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Loading Peptides into Dictyostelium discoideum Using Pinocytosis, Electroporation, Cell Penetrating Peptides, and Myristoylation

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Loading Peptides into *Dictyostelium discoideum* Using Pinocytosis, Electroporation, Cell Penetrating Peptides, and Myristoylation

By:

Lorena Lazo de la Vega

Thesis submitted in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Biochemistry

May 2014

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Dr. Janet F. Morrison: ______________________

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1. Acknowledgements:

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2. Abstract:

The phospholipid bilayer of the cell is fluid and allows transport of small, hydrophobic compounds across the membrane. However, larger molecules such as peptides cannot cross the bilayer as easily. Therefore, my goal is to identify and optimize a method for loading exogenous peptides, such as reporters, hormones, or drugs, through the cell membrane. For these studies, we are using *Dictyostelium discoideum* as a model organism to test four peptide loading methods: pinocytosis, electroporation, cell-penetrating peptides and myristoylation. Pinocytosis uses changes in osmotic pressure to load the peptides through vesicles. The cells were exposed to a hypertonic solution for 10 min, 30 min, 1 hour, 4 hours, 17 hours, or 21 hours to load the peptide. Electroporation uses an electric field to create pores in the cell membrane that allow the diffusion of the peptide into the cell. The three different buffers used were H-50, HEPES, and a sucrose phosphate buffer, which were tested at electric field strengths of 8.50, 1.25, or 3.13 kV/cm, respectively. To follow up on promising initial results, the HEPES and sucrose phosphate buffers were additionally tested at 0.50, 0.63, 0.88, or 1.0 kV/cm. Myristoylation takes advantage of the cell membrane fluidity; the myristoylated peptide inserts itself into the membrane to allow the peptide to flip into the inside of the cell. Similarly, the cell penetrating peptide helps transport cargo into the cell through endocytosis or direct penetration. The incubation times in the loading solutions ranged from 1 minute to 1 hour. Each of these methods was tested for cell viability and highest percent of fluorescent peptide loading, which was measured under fluorescence microscopy. Pinocytosis stresses the cell through the varying osmotic pressures, and loading was very slow. Although electroporation showed efficient loading, this method disturbs the cell membrane and stresses the cells. Incubation of cells in myristoylated peptide and cell penetrating peptides load the peptides more gently. In fact, a 20 minute incubation of cells with the myristoylated peptide showed up to 99% of fluorescent cells with no effect on cell viability. Therefore, future work will use the myristoylated peptides as reporters to learn more about enzymatic activity and better understand signaling pathways implicated in disease.
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3. Introduction:

Signal propagation within cells occurs through the binding of a ligand to a receptor or an enzyme to its substrate, resulting in a cellular response. A pathway can be activated by a G protein-coupled receptor, a member of a large protein family that binds molecules outside of the cell.\(^1\) Signals inside the cell are then commonly carried by kinases, enzymes that transfer phosphate groups to a specific substrate in a process called phosphorylation.\(^1\) Cell signaling is important because aberrant signaling can lead to various pathologies, such as cancer.\(^1\) Therefore, the long term goal of this work is to probe signaling pathways by assaying enzyme activity. However, this project focuses on optimizing methods for loading reporter peptides into the cell.

A. Protein Kinase B

One of the pathways of interest involves the enzyme protein kinase B (PKB), which regulates many cell functions such as survival, cell proliferation, and apoptosis.\(^2\) Therefore, PKB is involved in many conserved signaling pathways implicated in human diseases and tumor regulation.\(^3\) The pathway is activated when a signaling molecule is detected by the G-coupled protein receptor, which phosphorylates the kinase Rat sarcoma (Ras). This leads to the activation of phosphoinositide 3-kinase (PI3K), which then activates PKB.\(^3\) Since Ras, PI3K, and PKB are all implicated in multiple human cancers, they are all important research targets.

The study presented here is designed to advance research on PKB using the social amoeba *Dictyostelium discoideum* as a model organism. Researchers often employ *Dictyostelium* as a model organism to study normal and aberrant cell signaling due to conserved cell signaling.\(^4\) These pathways are involved in stem cell differentiation and morphogenesis, chemotactic signals, and abnormalities in centrosomal proteins.\(^4\)\(^5\) Since
there are 24 classes of protein kinases conserved in human cells, *Dictyostelium* cells also allow for the study of human diseases.⁶ These cells have actually been shown to have a homologue of mammalian PKB. In fact, there is a high degree of amino acid sequence conservation that makes it indistinguishable from the PKB found in animals.⁷ Therefore, anti-cancer drugs that target kinases in humans have been found to similarly affect conserved kinases in *Dictyostelium*, suggesting that this amoeba is suitable for cancer related research.⁵ Since the signaling pathway involving PKB is activated through a pathway also found in human cells, the present study employs *Dictyostelium* cells as the model organism.

In addition to their similarities to human cells in cell signaling, *Dictyostelium* cells also have a number of advantages that make them a convenient model organism. Not only do *Dictyostelium* cells contain a haploid genome which has been completely sequenced, but the cells are also easy to collect and culture because they develop quickly.⁸ Similarly, the yeast *Saccharomyces cerevisiae* is also a convenient, fast-growing model. However, yeast evolutionarily diverged from human cells earlier than *Dictyostelium* which limits its use in research.⁹ For example, researchers identified 41 proteins that are conserved in *Dictyostelium* and human cells but are not found in yeast.⁹ Although a similar homologue to PKB, Sch9, is found in yeast,¹⁰ PKB is not present in yeast which is another reason why *Dictyostelium* make a better model for studying the PKB signaling pathway.

