High-Throughput Cell Penetration Assay for the Discovery of Cell Penetrating Cyclic Peptides

by

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Cell-penetrating cyclic peptides (CPPs) are a promising tool in the delivery of therapeutic drugs to cells[1] and can act as therapeutics themselves. These therapeutic agents are aimed at the treatment of diseases such as HIV, cancer, and multidrug-resistant bacterial diseases. Their large surface area and higher conformational rigidity and stability relative to their linear counterparts[2] potentiate them to have a higher affinity and specificity for any target of interest. Additionally, CPPs have higher resistance to degradation, unlike their linear counterparts. Thus, for innovative developments in the peptide therapeutics field, it is paramount to have tools for studying and accelerating the discovery of CPPs. In this project, we aim to unravel the underlying rules of cell permeation of cyclic peptides and develop novel high-throughput methods for screening their cell penetration. Additionally, we aim to develop computational methods to predict the permeability of cyclic peptides based on their sequence. Targeting specific organelles can mitigate side effects and toxicity of the drug to cells. Understanding the core features of cell-penetrating peptides will allow for great advancements in therapeutics and the development of less invasive oral bioavailable treatments.

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Table of Contents

List of Figures

List of Tables

Introduction

Cell-penetrating peptides (CPPs) are composed of short chain amino acids and can be a tool to carry therapeutic drugs across cell membranes[3]. They have the ability to enhance the permeation of large biomolecules, which makes them promising for drug delivery. Additionally, some of these CPPs can carry out site-specific delivery of their cargo to organelles within the cell[4]. The ability to both permeate the cell and direct cargo to the target is especially exciting for therapeutic drug developments. Cell membranes are made up of phospholipid bilayers and membrane proteins, cholesterol, and sugars depending on the type of cell[5]. In addition, some cells have a cell wall or two bilayers. The amphipathic phospholipid bilayers layers stop surrounding molecules from entering the cell, which leaves only small, hydrophobic molecules capable of permeation by membrane diffusion capable. Larger or hydrophilic molecules therefore should pass through selective permeation via transmembrane proteins and ion channels, or particle engulfment mechanisms like endocytosis and pinocytosis. This project aims to test the capacity of some well-known CPPs on E.coli cells, as a gram-negative representative bacteria, and assess the possibility of using large libraries of peptides for discovery of novel CPPs in a high-throughput way.

There has been a breadth of research done on linear peptides; however, cyclic peptides are even more promising into this field as they are more stable and more hydrophobic due to their lack of reactive termini. Linear CPPs tend to be broken down by the proteolytic enzymes before they can make it through the cell membrane, and their additional H-bond acceptors and donors at termini make it less soluble in hydrophobic environment of the membrane. Current research has shown that many CPPs are mostly composed of basic amino acids (e.g. Arginine and lysine). It is proposed that the partial negative charge on the outer membrane absorbs the peptide, which is then able to penetrate into the membrane[6].

CPPs can be effective at killing bacteria, viruses, and fungi. There are many naturally occurring peptides that make up an immune system and target harmful pathogens[7]. Antibiotics are helpful in fighting these pathogens, but they have been grossly overused which has led to the development of antibiotic-resistant bacteria[8]. This has led to a crisis in the pharmaceutical industry and there are insufficient tools to develop new antibiotics as fast as the bacteria has mutated and become resistant. Discovering more CPPs that have antimicrobial activity will help address this crisis and aid in combating infectious pathogens. CPPs have the ability to both be used as an antimicrobial agent itself and also as a cargo delivery tool which is especially helpful for uses in cell transformation.

There has been an emergence of the use of peptide libraries, such as cDNA display and mRNA display[9,10]. They are *in vitro* screening techniques which can screen trillions of peptides for their binding affinity against a specific target[11]. Our research will use mRNA display libraries in a revolutionary way. We will use this screening tool to discover novel CPPs and scale up the membrane penetration assays. The existing methodology for discovering cell penetration is low throughput and requires each peptide to be tested individually.

Permeability assays are currently limited to using 96-well plates and screening using mass spectrometry or ultraviolet (UV) absorption. This methodology limits the number of peptides that can be screened at once. Our research investigates whether we can use mRNA display technology to develop a high-throughput permeability assay for the discovery of cyclic CPPs. mRNA display technology is a technology used to create diverse peptide libraries *in vitro*. The peptide libraries can be directed to create peptides with specific features or can be randomized. Theoretically, during the incubation of cells with mRNA library, CPPs will penetrate the cell, while non-penetrating peptides will remain in the buffer. Therefore, after separating cells from the buffer, we should be able to retrieve DNAs attached to peptides and find out which peptides have penetrated the cells. However, we first need to establish the assay using single peptides and confirm positive and negative controls for it. This project aims to test a few peptides inspired by previously reported CPPs and assess the possibility of using them as positive and negative controls for the proposed assay.

The existing research hasn't yet introduced a high-throughput assays for testing the cell penetration of cyclic peptides. They use methodology that limits the testing to a smaller scale, requiring ample time and resources for testing the cell-penetration of cyclic peptides. This makes it an exciting and innovational project to take on, and one that we are still in the process of developing adequate technology to perform.

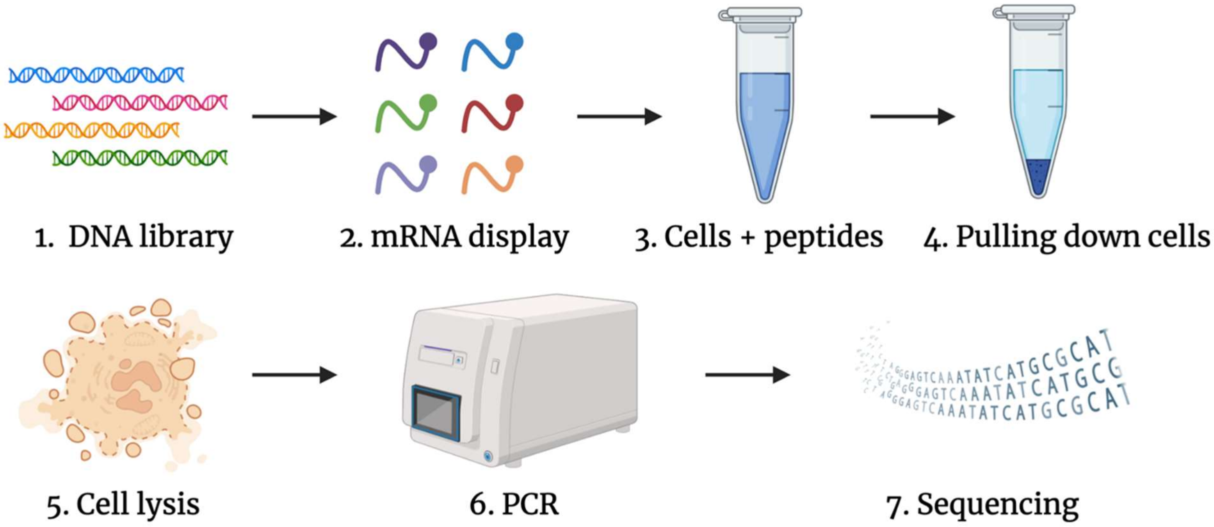


Figure 1: A simplified general workflow

We will design a DNA library and use mRNA display to create and cyclize the peptides. Then we will add the cells and peptides together and pull them down using centrifugation. The cells will be lysed and then PCR will be done in order to amplify the peptides within the lysed cells. Sequencing will be performed to determine the peptides that are penetrating in the cell versus the peptides that remain outside the cell.

