January 2016

Chloroplast Photo-Relocation Responses in model P. patens

Jason A. Meunier
Worcester Polytechnic Institute

Phytochrome Mediated Red Light induced Chloroplast Photo-Relocation Responses in *P. patens*

A Major Qualifying Project Report:

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

Jason Meunier

December 23, 2015

Contents

List of Figures

Abstract:

Chloroplasts are a vital component in plant cells due to their necessary role in photosynthesis. Here, we attempt to demonstrate a phenomenon known as photo-relocation which has been observed in chloroplast containing cells. Mediated by the phytochrome photo-transduction pathway under low intensity unilateral red light conditions, the model moss *Physcomitrella patens*' responses are analyzed. A model photo-relocation assay was used in which the unidirectional light vector was rotated 90° in order to isolate the chloroplast photo-movement response. Alignment of chloroplasts along cellular flanks as opposed to cellular cross walls may help indicate the role of certain molecular motors. Specifically, kinesins that have no involvement in cytokinesis are thought to play a major role in the regulation of chloroplast photo-movement. This study should emphasize the mechanisms and response tendencies with respect to chloroplast motility.

Chapter 1: Introduction

1.1: Project Introduction and Overall Perspective

The design of this MQP attempted to reproduce specific chloroplast responses to red light. In one case a banding pattern occurs in chloroplast containing cells under unilateral red light conditions. In another case, protenamata undergo a photo-response when a 90° light rotation is applied. Chloroplast banding patterns, or linearly arranged chloroplast accumulation along cellular cross walls, have been observed in experimental conditions using low unilateral red light (Kadota et al., 2000). Chloroplast banding patterns are believed to be an arrangement that is indicative of the photo-accumulation response. With the application of a 90° light rotation, chloroplast photo-movement may be better measured based on more obvious chloroplast orientation response. The molecular motor proteins that are responsible for photo-movement are believed to be a certain family of kinesins in *P.patens*. However, much of the specific biochemical pathways and genes involved in this response factor are still unknown. Developing a reproducible assay to evaluate photo-responses will help with understanding the participation of kinesins in chloroplast transport.

1.2: *Physcometrella patens* as a Model Organism

In the context of life science, in particular experimental biology, certain species of organisms have specific traits that lend themselves to being valuable assets for different applications. One of the most recognized model organisms in plant sciences is the moss *Physcometrella patens*. *P. patens* is a bryophyte, one of the oldest known divisions of plants. It is largely believed that bryophytes were the evolutionary descendants of algae (Rensing et al., 2009). This information would thereby suggest that moss-like species similar to *P. patens* were amongst the first land organisms. Furthermore, all land plants evolved from ancestral plants with similar characteristics to bryophytes. Scientists can therefore utilize the genetic traits of model moss species by considering relevant research pertaining to the mosses of interest by using it as a comparative system.

Since *P. patens* has been studied intensely, several aspects of the plants traits have been deemed useful for experimentation purposes. Most notably, the life cycle of this moss is mainly in the haploid stage. This indicates that only one copy of each chromosome is present in the plant's DNA for the majority of its existence (Cove et al., 2009). As opposed to a diploid stage, the haploid stage simplifies mutagenesis experiments because only one chromosomal copy is required to be mutated in this stage. This permits cellular mutational targeting to be carried out much faster and therefore poses as a useful aspect for the scope of this MQP. *P. patens* also has tremendous use in genetic editing through homologous recombination (Cove et al., 2009). By using gene targeting techniques, plasmids have been successfully integrated into the moss at which point foreign DNA was observed in the moss's genome (Schaefer, 1997). Therefore, *P. patens* is a viable specimen for conducting experiments using gene knockouts. An underlying reason this model moss is suitable for certain genetic techniques is that it has been fully sequenced (Cove et al., 2009). That is, *P.patens*' entire genetic code has been made available through the advancement of modern day sequencing techniques. Having this information about the moss genome is highly beneficial to research especially with respect to comparative genomic analyses. For example, four genes found in the complete DNA sequence for the chloroplast of the liverwort *Marchantia polymorphia* and the hornwort *Anthoceros formosae*, the close relatives of *P.patens*, are not present in our model mosses chloroplast genome (Sugiura et al., 2003). This suggests that there are some mutational variants present in the chloroplast genome of *P. patens*. Lastly, the mosses development is fairly simple and well understood. Even though it does not possess many of the physical structures that vascular plants hold, most of the signaling pathways found in vascular plants are also present in *P.patens* (Cove et al., 2009).

This MQP will attempt to utilize some of the unique characteristics that make *P.patens* useful as a model system. Of particular interest are the moss's light response mechanisms under red light induced conditions. Since *P.patens* has been fully sequenced and is a viable organism for recombinant DNA techniques, in the future we should be able to examine the primary genes that dictate these light response mechanisms under the experimental conditions. A distinct recombination technique could also be used in the future to support evidence of a given target gene function. This technique of discussion is known as RNAi.