In *Dictyostelium* aggregation the activation of PKB starts after starvation, which causes the cells to release pulses of cyclic AMP that are detected by surrounding cells (Figure 1).³ When cyclic AMP is detected, the downstream PKB pathway is activated.
This pathway regulates actin organization, induces cell polarization, and causes cells to move toward an aggregation center to form a multicellular body designed to disperse cells to more favorable environments. The multicellular aggregate of about 100,000 cells is formed by chemotaxis, the directed movement of the cells along a chemical gradient. These cells form a tipped mound, then a phototactic slug, followed by a culminant that has a stalk containing 20% of the cells and a spore head containing the remaining 80% of cells as spores (Figure 2). Therefore, this process is dependent on differentiation and allows only 80% of the cells to remain as spores, which will germinate after they spread to favorable conditions to form new amoebas. Due to the conserved PKB signaling pathway that *Dictyostelium* uses during its development and shares with human cells, these cells will be used to conduct enzyme assays after optimizing methods to insert reporter peptides.

**Figure 1:** Pathway of the activation of the cAMP pathway after starvation (adapted from Aubry, L. & Firtel, R.).
Figure 2: The developmental cycle of Dictyostelium discoideum (adapted from Aubry, L. & Firtel, R.).

B. Peptide Substrate Reporters

A peptide substrate reporter is a fluorescent, exogenous peptide that is a substrate for an enzyme of interest. To directly report on the activity of a kinase such as PKB, a reporter peptide is loaded into the cell. Membrane-bound proteins or enzymes in the cell modify the substrate if the cell contains the active enzyme, ATP, and other cofactors. The cell is then lysed, and its contents separated, to quantify the ratio of unmodified and phosphorylated forms of the reporter peptide. Therefore, the modification of the peptide after it is introduced into the cell is an indicator of enzyme activity and provides insight on how the cells might respond to perturbations such as inhibitors.

Previous methods to detect enzyme activity include immunocytochemistry, phosphoflow, and image cytometry; however, these methods are limited by the need to...
raise antibodies against enzymes of interest, non-specific binding, and the possibility of differences in enzyme activity despite similar levels of antibody binding.\textsuperscript{2, 12} Peptide substrate reporters complement these methods and can be developed to assay PKB activity at the single-cell level to understand the heterogeneity of cells that results in different biological responses.\textsuperscript{11}

<table>
<thead>
<tr>
<th>6FAM-GRP-MeArg-AFTF-MeAla-NH$_2$</th>
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<tr>
<td><strong>Figure 3:</strong> Sequence of PKB reporter peptide.\textsuperscript{11}</td>
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Recently, an optimized reporter has been developed for PKB (Figure 3).\textsuperscript{11} The peptide contains an amidinated carboxyl terminus as well as a 6FAM fluorescent label for detection of the peptide during an enzyme assay.\textsuperscript{11} The non-native residues N-methylarginine and N-methlyalanine make the substrate more resistant to various peptidases found in cells.\textsuperscript{11} The threonine residue on the peptide is phosphorylated by PKB present inside the cell. The peptide sequence has been optimized for the highest resistance to peptidases in the cell because this peptide has only been observed to fragment into two pieces in lysates. In-vitro experiments showed that it took 34 minutes for 50\% of the peptide to be phosphorylated in the presence of purified PKB and that the peptide had a half-life of 92 minutes in cytosolic lysate.\textsuperscript{11}

In the previous study of this PKB reporter, microinjection was used to insert the peptide into cells; \textsuperscript{11} however this method requires a high skill-level and practice. Since this method limits the number of cells analyzed, the statistical power will also be limited. Microinjection or another loading method is required because although the phospholipid bilayer of the cell is fluid and allows transport of small, hydrophobic compounds across the membrane, larger molecules such as peptides cannot cross the bilayer as easily. In the
present study, *Dictyostelium discoideum* will be used to optimize other methods for peptide loading. Several methods which use different mechanisms are available for loading membrane-impermeant species into cells. These methods include: pinocytosis, electroporation, conjugation to cell-penetrating peptides and myristoylation. Pinocytosis is a biological mechanism that uses endocytosis; electroporation uses a physical phenomenon because it facilitates diffusion; and cell-penetrating peptides as well as myristoylation take advantage of chemical interactions with the membrane for transport.

After optimization, one or more of these loading methods can then be utilized to transport exogenous peptides, such as substrate reporters, across the cell membrane to assay the activity of enzymes such as PKB. Other applications include transport of hormones, drugs, and reporters, which can facilitate a better understanding of cell signaling pathways implicated in disease.\(^\text{11}\)

**C. Methods of Peptide Loading:**

*Pinocytosis.* Pinocytosis is a form of endocytosis in which vesicles are formed through an invagination to bring particles into the cell. Previous research has shown that pinocytosis allows proteins and polysaccharides to enter mammalian cells after being engulfed by the cell membrane and then invaginated into a cytosolic vesicle by varying the osmotic strength of the medium in which the cells are incubated. In pinocytosis, the cells are exposed to a hypertonic loading medium to allow endocytosis to initiate.\(^\text{13}\) Since the hypertonic medium contains the reporter peptide (or molecule of interest), the vesicles that form engulf the peptide until it fully enters the cell (Figure 4).\(^\text{13}\) In this case, there is a low water concentration outside of the cell which causes the water to leave the cell as the solute enters the cell down its gradient.\(^\text{13}\) After the subsequent addition of the
hypotonic solution, the vesicles inside the cell bursts as water rushes in due to the low concentration of water inside the vesicle compared to the cytoplasm of the cell. Therefore, the process of osmosis causes the peptide to be released into the cytoplasm (Figure 4).