Broader Implications

Developing a high-throughput assay for the discovery of cell-penetrating cyclic peptides will increase the efficiency of testing peptides for their use as antimicrobial agents and cargo delivery tools . If we can develop a successful methodology for this assay, the discovery of specific peptides will be streamlined, and their potential for use in antibiotics will be revealed. These peptides can further be attached to peptides binding a specific target on cell surface, to guide the CPPs to cell type of interest[12]. Additionally, being able to test cyclic CPPs with high-throughput methodology will allow for a greater understanding of the rules of penetration into the cell membranes, which will allow further advancements in rational design of cell penetrating molecules.

Experimental

Materials

Peptides

Name	Sequence	Cyclization Type	Permeable
L1	WWRRWWRR	Linear	No
C1	WWRRWWRR	Head-to-tail	Yes
L1-K	WWRRWWRK	Linear	No
C1-K	WWRRWWRK	DSG	Yes
L-A7K	AAAAAAAK	Linear	No
C-A7K	AAAAAAAK	DSG	NO

Table 1. List of Peptides and cyclization type.

DSG is a chemical crosslinker that is used to cyclize peptides in mRNA display. Lysine (K) is added to the end for DSG.

Cargo

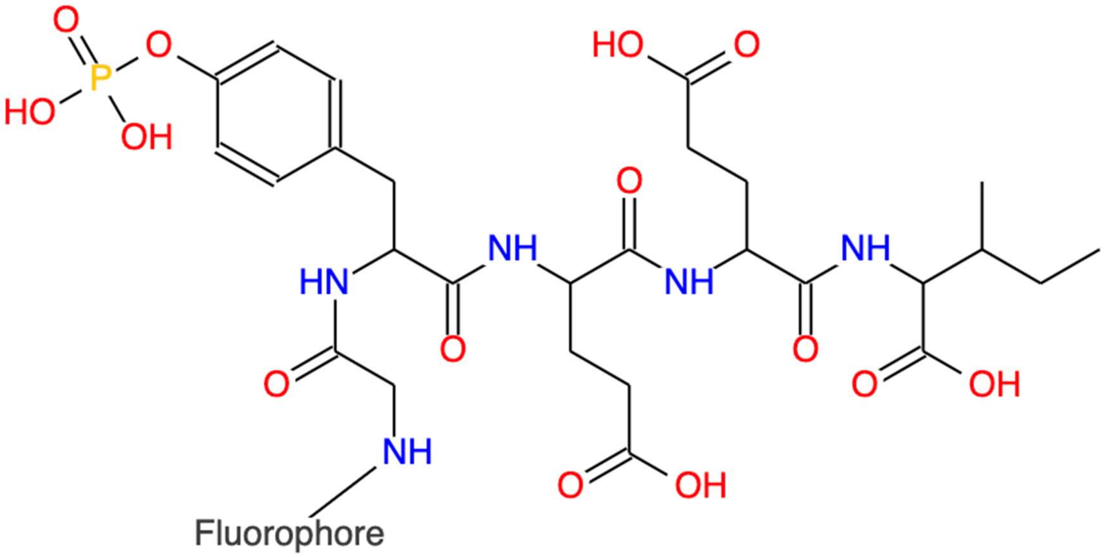


Figure 2. GpYEEI molecule attached to fluorophore.

For E. Coli cells fluorophore was fluorescein. For Yeast Cells fluorophore was Texas Red as yeast has autofluorescence in the same range as fluorescein.

Methods

Project Overview

We started by finding positive and negative control candidates for cell-penetrating cyclic peptides by a literature review to test whether the assay will work. We wanted to use 8mers specifically and found a positive control that alternates arginine and tryptophan residues that has worked for cellular delivery on several cell types[13]. Our negative control consists of a alanine, a hydrophobic molecule which will not penetrate the cell on its own. A phospopeptide attached to fluorophore is used as the cargo in these experiments. This phosphopeptide had been previously used by Kim et al. in their research on molecular cargo delivery.

Peptide Synthesis

Peptides were synthesized by WuXi Tides using Solid Phase Peptide Synthesis (SPPS).

MALDI-TOF

Matrix-assisted laser desorption/ionization (MALDI) is a mass spectrometer imaging technique. We used MALDI-TOF (time of flight), a specific type of MALDI that separates molecules based on their mass to charge ratio, to confirm that the experimentally validated masses of the peptides were consistent with the expected molecular weight of the peptides[14]. The corresponding figures are in the results section.

Spectral Flow Cytometry

A Spectral Flow Cytometer is a machine that uses light lasers to detect fluorescent signals to analyze cells in a solution. The Cytek Aurora spectral flow cytometer has five lasers, three FSC/SSC detectors, and 64 fluorophore detectors that allow it to detect many different fluorescent dyes[15]. We use the flow cytometer to determine a cell count of our samples and to show the fluorescent signal that is received within the cells from our fluorescent dyes. If the peptide has allowed for the fluorophore to enter the cell, then we will have a fluorescent signal, thus allowing us to determine whether the peptides are cell penetrating.

Yeast and E. Coli Cell Count Protocols

A series of experiments was conducted to optimize cell growth for flow cytometry to make sure the cells were viable and at a sufficient concentration. We transfer a single colony from an agar plate to 5 mL of Luria-Burtani Broth (LB) for E. Coli cells and 5 mL of Yeast Peptone Dextrose (YPD) for yeast cells. The tubes with E. Coli cells are placed in a 37-degree Celsius shaking incubator. The tubes with yeast cells are placed in a 30-degree Celsius shaking incubator. For E. Coli cells we start the growth at 9 am the day before we want to do Flow Cytometry and for Yeast Cells, we start at 5 pm. At 5 pm we transfer 1 μL of the E. Coli media into 5 mL of fresh LB and place back in shaking incubator. The cells are left to grow overnight, or approximately 18 hours.

