of our project there is nothing direct we could do to determine their functionality after modification. The functionality of the exosome is an area that should be considered with follow on testing.

This modification and imaging of the project proved promising, particularly with the use of high power microscopes even with the issue of bleaching. While cells were in living and fixed conditions, they depicted the modifications both of the nucleus and the exosomes.

3.2 Project Future

This initial project was successful which prompts the need for further investigation into the specific surface expression capacity of exosomes and their overarching ability for drug delivery. The future of this project should begin with analyzing the surface expression of exosomes. It should continue with a variation of modifications on CD63, applying these techniques to new membrane proteins.

The first step to carry on this project is to verify the exact location of the GFP on the cell. The project time did not allow us to verify the exact location, we were able to verify that the GFP was in the exosomes but not the exact location on the membrane. If the GFP is not located on the C Terminus, other techniques will need to be employed to rectify it. In addition, the CD63 tetraspanin could be modified to better take on GFP or other proteins. The CD63 could be altered from the M shape it is currently to an N shape, potentially serving as an easier target for protein attachment. Many other types of proteins could be used, particularly those proteins specific to certain types of cells. Due to the size of exosomes, they are able to cross the blood brain barrier, a cell type to target would be neural cells. A protein that is key to the surface of neural cell attachment is NCAM or neural cell adhesion molecule which is a homophilic glycoprotein. NCAM is an example of molecules specific to certain cell types. Looking further into exosome targeting for specific cells could provide a greater amount of information on the potential of exosomes to carry pharmaceuticals to various parts of the body.

Other follow on research could be conducted on the functionality of exosomes after modification. If they cannot maintain their biological integrity after modification of surface expression or attachment of tags, they lose their purpose. Drug delivery using exosomes relies on their functionality and capacity to deliver cargo within cells and ability to travel between cells. Further, functionality can be tested with an addition of a pharmaceutical within the exosome to deliver to specific cells. The exosome should be able to deliver the drug and maintain its functionality afterward. Toxicity of delivery can be tested and analyzed alongside this type of testing as well. Surface modifications and drug delivery can change potentially change the chemistry of exosomes and may lead to a change in toxicity of exosomes.

3.3 Lessons Learned

This project taught us a great deal about the importance of quality images from microscopy and scheduling. The Leica microscope proved to be a difficult microscope due to its limited magnification strength and the amount of other users. The Zeiss and Confocal microscopes were

far superior to the Leica but much harder to access because they are located in the Biology Department. In order to use the Zeiss and Confocal microscopes we were required to be trained on the microscopes but it was difficult to combine each person's schedule. It was not until late into the timeline that we were able to use the microscopes. If we were able to improve our scheduling capacity we would have been able to enter in earlier and potentially take more images of the cells.

Chapter 4: Engineering Standards

4.1 Ethical Considerations

The success of any project or research relies on the maintenance of ethical standards throughout the project and after. As representatives of Santa Clara University and as future members of the scientific and engineering communities, it is our duty to uphold these standards. The foundation of ethical conduct is honesty and integrity. In addition, Santa Clara University has its values that we must honor, the three C's: conscience, competence, and compassion. These values, along with honesty and integrity, are the framework in which to conduct our research. Conscience is the first value, it requires us to identify and address the social issues or concerns affiliated with this type of project. The second value, competence, requires us to truly understand the subject matter of our research and how to conduct it. The third, compassion, is the basis of the project, as we are working to improve treatments for cancer, in order to improve the lives of patients. Each stage of the project and interactions among ourselves and with our advisors must adhere to these values.

A comprehensive analysis of the ethics of our project requires us to consider the legal, environment, safety, manufacturability, and social concerns of our research.

4.2 Legal Considerations

The legal considerations of this project are minimal. Legal concerns encompass patent or intellectual property right infringement, additionally they should address the potential rights of this project. This system currently is entirely experimental with the data to be published within the scientific community. This project is academic with the goal to provide baseline research for the potential of exosomes to drug delivery systems.

This stage of the project would not apply to any intellectual property or patenting concerns as it not at the level for corporate application or interests. If this project continues further, particularly if the research reflected successful engineering of the outside of the exosome and a biomaterial or therapeutic insertion, this would be the point for patent application. The current state of this project is not applicable for this level of legal concern but it is a possibility in the future of this project.

Additionally, business ties are not relevant to this project as there is no corporate involvement. The sole organization involved in this project is Santa Clara University, allowing us the freedom to research in an academic environment. The university may hold interest in publications but those provide benefits to the university, professor, and students but the university would not have an interest past that point.