1.3: RNAi

Interference RNA, or RNAi, is a naturally occurring mechanistic response to gene expression that employs double stranded RNA (dsRNA) as a means of gene knockdown (Zamore, 2000). Since its recognized use in experimental biology, RNAi has become more refined as a technique used to exploit loss of function responses in model systems. RNAi is a highly evolved mechanism by which unstable parts (repetitive sequences, transposons, ect.) of the genome may be recognized and blocked during expression. Occurring post-transcription, RNAi is ATP dependant but uncoupled from mRNA translation (Zamore, 2000). Further, dsRNA cleavage occurs at 21-23 nucleotides apart, resulting in fragments that guide mRNA cleavage (Zamore, 2000). The knockdown that occurs is a direct result of the lack of gene expression for the target sequence, demonstrating a loss of function response. *In vitro*, RNAi is apparent through creating a complimentary sequence for a target gene, introducing it into the organism which is recognized as exogenous genetic information, in turn synthesizing specialized dsRNA. This foreign DNA recognition will activate the RNAi pathway (Daneholt, 2007). With respect to this MQP, combined with the motility assay described, RNAi would be a technique that could allow proof of principle for the genes that are responsible for molecular motors directing chloroplast motility in *P.patens*. By targeting the genes thought to code for proteins involved in chloroplast photo-accumulation factors, an RNAi knockdown series could provide enough evidence for this notion to be supported. Future work concerned with the regulatory mechanisms of chloroplast photo-movement should focus on targeting the genes thought to be responsible for this phenomenon.

1.4: Polarized Cell Growth and Tip Growth

The main goal of all biological systems is to pass on genetic material. In all plant species, growth through cellular replication serves as the foundation to survival and reproduction. Two main classifications of cellular growth types are polarized growth and diffuse growth (Yoo et al., 2012). Diffuse growth allocates new growth material continuously along the expanding cell track, whereas polarized growth effectively isolates cell wall precursors to a specific point along the generated cell (Smith, 2005). One of the main challenges of this MQP will be designing a way to promote low density polarized growth in the protonemal cells of interest. This property of polarized growth has a propensity to yield an elongated shape where new targeted growth has occurred.

Polarized growth is a specialized plant function that enables certain cellular tasks to develop in that area. Further, spatially focused cell expansion through a particular type of polarized growth known as tip growth, has been observed in pollen tube cells and root hair cells (Cole, 2006). Tip growth, aids in the development of the species by promoting cell augmentation, or the cellular maturation and expansion. In mosses, protonemal cells are specialized cells whose primary function is the extension into surrounding environments for nutrient uptake (Hepler et al., 2001). In bryophytes, protonemal expansion is defined by the same tip growth phenomenon as previously mentioned-polarized growth.

Actin filaments play a key a role in the function of tip growth in *P.patens*. These linear polymers comprised of globular subunits serve multifunctional purposes including proper cell growth and division as well as defining cell morphology (Harries et al., 2005). Moreover, Harries et al. demonstrate that by using loss-of-function mutants by RNAi, severe defects in cell shape and division patterns occur. By blocking the expression of the actin-related protein2/3 (Arp2/3) complex, responsible for normal dynamics in the actin filaments, absence of caulonemal cell type (normally found in protenamal cells in P.patens) ensues (Harries et al., 2005). Hence, it is apparent that without proper expression of certain proteins responsible for actin regulation, normal polarized cell growth is negatively affected. Additionally, cell polarity has been observed to be controlled by microtubules in *Physcomitrella patens* (Doonan et al., 1988). This section represents a complex network of actin filaments and microtubules which make up the molecular motor system responsible chloroplast motility. An understanding of polarized cell growth is important for this MQP as targeted cells can be isolated by using light-directed methods to encourage cell expansion in a unidirectional manner. The cell expansion concepts discussed above will be important in determining the *in-vitro* results explored later in the body of this paper.

1.5: Light Spectrum and the Physics of Light

A basic conceptualization of how light works is important to understand plant growth, reproduction, and biochemical processes. Since this MQP focuses on the molecular mechanisms of chloroplast motility, the main environmental stimuli (light source) responsible for influencing chloroplast response factors in *P.patens*, must also be explored. The mechanisms by which the plant harnesses light cannot be described without understanding the

fundamental principles of light. Light energy is simply the accumulation of electromagnetically charged particles passing through a distance in space, exhibiting traits of both rays and waves. Light units (quanta) are measured in photons, or the summation of all charged particles comprising the beam, which has no mass. Additionally, there has to be a source of light in which the originating radiation particles are formed. In a beam of light, an electromagnetic field is created through accelerating a particle like an electron, by an electrical field. This electromagnetic field constitutes an oscillating wave with a perpendicular electrical field and an opposite perpendicular magnetic field (Pecora, 2013).