![Diagram of pinocytosis](image)

**Figure 4:** Schematic of pinocytosis representing how the fluorescently tagged peptide (green) enters the cell.

Although varying osmotic pressure can stress cells, pinocytosis has been previously used in mammalian cells and osmotic lysis of the pinosomes did not diminish viability or growth rate.

*Electroporation.* Cells can also undergo electroporation, a method that exposes the cells to an electric field, which induces the formation of pores in the cell membrane. When the cells are exposed to an electric field, the lipids in the phospholipid bilayer are reoriented, creating a hydrophilic pore that increases the rate of diffusion through the membrane (Figure 5).
Figure 5: Schematic of the phospholipid bilayer before and after exposing the cells to an electric field. Exogenous molecules, such as the peptide reporter (green), then diffuse through the membrane.

Membrane recovery after the application of the electric field allows the membrane to seal and return to its normal state.\textsuperscript{16} Although electroporation is commonly used for DNA transformation, this method is also effective in loading peptides and other membrane impermeant species because a similar mechanism is needed for any charged oligomer to cross the membrane.\textsuperscript{17}

\textit{Cell-penetrating peptides.} Cell-penetrating peptides are short, water-soluble, and composed of a maximum of 30-35 amino acids with a net positive charge.\textsuperscript{18} These sequences help transport cargo into the cell at low concentrations with minimal interruption of the cell membrane.\textsuperscript{18, 19} This transport is achieved by either endocytosis or direct penetration, which includes inverted micelle formation, pore formation, or the carpeting or thinning of the cell membrane.\textsuperscript{18, 19} In all forms of transport, the process is started through the interaction of the positively charged CPP and the negatively charged components of the phospholipid bilayer.\textsuperscript{19} This attraction causes a destabilization of the membrane due to the folding of the peptide. The inverted micelle formation involves the interaction of the hydrophobic residues of the membrane and the peptide.\textsuperscript{18} Two other
descriptive models for pore formation include the barrel stave model, which involves hydrophobic residues close to the lipid chains and the hydrophilic residues facing the inside of the pore, and the toroidal model, which involves the bending of the lipids so that the head group is close to the CPP. The carpeting or thinning of the cell membrane is caused by interaction between negatively charged lipids and the positively charged CPPs. Although CPPs do not stress the cell as much as pinocytosis or electroporation, the synthesis of the peptide for each procedure is costly and time consuming.

**Myristoylation.** Since the lipid bilayer is fluid, myristoylation was investigated as the fourth method to transport the peptide across the membrane. Myristoylation of the peptides chemically modifies the peptide by covalently binding it to a lipophilic myristoyl group to allow it to penetrate the cell membrane. Specifically, myristoylation involves the addition of a hydrophobic myristoyl group, derived from the C14:0 lipophilic group myristic acid (Figure 6), to the reporter peptide (Figure 7) to make the peptide more permeable and easily transported into the cell after it inserts itself in the membrane (Figure 8). Past research suggests that this method takes advantage of lipids’ ability to flip between the inner and outer leaflet of the cell membrane.
To allow the peptide to diffuse throughout the cell, the original reporter peptide is further modified to contain a cysteine used to create the disulfide bond between the peptide and the cysteine on the new fragment. Therefore, the peptide contains the two new cysteines which create the disulfide bond used to attach a short lysine chain bonded to the myristoyl group (Figure 6).\textsuperscript{21}

The disulfide bond is broken by natural reducing agents in the cell. However, there are peptides that remain in the outer leaflet of the phospholipid bilayer. Therefore, a reducing agent added to the cell medium cleaves the disulfide bond which connects the myristoyl group and the peptide facing the outside of the cell. The removal of peptides
facing the exterior cell surface then allows the cells to be imaged with the resulting fluorescence signal coming only from peptide inside the cells.21

The optimized loading method will be determined after defining the success of the method by using microscopy to observe the labeled peptides. This will help determine the concentration and uniformity of the distribution of the peptide within the cytoplasm since it is essential to have uniform loading to obtain useful measures of phosphorylation. For example, if the peptide is sequestered in pinosomes, PKB enzymes within the cell may not have access to peptide substrate reporter molecules, which will not be phosphorylated to the same degree. The level of effectiveness of the different methods was also determined through viability of the cells after loading since the long-term goal is to study phosphorylation of the peptide in live cells. Depending on the results, the conditions were adjusted until each method was optimized for the loading process. Overall, optimization of these methods provides a variety of techniques to introduce exogenous peptides into Dictyostelium cells to be able to conduct enzyme assays with fluorescently labeled reporter peptides.
4. Experimental

A. Making Frozen Stocks of Dictyostelium discoideum

An agar plate containing the K-AX3 strain of Dictyostelium discoideum was received from the Dictyostelium Stock Center (Chicago, IL). The spores on the plate were obtained carefully and transferred to HL-5 medium. [HL-5 was composed of 14 g Protease Peptone No. 2, 7 g Yeast Extract, 1.5 g KH$_2$PO$_4$, 0.945 g Na$_2$HPO$_4$·H$_2$O in 1 L of distilled H$_2$O which was adjusted to a pH of 6.5 with 1 M NaOH. The medium was autoclaved and stored at 4 ºC but used at room temperature.] After expanding the cells in culture, the cells were centrifuged at 1000 × g for 2 minutes then resuspended in 10 mL of sterile HL-5 medium. The volume of this media was then doubled with HL-5 media containing 20% sterile DMSO, resulting in a final DMSO concentration of 10%. The cells were resuspended in 20 mL of media with a density of 2×10$^6$ cells/mL. The cells were then aliquoted to cryo-tubes in a volume of 0.5 mL and stored at -80ºC (Dan Dickinson, personal communication, 2013).