4.3 Environmental Considerations

For any type of laboratory research the largest environmental concern is biohazardous waste being disposed of properly. The materials used have a minimal impact on the environment. The disposal methods the Bioengineering Department are in line with safety requirements mitigating the risk. The use and disposal of any biologics can pose a threat to environmental safety but this project did not utilize any toxins or pollutants, removing a direct environmental concern. However, being mindful of the correct disposal areas was necessary to prevent any negative effect on the environment. Additionally, we minimized our waste by reducing and reusing the materials and tools we used.

4.4 Health and Safety Considerations

Protecting the health and safety of those working on and around the project is paramount. Prior to entering the lab we completed the lab safety training courses conducted by Santa Clara University. These courses gave us the basic knowledge for proper lab conduct but we were given

further information from our advisor who walked us through the lab space. Throughout the project all lab protocol and conduct was honored, including protective clothing, gloves, and eye protection. The hood and ethanol was used in conjunction in order to prevent contamination or health concerns for the lab space.

4.5 Manufacturability

For any device or biologic, manufacturability must be considered to predict its capacity for scalable production. The exosome drug delivery system has the capacity to be manufactured in a lab setting. The desired function of the exosome system can be specifically engineered based on the drugs inserted and tagging on the surface for location targeting. The specific disease target system could be expanded by a pharmaceutical level company and then used for various patients. Such as any drug in the early stages, manufacturability and scaled production is far down the road but this system would allow for manufacturing at some stage in its development. A product or device with limited manufacturability forces the price to sky rocket or can majorly limit its accessibility.

4.6 Social Considerations

The success of a targeted drug delivery system would greatly improve the treatment of diseases in populations across the world. However, social considerations apply to this project and type of research. The first consideration is the materials used for research. The use of human embryonic kidney cells could be a point of consideration because the use of any type of embryonic cell is still a political and social issue in the United States. Each material or cell line used for research must be fully understood and be the most robust choice for research due to the issues surrounding cell research. If there are less contentious options with the same degree of robustness, those should be used. Once the research can project this system closer to clinical testing considerations should be made about the use of animal models. Minimizing the amount of animals used but maintaining proper data collection is a necessary consideration. Once in patient

testing, the system must not cause damage or harm to the patient and be used only with patients who can give informed consent. Another major social consideration is the research cost and cost of the therapy upon introduction to the market. The development of disease treatment systems can cost an exorbitant amount, therefore the costs should be minimized and monetary funding used honestly. The next social monetary consideration is the cost of the system when introduced to market. Patients in need should be able to access it while compensating the parties involved in its development. The success of this system has the potential to treat various diseases, improving the quality of life and life expectancy for many people.

Appendix A- References

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Appendix B- Protocol

B.1 Cell Culture

Preparing Medium

Prepare the complete D-MEM medium containing 10% FBS supplemented with 0.1 mM MEM Non-Essential Amino Acids, 1 mM sodium pyruvate and 2 mM Lglutamine

1. Remove 1/10 of D-MEM and replace amount with 50 mL FBS.
2. Also add:
   - L-Glutamine
   - Non-Essential Amino Acids (mix)
   - Sodium Pyruvate
3. Sterilize the medium
4. Store at 4°C

Thawing

1. Remove the vial of frozen cells from liquid nitrogen and thaw quickly in a 37°C water bath.
2. Decontaminate with ethanol solution and transfer to sterile tube with PBS. Then centrifuge and resuspend.
3. Transfer cells to flask containing complete medium.
4. Incubate flask.
5. The next day aspirate off medium and replace with fresh medium.
6. Incubate cells and check daily for 80-90% cell adherence.

Freezing

1. Freeze cells at 3 x 106 viable cells/mL density
2. Prepare medium with 10% DMSO. Should be done immediately prior to use.
3. Culture desired quantity to 70-90% adherence.
4. Remove cells from tissue culture flask(s).
5. Centrifuge the cells suspension, remove medium and resuspend cells in the pre-determined volume of chilled freezing medium.
6. Dispense aliquots of suspension into cryovials.
7. Freeze cells in apparatus with temperature decrease of 1°C per minute.
8. Transfer vials to liquid nitrogen storage

B.2 Transfection

Check the confluency of your cells. They should be ~75-90% when transfecting

Preparing your materials:

1. Pre-heat cell media (dMEM+10%FBS)
2. After checking cell confluency, wipe down the under surface of your cell plate with 70% EtOH on a kimwipe and place in hood
3. Turn on the aspirator and spray EtOH on the tubing that will be inside the hood
4. Obtain proper pipettemans and pipette tips. Sterilize with EtOH
5. Obtain three 1.5mL centrifuge tubes from your instructor
6. Sterilize hands with EtOH before putting in hood.