Cellular Uptake Assay

A cellular uptake assay is done to determine which peptides are cell penetrating, and which are not. For testing the control peptides, we use the following protocol. After growing the cells with the appropriate growth protocol for E. Coli, we add the peptide and fluorophore together in centrifuge tubes and incubate at room temperature for 30 minutes. We add 35 μL of peptide, and 7 μL of fluorophore to each centrifuge tube. Then we add 0.5-5 μL of cells and LB to make the total volume 700 μL. For each peptide we also have a control without fluorophore or cells, to ensure the peptide by itself doesn't produce false positive signals. We have four additional controls: one with cells and fluorophore, without any peptide, as our unstained control for unmixing in flow; LB alone, LB with cells, and LB with unwashed fluorophore. Once these tubes are prepared, we wrap them in tinfoil to avoid photobleaching of fluorophore, and place on a Fisherbrand shaker plate at 60 speed for two hours. After two hours we pull down the cells by centrifuging them at 3,000 rpm for 5 minutes. The supernatant is then discarded and replaced with 700 μL PBS. The cells are then resuspended via vortexing. This process is repeated 3 times to wash the remaining fluorophore in solution which hasn't get into the cells. Then we fix the cells by adding 130 μL of 37% formaldehyde to make a X% final solution. The solution is transferred to FACS tubes to do flow cytometry. We run flow cytometry with the acquisition method set to 5 μL for each tube and analyze the signal of FITC channel in singlet cells (marked as B-2).

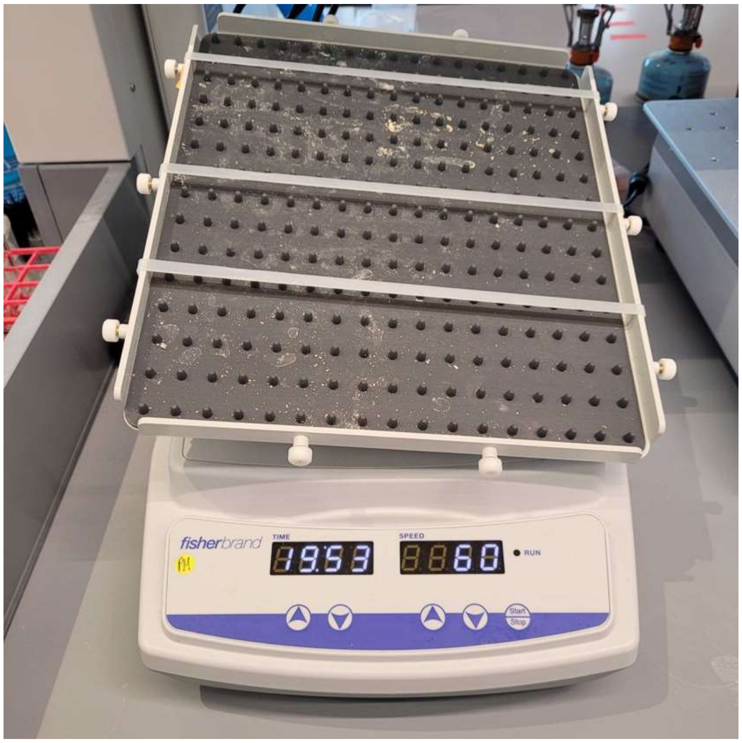


Figure 3: Fisherbrand shaker plate for reference

Results

The development of the high-throughput cell penetration assay is a work in progress that will continue beyond this thesis. Thus far, positive and negative controls have been established, and the protocols for cellular uptake are optimized.

MALDI Results

MALDI-TOF to confirm the purification of peptides. The peptides had experimentally confirmed masses consistent with their molecular weights.

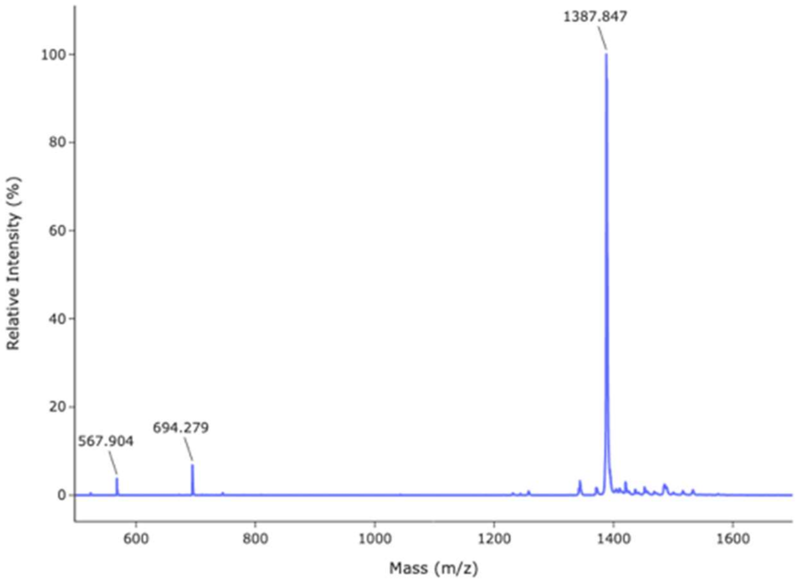


Figure 4: MALDI results L1

Expected molecular weight of 1387.63 g/mol confirmed in MALDI with molecule weight of 1397.847 g/mol shown

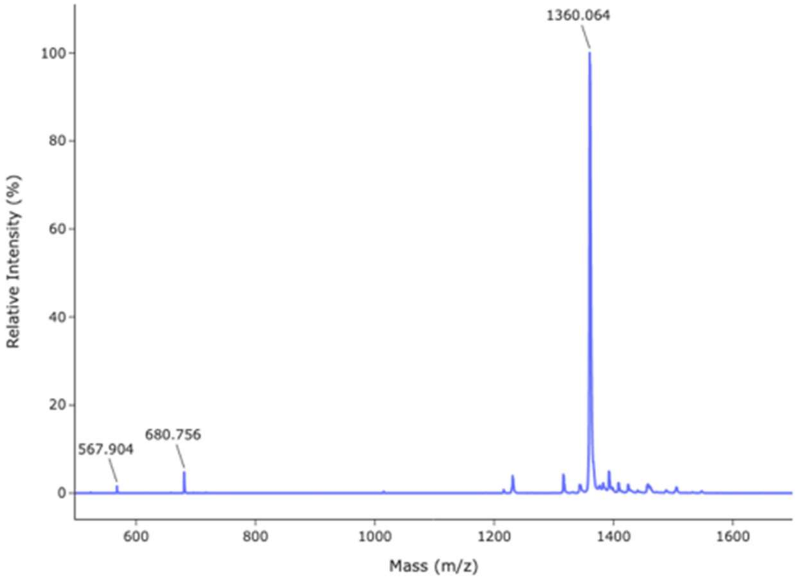


Figure 5: MALDI results L1-K

Expected molecular weight of 1359.61 g/mol confirmed in MALDI with molecule weight of 1360.064 g/mol shown