B. Dictyostelium discoideum cell culture

Dictyostelium discoideum cells (K-AX3) were grown in stationary, axenic cultures, i.e., in the absence of bacteria. The K-AX3 mutant is capable of feeding on liquid HL-5 media supplemented with antibiotics (10 µg/mL of ampicillin and 30 µg/mL of streptomycin) and glucose. New cultures were started every three weeks by adding 0.5 mL of the concentrate of cells from the freezer stocks to a total volume of 10 mL of media. The cells were cultured for at least one day before any experiments were
conducted. Since the cells double every 8-12 hours, they were regularly observed under a microscope and maintained at a cell density between $1 \times 10^5$ and $5 \times 10^6$ cells/mL.\textsuperscript{22}

C. Pinocytosis

To conduct pinocytosis experiments, preliminary conditions set by a Molecular Probes commercial protocol for mammalian cells were adapted for use with \textit{Dictyostelium} cells.\textsuperscript{13} In the first set of experiments, $1 \times 10^6$ cells were centrifuged for 2 minutes at 1000 $\times$g (2400 rpm) in HL-5. Once the pellet was obtained, the cells were suspended in 100 $\mu$L of hypertonic solution (0.5 g of polyethylene glycol (MW: 1000), 0.5 M sucrose, and 50 $\mu$L of 1 M HEPES in a total of 5.0 mL HL-5 media pH 7.4). A final concentration of 500 $\mu$M fluorescein was added to 100 $\mu$L of the hypertonic solution. The cells were then incubated for 10 min, 0.5, 1, 4, 17, or 21 h. After the incubation, the cells were washed in 500 $\mu$L of HL-5 at least 3× to remove free peptide. For some of the experiments, about 2 mL of the hypotonic lysis medium (normal culture medium diluted in water in a 6:4 ratio) was then added to the pellet and incubated for 1.5 minutes to induce the pinosomes inside the cell to burst and release the peptide into the cytosol. The cells were then transferred to HL-5 media for imaging.

D. Electroporation

To conduct electroporation experiments, three protocols that had been previously published and used for \textit{Dictyostelium} transfection were optimized for peptide loading. Each protocol used a different buffer and had different conditions for electroporation (Table 1).\textsuperscript{17, 23} To conduct electroporation, $1 \times 10^8$ cells were suspended in 1 mL of each of the three different sterile buffers: H-50 buffer, phosphate and sucrose buffer, and HEPES (Table 1).
For the HEPES or phosphate and sucrose buffer, approximately 500 µM of fluorescein was added to the solution. Subsequently, only 400 µL of this solution was transferred to the 0.4 cm gap cuvette. The H-50 buffer solution was similarly prepared; however, only 100 µL of the buffer with fluorescein and cells was added to a 0.1 cm gap cuvette. All samples were then chilled on ice for 15 minutes. The BIO RAD Gene Pulser II Electroporation System (Catalog # 165-2105) was set to the conditions described (Table 1), and the electric field was applied in two pulses with 5 s between each pulse. Since electroporation for these protocols involves adding an electric field through the cells, we also recorded the resistor-capacitor (RC) time constants, which were indicators of the resistance and capacitance of the sample, and compared them to literature values (Table 1). After exposing the cells to the electric field, the cells were resuspended in HL-5 medium and washed at least 3× with 500 µL of HL-5 media before they were imaged. Once the three published protocols were evaluated, different electric fields ranging from 0.50 to 1.0 kV/cm were tested with the most promising buffers, HEPES and phosphate

| Table 1: Conditions Used When Comparing Different Protocols<sup>17, 23</sup> |
|----------------------------------|------------------|------------------|------------------|
| Buffer Composition               | H-50 Buffer      | Phosphate and Sucrose Buffer | HEPES Buffer      |
| Buffer Composition               | 50 mM KCl        | 10 mM Na<sub>3</sub>P<sub>4</sub> | 1 M HEPES        |
|                                  | 5 mM NaHCO<sub>3</sub> | 50 mM sucrose     |                 |
|                                  | 10 mM NaCl       |                  |                 |
|                                  | 1 mM MgSO<sub>4</sub> |                |                 |
|                                  | 1 mM NaH<sub>2</sub>P<sub>4</sub> |             |                 |
| Capacitance (µF)                 | 25               | 3                | 25              |
| Buffer pH                        | 7.0              | 6.1              | 7.0             |
| Voltage (kV)                     | 0.85             | 0.60             | 1.25            |
| Electric Field (kV/cm)           | 8.5              | 1.5              | 3.1             |
| Literature Time Constant (ms)    | 0.6              | N/A              | 0.5-0.7         |

For the HEPES or phosphate and sucrose buffer, approximately 500 µM of fluorescein was added to the solution. Subsequently, only 400 µL of this solution was transferred to the 0.4 cm gap cuvette. The H-50 buffer solution was similarly prepared; however, only 100 µL of the buffer with fluorescein and cells was added to a 0.1 cm gap cuvette. All samples were then chilled on ice for 15 minutes. The BIO RAD Gene Pulser II Electroporation System (Catalog # 165-2105) was set to the conditions described (Table 1), and the electric field was applied in two pulses with 5 s between each pulse. Since electroporation for these protocols involves adding an electric field through the cells, we also recorded the resistor-capacitor (RC) time constants, which were indicators of the resistance and capacitance of the sample, and compared them to literature values (Table 1). After exposing the cells to the electric field, the cells were resuspended in HL-5 medium and washed at least 3× with 500 µL of HL-5 media before they were imaged. Once the three published protocols were evaluated, different electric fields ranging from 0.50 to 1.0 kV/cm were tested with the most promising buffers, HEPES and phosphate.
and sucrose buffer, to determine whether electric field had any effect on the percent of fluorescent cells or viability.