Transfections

1. Label your 1.5mL tubes for the transfection reactions you calculated
2. Add media to the tubes and then add the calculated amount of DNA. Mix thoroughly by pipetting
3. Add the proper amount of Fugene6 DIRECTLY to the reaction without touching the sides of the tubes.
4. Incubate for 10 minutes at RT inside the hood
5. Aspirate off old media from the cells and replace with 2mL of new media. Add media to the side of the plate while tipping to prevent detachment of the cells
6. Add the transfection reaction mixture dropwise to each well
7. Tilt the plate back and forth to distribute the mixture evenly
8. Put the plate in the 37C and check back at 24, 48, 72 hours to take fluorescent pictures

B.3 Cell Fixation

1. Culture cells by adding 500 µL of culture media containing approximately 5000 cells to the wells of a cell culture plate containing gelatin-coated coverslips.
2. When cells have reached the desired density/age, remove the culture media from each well and wash twice with PBS.
3. Add 300-400 µL of 2-4% Formaldehyde Fixative Solution to each well, and incubate for 20 minutes at room temperature.
   Note: Some cell types can be damaged by the change in surface tension that occurs when the culture medium is entirely removed and replaced with wash buffer. If this is the case, pre-fix the cells by adding 500 µL of 4% Formaldehyde Fixative Solution directly into the culture medium. After 2 minutes, replace the pre-fixation culture medium with 300-400 µL of 2% Formaldehyde Fixative Solution and incubate, for 20 minutes at room temperature.
4. Wash the wells twice with PBS and cover with 400 µL of wash buffer. The coverslips can be stored at 2-8 °C for up to 3 months or they may be stained immediately.
   Note: Fixation can result in hydrophobic cross-linking of tissue proteins. The time, temperature, pH, and fixative used will determine the degree of cross-linking. Once the fixation protocol has been optimized, the same procedure should be used consistently.

Appendix C- Project Management

C.1 Budget

Table B.1.1: Anticipated Budget

Item	Count	Cost ($)
Cell Line	1	400
Vectors	2	400
GFP RFP Genes	1	500
Cell Culture Media	1	400
Potential Necessary materials	1	350
		2,050

Appendix D- Senior Design Slides

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Exosomal Protein Engineering and Imaging

Joseph Losacco, Sophie McDevitt, and Zac Stickney

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Overview

- Background
- Experimental Goals
- Design
- Images
- Moving Forward

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Cancer in the United States

- Cancer is a leading cause of death
  - Over 590,000 deaths expected in 2016
- Limited success for chemotherapy and radiation
- Potential for protein drugs

American Cancer Society®

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Current Therapies

- Surgery
  - Complete removal of tumors
- Chemotherapy and Radiation
  - Degradation of cancerous and healthy cells

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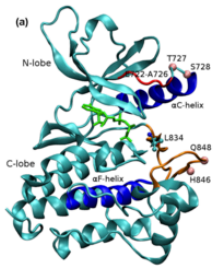
Developing Therapies

- Protein Drugs
  - EGFR
- Immunotherapy
  - Modified T Cells to attack tumors



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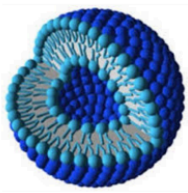
Subcellular Drug Delivery

- Liposomes
  - Lipid or aqueous based delivery
- Nanoparticles
  - Large binding surface capacity
- Exosomes



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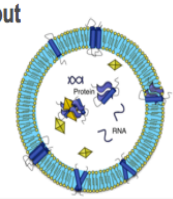
Exosomes

- Cell derived nano-vesicles
- Naturally forming in the body
- Transport various molecules throughout the cell and with other cells
  - High tissue penetrability



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Exosome Potential

- Communicative nature
  - Transportation
- Size
  - Blood-brain barrier relationship
- Potential to 'tag'
- Application to multiple diseases

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Design Process

- Modifiable
- Human Compatible
- Visualizable
- Maintain Functionality

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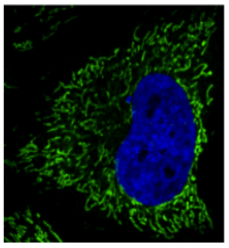
HEK 293