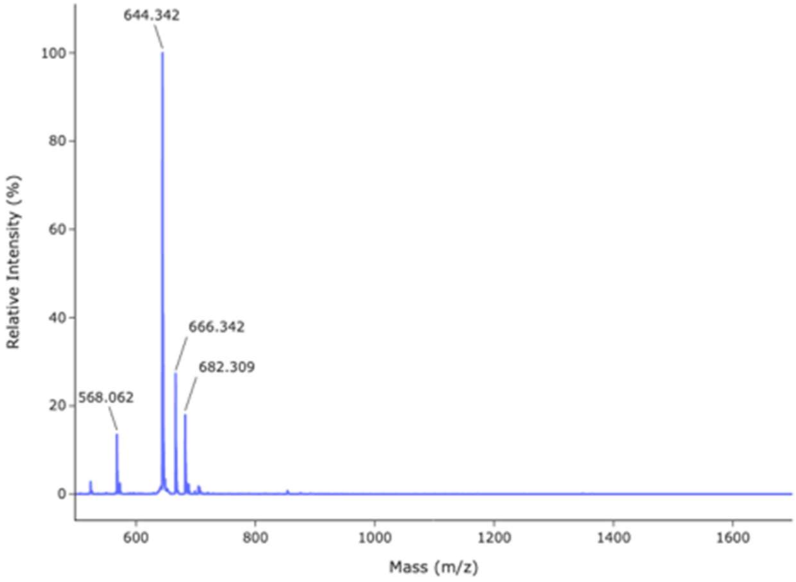


Figure 6: MALDI results LA7-K

Expected molecular weight of 643.74 g/mol confirmed in MALDI with molecule weight of 644.342 g/mol shown

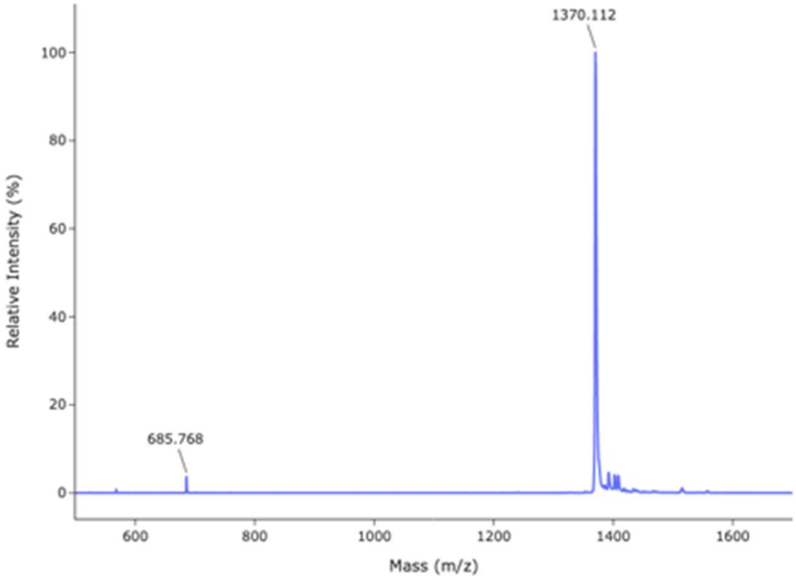


Figure 7: MALDI results C1

Expected molecular weight of 1369.61 g/mol confirmed in MALDI with molecule weight of 1370.112 g/mol shown

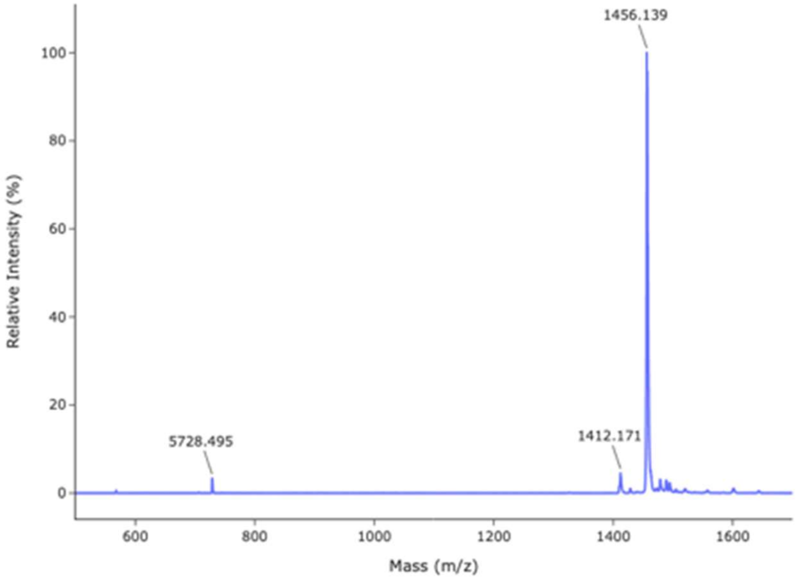


Figure 8: MALDI results C-A7K

Expected molecular weight of 1455.7 g/mol confirmed in MALDI with molecule weight of 1356.139 g/mol shown

Cellular Uptake Results

Flow cytometry has been performed to optimize the protocols for the cellular uptake assay that will be used for future peptide libraries. Throughout the process we have made modifications to the ratios of reagents and amount of time that the solutions sit in order to optimize this procedure.