E. Cell Penetrating Peptides

The 2 nmol F-Cup-Con cell penetrating peptide (CPP) stock from Cupid Peptides was used to make a 200 µM solution in distilled water. Although this CPP will be used to quantify peptide loading, it only contains the fluorescent tag and was not conjugated to the reporter.

Approximately 5x10⁵ cells were collected and centrifuged. The supernatant was removed to add 20 µM of the CPP in a total volume of 100 µL low fluorescence media (sterile 0.5 mM NH₄Cl, 0.2 mM MgCl₂, 0.01 mM CaCl₂, 0.05 mM FeCl₃, 5 mM K₂HPO₄, pH 6.5 and 61 mM glucose monohydrate) for an incubation of 1 hour. To stop peptide loading, the cells were centrifuged and washed 3x with HL-5 before imaging.

F. Myristoylation

For loading the modified peptide, approximately 5x10⁵ cells were collected and centrifuged. The supernatant was removed to add 20 µM of the myristoylated peptide from Anaspec (diluted from a 2 mM stock solution in DMSO stored at -80º C) in a total volume of 100 µL low fluorescence media.

In myristoylation experiments, the Dictyostelium cells were incubated with the peptide for 20 minutes to 2 hours at room temperature followed by an incubation period of 1 minute in tris(2-carboxyethyl)phosphine (TCEP) solution. Since TCEP is a membrane-impermeant reducing agent, this solution was meant to cleave the disulfide bond facing the outside of the cell connecting the myristoyl group and the reporter peptide. Removing the peptide facing the external cell surface would help obtain better
data to make sure only peptide loading is being measured.\textsuperscript{21} After incubation with TCEP, the cells were washed 3 times with 500 µL of HL-5.

\textbf{G. Image Acquisition and Analysis}

Fluorescence micrographs were taken immediately after at least 100 cells were washed and placed on slides. It was also very important to minimize exposure to the excitation light to minimize photobleaching.

A 1500M Thor Labs CCD camera was used to obtain the images for quantification on an Olympus IX73 inverted microscope. Micromanager 1.4.15 software was used to collect the images. For these images, the gain was kept at zero, and the readout rate was 7 MHz. The binning was set to 2, and the exposure time was set to 500 ms under fluorescence and 1 ms in brightfield. The illumination intensity of the LED excitation source was set to 16%.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Normalization of (A) fluorescent image by dividing (B) the illumination intensity to obtain (C) a normalized image from which the background noise can be calculated.}
\end{figure}

To obtain the intensity of the source, an image was taken of a uniformly fluorescent sample (Chroma Technology) using the FITC filter and an exposure time of 5 ms (Figure 9B). Image analysis was then performed using ImageJ. The image of the cells (Figure 9A) was then divided by the full field illumination for each day to normalize for
variations in excitation intensity across the field of view (Figure 9C); brightness maximum and minimum were subsequently adjusted to make sure the cells were clearly seen. The area, mean and standard deviation of an empty area were also obtained to set the detection threshold at 10 standard deviations above the mean background intensity.

After normalization and thresholding, the number of particles (i.e., fluorescent cells) was analyzed by setting the ImageJ program to find particles no smaller than 25 pixels$^2$, with at least a 60% circularity to make sure only cells were counted as opposed to any other debris on the slide.

Images were also taken under brightfield conditions to determine the total number of cells in the field of view to calculate the percent of fluorescent cells in each slide.

**Figure 10:** (A) The threshold is set after calculating the mean background intensity and adding ten times the standard deviation to obtain the thresholded image. (B) The particles are then counted according to the criteria described below.
H. Cell Viability Stain

The cell viability of *Dictyostelium discoideum* was determined using trypan blue in buffer. The sterile phosphate buffer was made with 5 mM H$_2$PO$_4$, 5 mM Na$_2$HPO$_4$, 2 mM CaCl$_2$, and 2 mM MgCl$_2$ adjusted to a pH of 6.5 in distilled H$_2$O (Dan Dickinson, personal communication, 2013). 1x10$^5$ cells were suspended in 500 µL of 0.4% trypan blue solution for 3-4 minutes. 10 µL of the cells in trypan blue solution were then added to the hemacytometer, where 100 cells were counted under the microscope. The cells that turned dark blue indicated cell death and cell counts were used to determine percent viability.

After the brightfield and fluorescent images were taken, the micrographs were analyzed to determine the percent of fluorescent cells and percent of viable cells after conducting each loading method. The brightfield images were used to obtain the total number of cells under the field of view and the standard thresholding procedure was used to determine the percent of fluorescent cells above the threshold.
5. Results and Discussion:

A. Pinocytosis:

For pinocytosis, 6 incubation times were used that ranged from 10 minutes to 21 hours. The brightfield and fluorescence images are shown for two representative time points, 30 minutes and 4 hours (Figure 11).
Figure 11: (A, C) Brightfield and (B, D) fluorescence images under 10X magnification. Images showing (A, B) 30 minute and (C, D) 4 hour incubation period for pinocytosis. Fluorescence images are under the same black: white scale.
Micrographs after the 30 minute and 4 hour incubation showed that the fluorescent dye loaded into the cell (Figure 11). Although the intensity of the fluorescent cells was not quantified, qualitative examination suggested that the loading for the 30 minute incubation was fairly uniform with minor exceptions (e.g., two very bright cells in the field of view in Figure 11b). Additionally, there was no visual effect of incubation on cell morphology. The cells were intact and did not show a change in circularity (Figure 11a).