- Transfectability
- High growth rate
- Ease of culture



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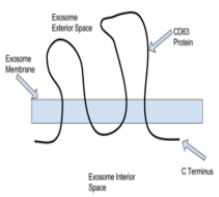
CD63

- Surface protein
- Viable for surface display
- Potential use as a scaffold with transmembrane domains



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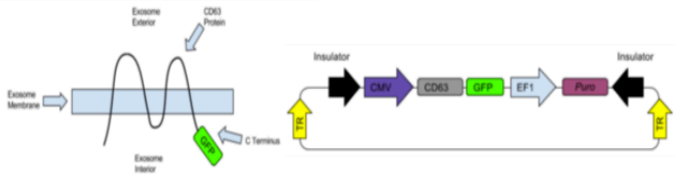
Engineered Cell Line

- CD63 with Fluorescent protein at C-terminus



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Types of Imaging

- **Live Cell Imaging**
  - Visualize cells in real time
- **Fixed cellular Imaging**
  - Fix cells onto coverslips using 2% Paraformaldehyde

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Analysis of our Imaging Platforms

- **Leica DMI 3000 20x Inverted Scope**
  - Focal Length
  - Low Magnification
- **40x Zeiss Axioscope Fluorescence Microscope**
  - Small Focal Length
  - Exclusively Fixed cells
- **Confocal Microscope 63x**
  - Ability to planar images
  - Ability to use live or fixed cells

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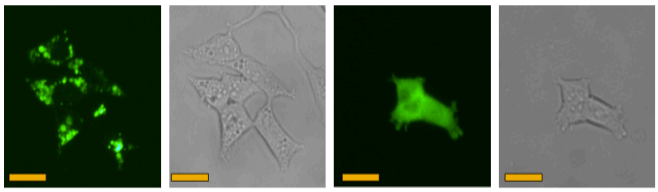
Leica Inverted Scope
Exosomal vs Cytosolic Protein Tagging



Under Argon Laser | Under White light | Under Argon Laser | Under White light

Exosomal GFP Tagged CD63 | **Cytosolic GFP**

Represents Approximately 10 Micrometers

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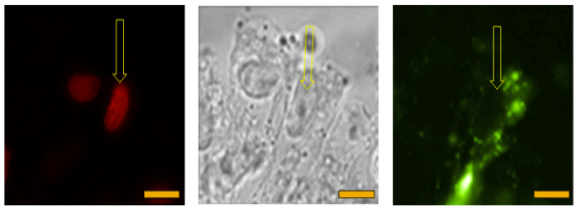
Zeiss Axioscope Fluorescence Microscope
Exosomal and Nuclear Protein Tagging



RFP Tagged Nuclear Protein | White Light | GFP Tagged Exosomal Protein

Represents Approximately 10 Micrometers

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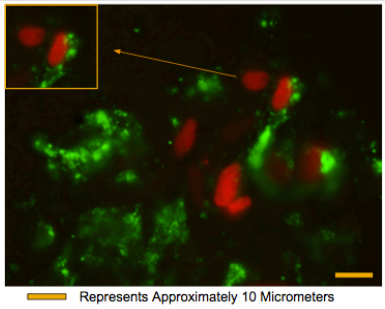

Represents Approximately 10 Micrometers

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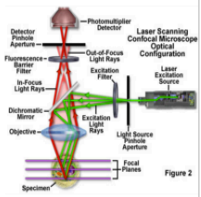
Confocal Microscope

- High magnification and resolution
- Ability to dissect the cell in a series
- Ability to create 3 dimensional images
- Enhanced visualization of exosomes and multivesicular bodies



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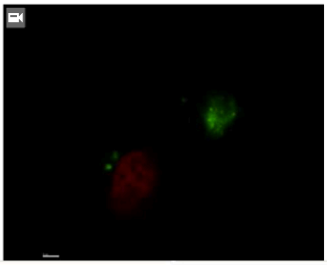
Confocal: Imaging Live cells



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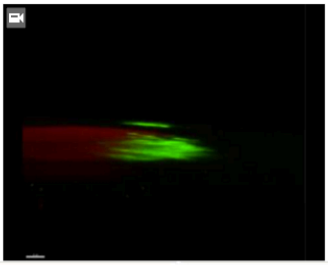
Confocal: 3D Model



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Next Steps

- Evaluate the advantage of these different scaffolds compared to CD63
- Explore new modifications of tetraspanin CD63
- Transfer the techniques applied to this novel display system to a new membrane proteins

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