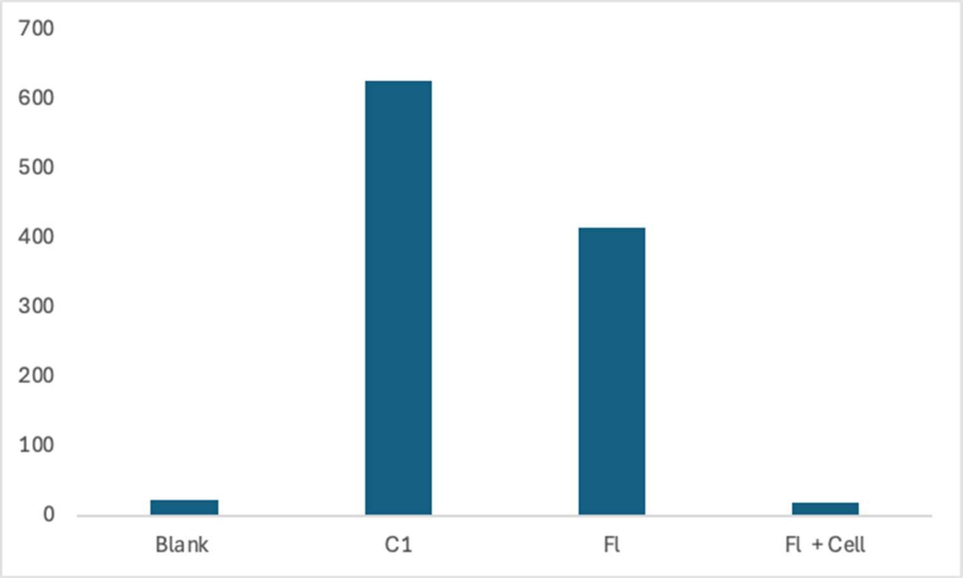


Figure 9: Signal of Phosphopeptide attached to FITC

C1 significantly increases the uptake of fluorophore into the cell. C1 is the selected positive control. Blank has LB and E. Coli cells.

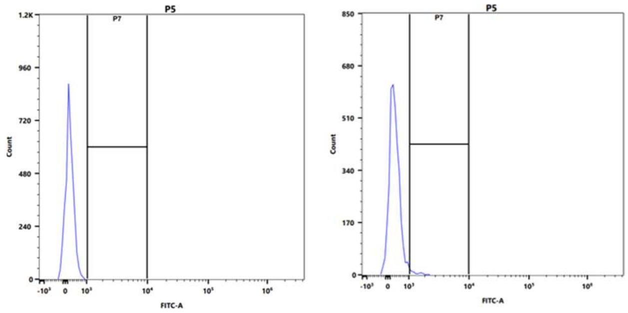


Figure 10: Fluorescent signals with and without peptides

There is high fluorescent signal seen in the presence of C1(right figure), and low fluorescent signal in without peptide present (left). Fluorescent signal determined with flow cytometry.

After determining that C1 was a proper control, we altered the peptide concentration to find the limit of detection in this assay, since the concentration of peptides in mRNA display is in nano molar range. Even though the peptide signal in mRNA display would be largely amplified after PCR, we were still curious to find the least amount of peptide which can produce a detectable signal in fluorophore uptake assay.

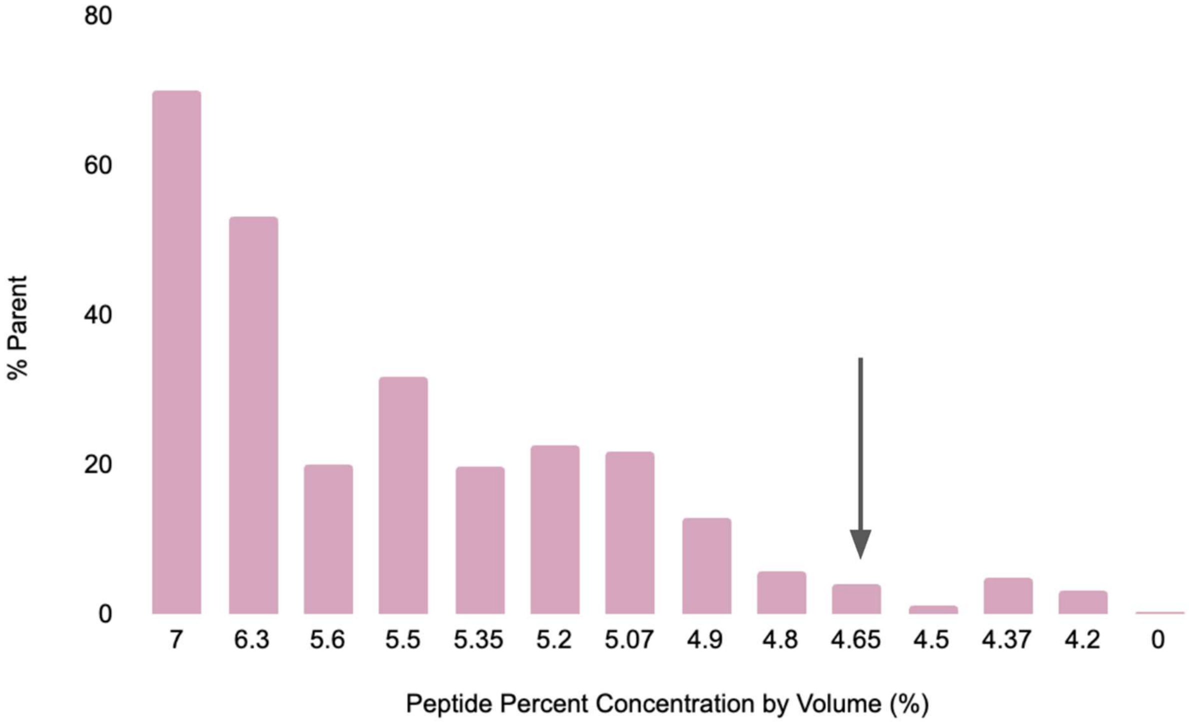


Figure 11: Limit of detection for peptide C1.

We determined that the limit was 4.65 percent of peptide solution.

After finding the limit of detection for peptide C1 we increased the fluorophore concentration to see if that increased signal.