To compare loading of the dye, fluorescent micrographs for three incubation time points were compared on the same brightness (white/black level) scale (Figure 12). The image taken after 30 minutes showed a few bright cells; however, at 4 hours the loading was more uniform between cells. At 21 hours, the loading was both more uniform and brighter than the shorter incubation times. Although the calculations determined the number of cells that were above the threshold, quantitative comparisons of the intensity of the fluorescence were not obtained.
Analysis of the fluorescent images showed a positive correlation between the percent of cells that were fluorescent and incubation time (Figure 13), meaning that loading increased with time. However, this relationship had a weak correlation ($R^2 = 0.62$). The percent of cells that loaded the fluorescent dye was very low during incubation times up to 4 hours (5% to 45%). Loading for 21 hours was more effective, and for one trial the percent of fluorescent cells approached 100%. However, these results were inconsistent since the other two 21 hour trials showed significantly lower percentages of fluorescent cells (36% and 84%).

Figure 12: Fluorescence images under 10X magnification of the three time points A) 30 minutes, B) 4 hours and C) 21 hours adjusted to the same intensity scale. Images all had the same white: black level.
Since *Dictyostelium* cells feed through endocytosis, longer incubation times suggest that this consistent feeding engulfed not only the nutrients in the media but also the dye.\textsuperscript{14} However, the percent of cells that were loaded with dye above the detection threshold varied substantially between trials (Figure 13).
The limited loading of the dye by pinocytosis may be related to the robustness of *Dictyostelium* to osmotic pressures. Mammalian cells are readily loaded with substrates by pinocytosis. However, *Dictyostelium* cells live in soil and may be more resistant to changes in osmotic pressure, which makes pinocytic loading more challenging. This possibility was supported by the viability data. Although the percent of fluorescent cells increased with longer incubation times, trypan blue staining indicated the overall cell viability was minimally affected. Under normal culture conditions, 96% of the cells were viable based on a trypan blue stain. The viability of the cells was determined after pinocytic loading at each incubation time and compared to this baseline. Despite long incubation times in hypertonic media, the viability was not strongly affected (Figure 14). The slight decrease in viability that was observed could be due to osmotic pressure or because long incubation periods of large numbers of cells in a small volume (100 µL) stress the cells.

**Figure 14:** Percent viability of cells for the pinocytosis method after fluorescence images were taken. The line is the cell viability in a normal cell culture.
While pinocytic loading resulted in acceptable levels of viability, loading was variable between trials and between individual cells. For most incubation times, the majority of cells did not load detectable levels of dye. In fact, future work will involve measuring the variability in the intensity of the fluorescent cells due to the high variation observed. While the longer incubation times showed some promise, a 21+ hour incubation period is not feasible for enzyme assays. In human cells, the reporter peptide has a half-life of 92 minutes, determined through analysis using lysate, meaning that the peptide is significantly degraded by cytosolic peptidases on this timescale;\textsuperscript{11} consequently, the reporter peptide should be loaded and analyzed within ~1 h because the peptide may degrade after long incubation periods.

The long-term goal of this work is to load reporter peptide for single-cell enzyme assays. In these assays, the amount of peptide loaded into each cell will be quantified after the separation of the phosphorylated and unmodified forms of the peptide. Because this detection method accounts for peptide quantity, uniform loading is not essential; however, it is problematic if a large percent of the cells do not load a detectable quantity of the peptide, since single-cell analysis of these cells will yield no useable data.
B. Electroporation:

Initial experiments on electroporation of the *Dictyostelium* cells utilized three protocols that had been previously developed for transfection. We needed to optimize these conditions to load the exogenous peptide composed of amino acids, or our model dye molecule, instead of large nucleic acid-based molecules. The first protocol used the H-50 buffer\(^{17}\) while the other two protocols were adapted from the electroporator manual and used either the HEPES or phosphate and sucrose buffer\(^{23}\).

<table>
<thead>
<tr>
<th>Table 2: Conditions Used When Comparing Different Protocols</th>
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<tbody>
<tr>
<td>H-50 Buffer</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Capacitor (µF)(^{17, 23})</td>
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<tr>
<td>Voltage (kV)(^{17, 23})</td>
</tr>
<tr>
<td>Electric Field (kV/cm)(^{17, 23})</td>
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<tr>
<td>Time Constant 1 (ms)</td>
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<tr>
<td>Time Constant 2 (ms)</td>
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</tbody>
</table>

After an initial comparison of the three published protocols, the first protocol using the H-50 buffer did not work well. The condition of the cells after observing them under the brightfield view was poor. Some cells were clumped into groups while others had lysed (Figure 16A). However, the resistance was very similar to the accepted value (Table 2).\(^{17, 23}\) The remaining two protocols were more promising because the cells were circular, intact, and had not formed clumps (Figure 16B, C); consequently, these two protocols were further optimized for the effect of the electric field on loading and viability. However, the time constant for the HEPES buffer was almost 4× larger than the literature value.
Although the published protocols suggested specific voltage settings, it was important to optimize these protocols for this application to obtain the highest percent of fluorescent cells while maintaining viability. Therefore, the two different protocols were tested at different electric field strengths (Table 3&4). In fact, the time constants for the HEPES buffer was still higher for these trials when compared to the literature value of 0.5-0.7 ms. Therefore, this indicated that the pulse applied to the cells took longer to dissipate. These further studies with the HEPES and phosphate buffers showed the best conditions for electroporation.

**Figure 15:** Brightfield images under 10X magnification of cells treated according to the three different protocols for electroporation. A) Image of cells exposed to the H-50 buffer and 85 kV/cm in a 0.1 cm gap cuvette. B) Image of cells exposed to the HEPES buffer and 3.1 kV/cm in a 0.4 cm gap cuvette. C) Image of cells exposed to the phosphate and sucrose buffer and 1.50 kV/cm in a 0.4 cm gap cuvette.