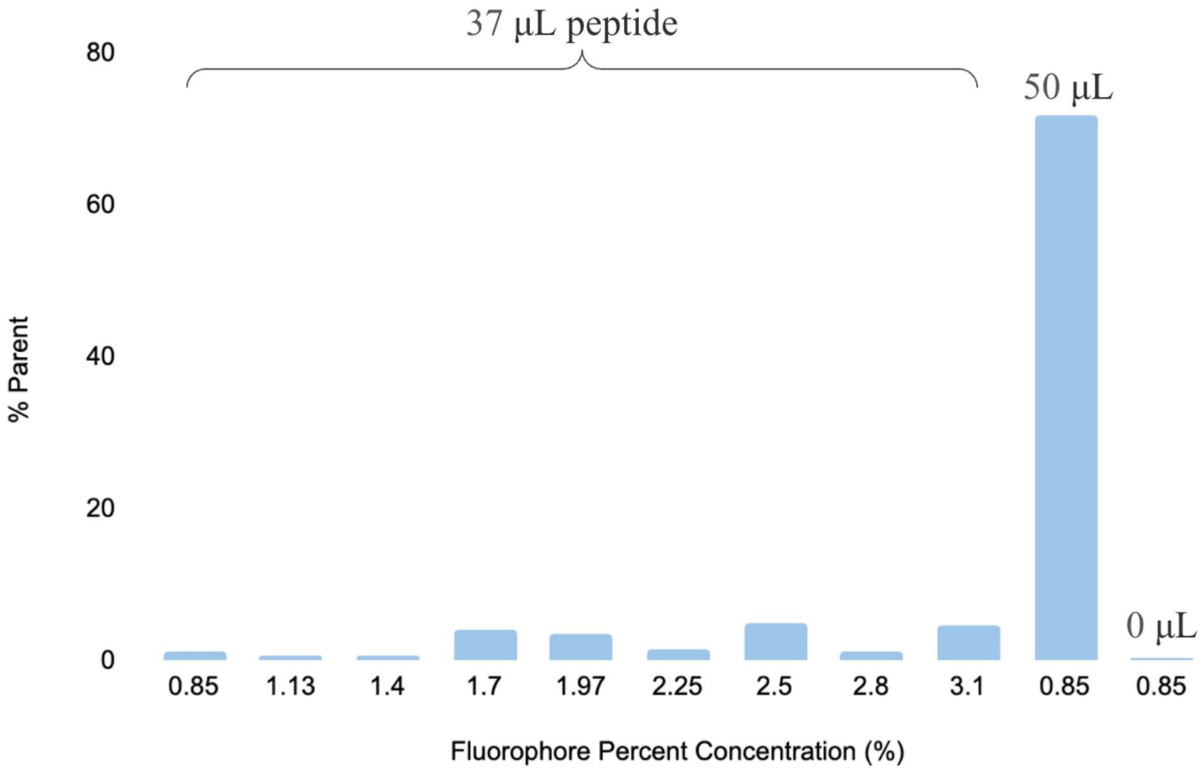
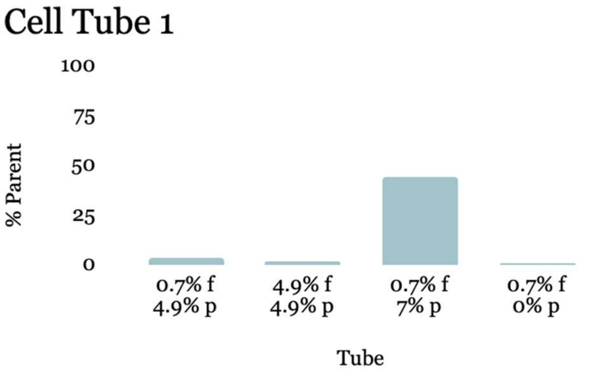
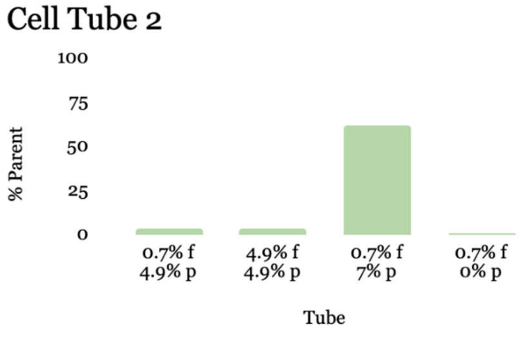


Figure 12: Increasing fluorophore concentration

We found that increasing the fluorophore concentration has no significant effect on the signal seen.





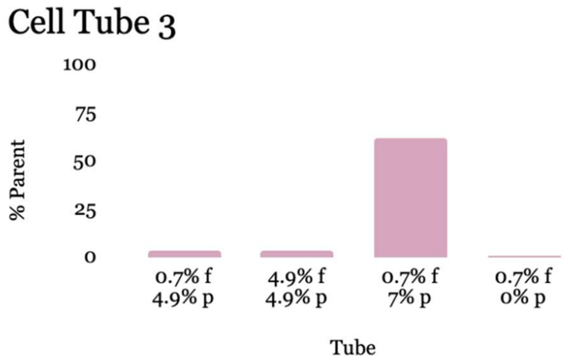
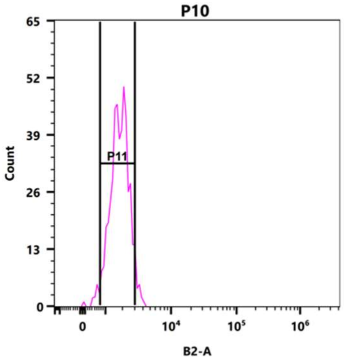


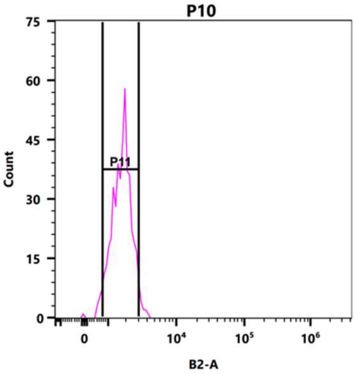
Figure 13: Increasing fluorophore concentration from 0.7% to 4.9% in E. Coli cells

In *E. Coli* increasing the fluorophore concentration does not impact signal, but increasing peptide concentration does.

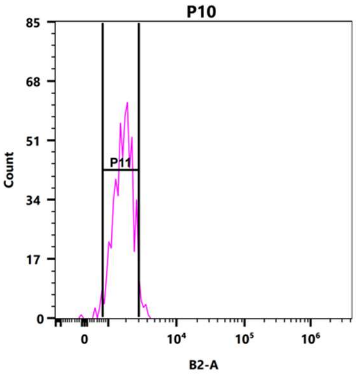
When optimizing the cellular uptake assay for yeast cells, we ran into issues with not finding a difference in signal from the positive and negative controls. After doing some research we found that the autofluorescence of yeast interferes with the FITC signal.



Peptide, Fluorophore, and Cell



Fluorophore and Cell



Cell only

Figure 14: Fluorescent signal of peptide, fluorophore, and cells combined, fluorophore and cells combined, and cell only in yeast cells.

The signal of the different tubes when we do flow cytometry are the same for all three combinations of reagents. For this assay to be functioning properly, we should only see signal when the peptide, fluorophore, and cell are all together as the peptide should make the cell more permeable for the fluorophore. However, because the yeast autofluorescence interferes with the FITC fluorescence, we are unable to tell how much fluorophore has made it into the cell. For future experiments yeast we have ordered a different fluorophore attached to the phosphopeptide (GpYEEI) molecule, called Texas Red that functions under a different channel, however this fluorophore has not arrived thus far, and we have continued the project working with E. Coli for the time being.