![Brightfield images](image)

<table>
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<tr>
<th>Voltage (kV)</th>
<th>Electric Field Strength (kV/cm)</th>
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Table 3: Time constants (ms) for electroporation using phosphate and sucrose buffer.*
Table 4: Time constants (ms) for the electroporation using HEPES buffer.*

<table>
<thead>
<tr>
<th>Voltage (kV)</th>
<th>Electric Field Strength (kV/cm)</th>
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</table>

*Note: Used with a 0.4 cm gap cuvette at 3 µF. The time constants were obtained after the first pulse (1) and the second pulse (2).

The micrographs for the phosphate and sucrose buffer and the HEPES buffer are shown for an electric field of 0.50 kV/cm (Figure 16). These images showed that the fluorescent dye loaded into the cells but there were more bright cells in the phosphate and sucrose buffer (Figure 16). However, there was more uniform loading of the dye for the trials using the HEPES solution. In both cases, the cells were intact and remained circular, indicating that the cell viability had not been visibly affected.
Figure 16: (A, C) Brightfield and (B, D) fluorescence images at 10X magnification of cells exposed to 0.50 kV/cm with the (C, D) HEPES buffer and the (A, B) phosphate and sucrose buffer.
To compare loading of the dye, fluorescence micrographs for three different conditions were compared at the same level of brightness (Figure 17). The images compare the HEPES buffer to the phosphate and sucrose buffer and also compare electric fields of 0.50 kV/cm to 0.88 kV/cm using the HEPES buffer. The HEPES buffer resulted in a greater number of cells loaded with a higher overall intensity. The sample exposed to the HEPES buffer also showed that the intensity of fluorescence in the cells was generally higher in the 0.88 kV/cm electric field than in the 0.50 kV/cm field.

![Fluorescence images](image)

**Figure 17:** Fluorescence images of three different conditions that were tested in 0.40 cm gap cuvettes. A) Image of cells exposed to the HEPES buffer at 0.50 kV/cm. B) Image of cells exposed to the HEPES buffer at 0.88 kV/cm. C) Image of cells exposed to the phosphate and sucrose buffer and 0.50 kV/cm. All images had the same black/white ratio.
The effect of electric field on the percent fluorescent cells (Figure 18) and the cell viability (Figure 19) was determined for both buffers. No trend in fluorescence was observed as the voltage was increased. However, the HEPES buffer consistently had a higher percent of cells loaded with peptide compared to the phosphate and sucrose buffer (Figure 18).

![Graph showing percent of fluorescent cells vs. electric field](image)

**Figure 18:** Percent of fluorescent cells for each electroporation method. Trials tested the same protocol run with phosphate and sucrose buffer versus HEPES buffer. Each protocol was then tested at different electric fields: 0.50, 0.63, 0.88, and 1.1 kV/cm. Cuvette gap was 0.4 cm for all trials.

When the cells were exposed to 0.50 kV/cm, only 12 to 45% of the cells in phosphate and sucrose buffer loaded the fluorescent dye. On the other hand, 39 to 59% of the cells in HEPES buffer loaded the dye at 0.88 kV/cm. A major difference observed between the two different protocols was the difference in time constants between the two buffers. The HEPES buffer had higher time constants than the sodium and phosphate...
buffer. Therefore, the length of time it took for the pulse to dissipate in the solution may have been a factor in the difference in the percent of fluorescent cells.

![Graph](image)

**Figure 19:** Percent viability of cells for each electroporation method. Trials test the same protocol run with phosphate and sucrose buffer versus HEPES buffer. Each protocol was then tested at different electric fields: 0.50, 0.63, 0.88, and 1.1 kV/cm. The line across is the cell viability in culture.

Although the HEPES buffer produced a higher percent of loading, it also negatively affected the cell viability (Figure 19). For example, at 0.88 kV/cm the cell viability ranged from 89 to 94%, which was slightly lower than the 96% cell viability for untreated cells. This may indicate that in electroporation as the loading increases the cell viability is decreased due to the stress on the cell. However, since the loading was generally higher when using the HEPES buffer, it was determined to work the best (Figure 18). If the cells are stressed, the PKB activity may be affected since it is involved in stress responses such as cell survival. Therefore, our measurements of PKB activity in future single cell enzyme assays may not be representative of the true PKB activity in the cell.
After testing the three different protocols, the two buffers that were established to work the best were the HEPES and the phosphate and sucrose buffer. Then, since there was a lot of variability in the percent of fluorescent cells after varying the electric field, the optimized conditions include the lowest electric field to potentially lower the stress on the cell membrane. Although the HEPES buffer at 0.88 kV/cm gave the highest percent of cells, 59%, this percent was still not ideal. Since these trials were conducted using the fluorescent dye, the trials with the actual larger peptide may behave differently. Due to its larger size, it may be more difficult for the peptide to enter the cell and will therefore take longer to diffuse through the cell membrane. Therefore, the ideal percent of fluorescent cells should be as high as possible to ensure that the loading can still take place with the reporter peptide.
C. Cell Penetrating Peptides:

The brightfield and fluorescent images were taken after the 1 hour incubation with the cell-penetrating peptide provided by Cupid Peptides. The cells under the brightfield image were intact and did not seem to have been affected by the 1 hour incubation. In the fluorescence images, some cells were very bright (arrow, Figure 20), while other cells were very dim and did not show up in the fluorescence image. This was reflected in the quantitative results for percent of cells that were fluorescent (Table 5).