After switching back to *E. Coli* cells, we tweaked our cellular uptake assay protocol for efficiency but found that this new protocol failed to show differences between the positive and negative controls as we had seen in the past. We had omitted the step of letting the fluorophore sit with the peptide for 30 minutes and had started growing the peptides the night before, instead of starting them the morning before. We think that the cells were too young and the positive and negative charges on the phosphopeptide and C1 did not have enough time to sit together and attract with the omitted step.

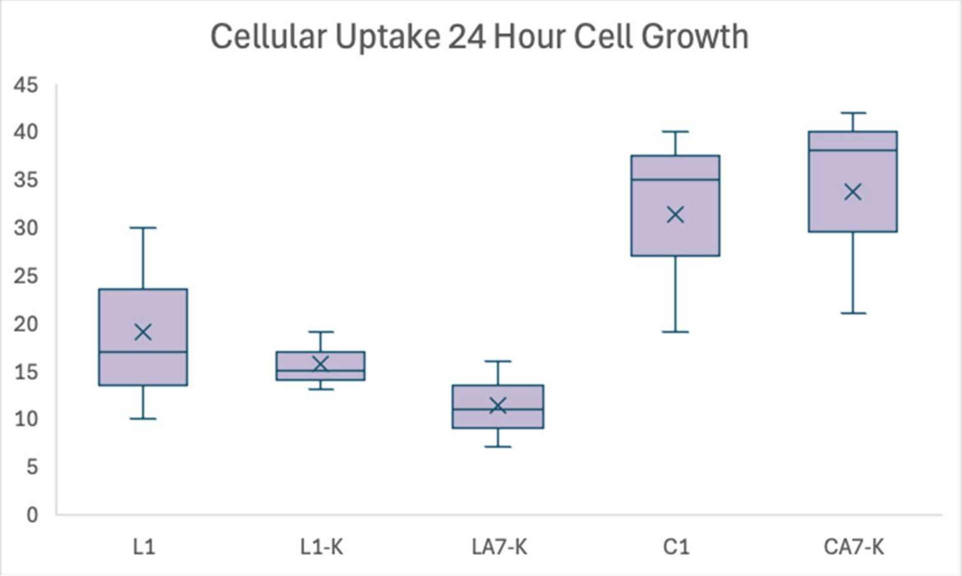


Figure 15: % Parent and Cell Count of E. Coli Cellular Uptake 24-hour cell growth

Results after changing cell growth from 16 hours to 24 hours. Older cells increased uptake of C1 slightly compared to previous experiments.

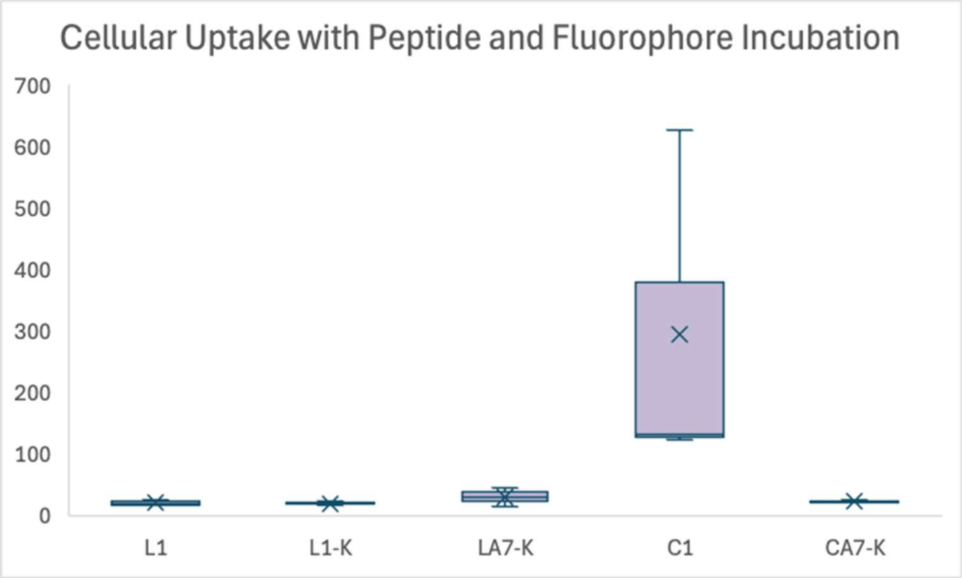


Figure 16: % Parent and Cell Count of E. Coli Cellular Uptake peptide and fluorophore incubation.

Results after changing cell growth from 16 hours to 24 hours and incubating peptides and fluorophore for 30 minutes before adding cells. This significantly increased the uptake of C1 however there is unexplained variation between the signal seen in the C1 used for unmixing vs the C1s that were not used for unmixing.

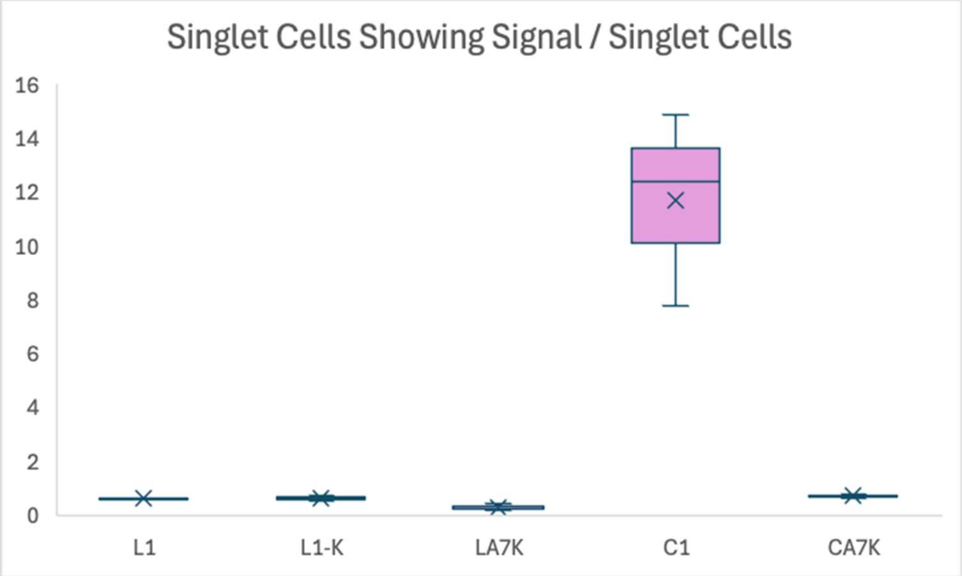


Figure 17: Singlet Cells Showing Signal/Singlet Cells for each peptide

We show that the C1 peptide significantly increases the signal seen compared to the other peptides and the control. The first L1 in this case is an outlier as the overall cell count was low and the amount of signal seen within the count was higher than we see in the other L1s.

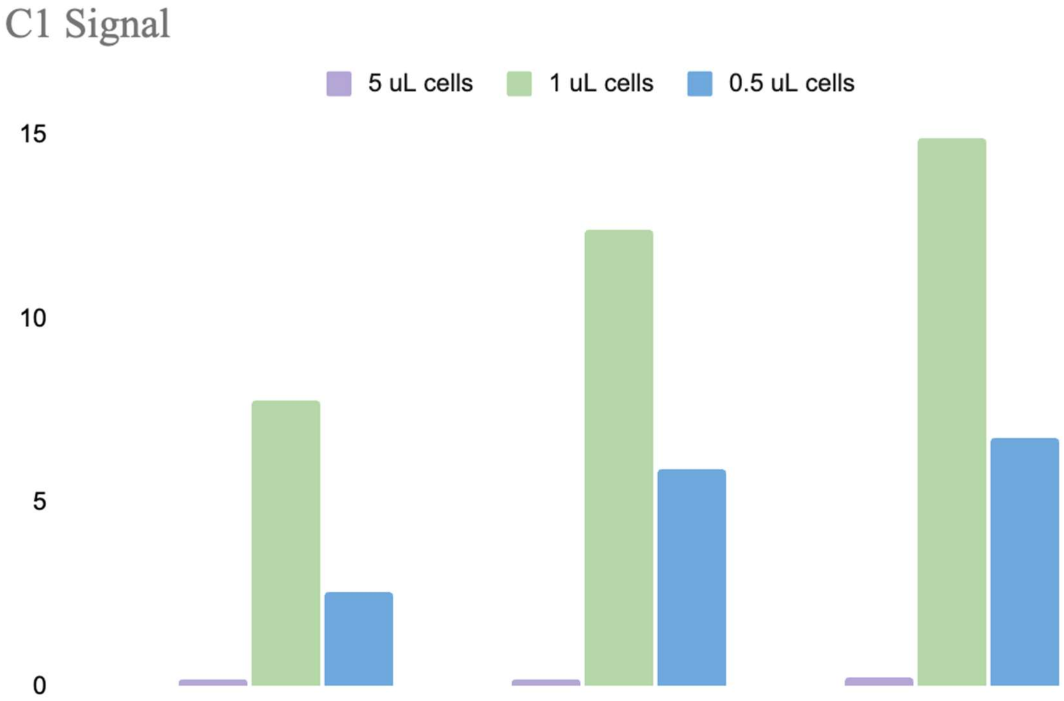


Figure 18: Reducing Cell Concentration

Reducing the cell volume to 1 µL increased signal, but when we decreased it further to 0.5 µL the signal decreased from 1 µL but is still significantly more than we see when we add 5 µL of cells. This also shows that peptide penetration is irreversible.

Discussion & Future Directions

In our results so far, we have optimized our cell growth protocol for E. Coli to achieve the correct OD reading and have a sufficient cell count for flow cytometry. MALDI-TOF verified that the peptides that we purchased were pure. All the ones that we have received were purified as the molecular weight was consistent with the experimentally validated weight. As we ordered the peptides externally, there have been issues with purifying some of the peptides on their part, as well as there have been delays of the peptide that is attached to the fluorophore directly that we ordered. In our first order of peptides the peptide named C1-K was unable to be purified and we were unable to do tests on it. For the initial control tests this is okay, but in the future, we need to find a solution for the peptide C1-K. This is the cyclic peptide that has the lysine on the end of it to attach to the DSG crosslinker. We need to confirm that C1-K has similar permeability levels to C1 and is an acceptable control for our cellular uptake assay when we use mRNA display to cyclize the peptides. We have used flow cytometry to show that our positive control C1 does increase the permeability, as there is significantly more signal seen in the presence of C1, and significantly less signal seen in the presence of the other peptides. We have shown this many times as we have navigated through optimizing this protocol and have had to adjust for issues such as decreased signal seen under certain conditions. We found that adding the 30 minute incubation time for the peptide and the fluorophore before adding cells and media is important for the increase in signal as the negative charge on the phosphopeptide F-GpYEEI likely interacts with the positive charge of peptide C1 while the neutral, negative, or less positively charged molecules interact with the F-GpYEEI molecule less, increasing signal for C1 while keeping the signal low for other peptides. We also found that increasing the growth time of

E. Coli makes a difference in signal, possibly because older cells have more permeable membranes.

This is ongoing research and will be continued. The next steps are to synthesize the peptides in house. We have only been using 8mers, but we want to make a 10mer and see if this increases the amount of signal seen. We want to try incubating the peptide and the fluorophore overnight to see if the sitting time makes a difference in signal seen. We have ordered the phosphopeptides Fluorescein and Texas Red attached to C1 and we want to see if the attachment makes a difference in signal. We will be unable to do this experiment until these peptides have arrived. Additionally, once the Texas red phosphopeptide arrives we need to optimize our protocol for yeast as we were unable to do that with our current phosphopeptide.

After all the controls have been set up and optimized, we can move on to mRNA display and using it to create our random peptide library. Each of the steps within the process of the assay will need to be tested and optimized as well. We will do similar steps to cellular uptake for the actual permeability assay, but we will do PCR on the cell solution to determine which peptides have made it into the cell. If the mRNA is successful, we can down the line tailor it in order to select for certain qualities in the cyclic peptides, ultimately enabling the discovery of more cyclic peptides, and the underlying features that allow them to be that way.

Conclusion

There is significant progress to be made in the field of peptide and therapeutic discovery. The high throughput permeability assay for cyclic peptides we aim to develop will enhance therapeutic drug development as it enables a drug to be directly delivered to specific organelles by a stable and rigid peptide. Thus far we have shown that the peptides we have selected for positive and negative controls are experimentally valid controls and have developed a cellular uptake assay for these peptides in E. Coli cell lines. While we still have many steps to go in the full development of our high throughput assay, we have made progress in the cargo delivery characteristics of $(WWRR)_n$ peptides in E. Coli cells and aim to publish these preliminary results in a low impact journal. To get the results that we have as of now we have had to navigate through many roadblocks as we are developing novel experiments. This is an ongoing project that will overall reveal much of the unknown qualities of cell penetrating peptides and aid in unveiling a new realm of drug discovery and microbial resistance. The permeability assay for cyclic peptides has the potential to enable therapeutic drug discovery to become streamlined, as well as help the development of antibiotics which is increasingly important with the antibiotic resistance crisis. Every day we get closer to the development of this assay, and with it the potential to help countless individuals suffering from insufficient therapeutics and antibiotics.

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