![Figure 20: Brightfield (A) and fluorescence images (B) of 1 hour incubation period in CPP for trial 1.](image)

In the first trial, the percent of fluorescent cells was 68%, while in the second trial the percent of fluorescent cells was 50%. For both trials, the viability remained at a normal level of 99% and 100%, which showed that the cell viability was not affected by the loading. Although only two trials were conducted, the variability in the percent fluorescence was high and therefore the loading of the CPP was not consistent.
Considering the low CPP concentration, these results are quite promising, since higher concentrations could result in loading of greater numbers of cells. However, previous research has shown the addition of cargo to alter the CPP uptake pathway.\textsuperscript{18} Specifically, the type, size, and binding of the cargo have been shown to influence the CPP translocation mechanism.\textsuperscript{18} Therefore, the addition of the PKB substrate peptide to the CPP as cargo will likely affect loading and may make it harder for the peptide to cross the membrane.
D. Myristoylation:

The brightfield and fluorescence images showed substantial loading of the myristoylated peptide at 20 and 40 minute incubation periods (Figure 21). In fact, the loading of peptide into the cells was extremely rapid with detectable fluorescence after 1 minute. Although the intensity was not measured, qualitative comparisons show that the intensity of the fluorescence was substantially higher compared to the previous methods. Since the range of the incubation periods was short and since this is a gentle method, the cells were found to be intact. In fact, the cell viability after observation of the cell morphology was not affected.
Figure 21: (A, C) Brightfield and (B, D) fluorescence images at 10X magnification of (A, B) 20 minute and (C, D) 40 minute incubation period using the myristoylated peptide.
After 1 minute incubation, the percent of cells that loaded the peptide ranged between 75% and 84% (Figure 22). This percent increased until the 20 minute incubation, which showed that 93% to 99% of the cells loaded the peptide (Figure 24). This incubation time also had the most consistent loading across three trials. For incubations longer than 20 min, the percent of fluorescent cells was the same as that for 20 minutes within the range of noise.

**Figure 22:** Percent fluorescence of cells after myristoylation for their respective incubation periods of 1, 5, 10, 20, 30 and 40 minutes.
Although loading was very high, the cell viability was not affected (Figure 25).

Therefore, myristoylation showed that the cell viability was not affected by the loading of the peptide into the cell so it was determined to have the highest percent of fluorescent cells without affecting the cell viability.

![Figure 23: Percent viability of cells after myristoylation, after fluorescence images were taken, for the different incubation periods of 1, 5, 10, 20, 30, and 40 minutes. The line across is the cell viability in culture.](image)
6. Conclusions:

Overall, four different methods were optimized and analyzed for success in loading exogenous dye or peptide as well as for high cell viability. For pinocytosis, the optimal incubation period was 30 minutes. Although this incubation time did not yield the highest percent of fluorescent cells, it reflects the most useful incubation time for an enzyme assay. The optimal voltage and buffer for electroporation were 0.35 kV with the HEPES buffer. The 20 µM cell penetrating peptide was used with an incubation period of 1 hour, as per manufacturer instructions. Due to the limited sample available, the loading conditions for this peptide have not been optimized. The optimal incubation with 20 µM of the myristoylated peptide was 20 minutes with no effects on cell viability.
After comparing all these methods, there was a clear trend (Figure 26). The viability was not strongly affected by any of the loading methods tested. The percent of fluorescent cells was maximized with myristoylation since the loading was as high as 97% in only 20 minutes. Therefore, myristoylation has been shown to be the optimized method because it loads the highest percent of fluorescent cells and loading of the exogenous peptide does not affect the condition of the cell. Since the myristoylated peptide with the reporter also loads consistently in all cells after a short incubation period. Therefore, myristoylation will be used in future projects involving the single cell enzyme assays because it has shown promising results.
7. Future Work

Future work will examine the loading of different concentrations of the myristoylated peptide and the CPP to be able to make more concrete conclusions on the difference in effectiveness of these two methods. We could also potentially use the 0.51 mM stock peptide (courtesy of the Allbritton lab at UNC) for future optimization trials to determine if it loads into cells differently than fluorescein. However, a trial run with pinocytosis was conducted and there was not a clear difference between the loading of the peptide and the dye.

![Image after 1 minute incubation with myristoylated peptide showing the distribution of peptide within the cell.](image)

**Figure 25:** Image after 1 minute incubation with myristoylated peptide showing the distribution of peptide within the cell.

Ultimately, the myristoylated peptide will be used to load the exogenous peptide for enzyme assays testing for PKB activity since it was shown to have the highest percent of fluorescent cells with no measurable effect on cell viability. However, despite the effective loading of the myristoylated peptide, there was an uneven distribution of the peptide within the cell, which must be addressed before proceeding. As indicated by the arrows (Figure 23), some regions of the cells were brighter than others. In the past, this distribution has been observed in mammalian cells because of myristoylated peptide
being concentrated in membrane bound organelles. Therefore, this distribution is hypothesized to be due to the membrane bound organelles that could be uptaking the peptide, such as Golgi apparatus, lysosomes, or the endoplasmic reticulum. If the peptide enters any of these organelles, it will not be accessible to PKB for phosphorylation. Therefore, when the enzyme assays are conducted this challenge may cause PKB activity to be underestimated. If the peptide enters a lysosome, the peptide might be degraded upon entering; consequently, before continuing with single cell enzyme assays, this limitation should be further investigated to determine if there are any conditions that could be changed to minimize this effect.

Another project could also include increasing the concentration of the cell penetrating peptide to see if localization of the peptide can be avoided. Since we originally only used 20 µM, as suggested in the Cupid Peptides protocol, this concentration was low compared to the high concentration of dye (500 µM) used for pinocytosis and electroporation. By optimizing the loading of myristoylated peptide into the cell, the next step is to further analyze PKB activity through analysis of the ratio of phosphorylated to nonphosphorylated peptide in the cell.
8. References