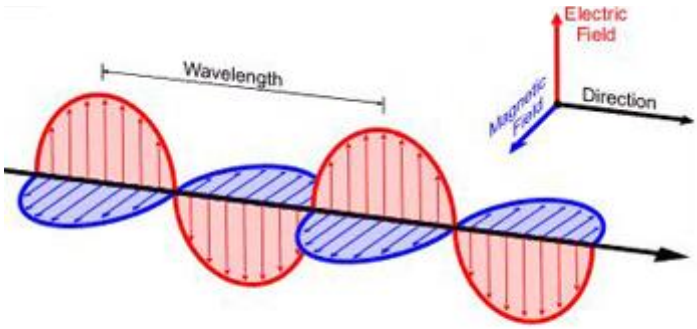


Figure 1: Electromagnetic waves (NOAA, 2015)

The measurement of the apexes of the sinusoidal waves describes what is known as wavelength. Wavelength can therefore be calculated by subtracting the crests of two successive waves, which is measured in nanometers (nm.).

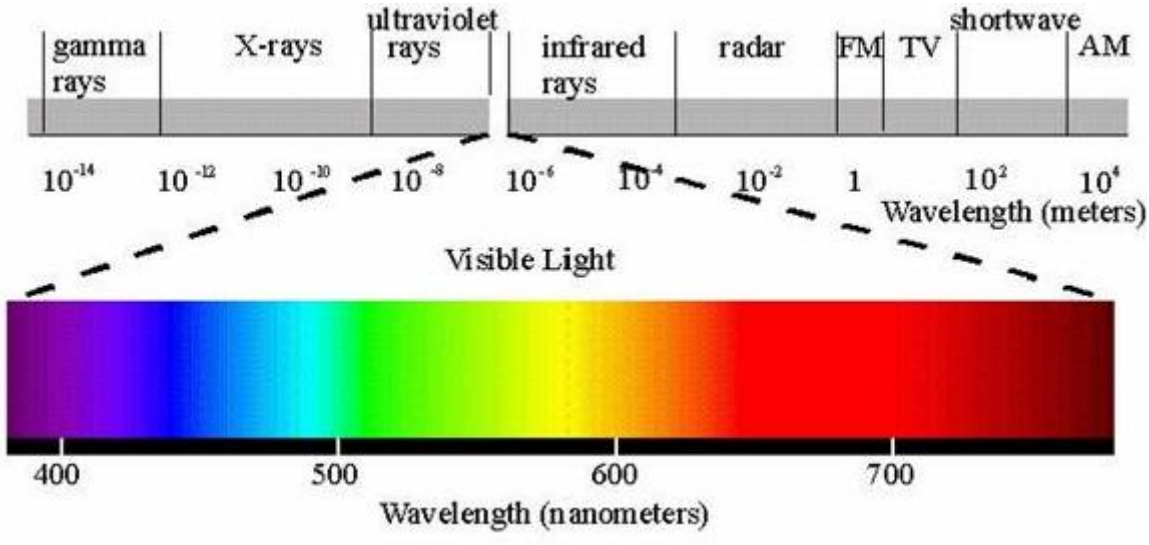


Figure 2: Light Spectrum (Beer Color Laboratories, accessed 2015)

In *Figure 2* above, we see that the high frequency rays (short wavelengths) like gamma rays fall out of the visible spectrum of humans. The high wavelength has an inverse property with frequency and energy, as long wavelength rays have low energy and frequency. Likewise, short wavelengths tend to have high energy and high frequency values. For the scope of this MQP, it is the small area in figure 2 known as the visible light spectrum that we are concerned with. Visible light consists of the seven main colors recognizable by the human eye: red, orange, yellow, green, blue, indigo, and violet. The visible spectrum falls between the ranges of 390-700 nanometers (Starr, 2005). Like humans, plants too are able to decipher energy in this range. Sunlight, the plants main energy source, gives off what we know as white light. Through experimentation with prisms, scientists were able to split white light to emit the seven colors comprising the visible light spectrum. Hence, white light is the combination of all wavelengths in the visible light spectrum. Plants have photoreceptors that are able to decipher energy in this range and react with respective biochemical responses. However, since natural light is an uncontrollable and inconsistent source, experimentation utilizing natural light as the main source is severely limited. It is therefore imperative that artificial lighting is used under experimental methods. Not only can exposure time be regulated, but specific wavelengths can be isolated at smaller ranges to analyze the effect of a specific color on the plant's photochemical responses.

1.6: Review of Basic Chloroplast Function and Physiological Responses

All plants have a general structure for capturing light, metabolizing light energy to useful chemical energy, and regulating the processes involved with photosynthesis through adaptation. For example, plants have organelles known as chloroplasts which contain the main pigments responsible for capturing light. They can be visualized as mini solar panel cells that are directed by biochemical signals to optimize light intake by synchronizing movement with light source angles. Evolutionarily, plants have been selected for various methods to cope with the perpetual fluctuation of light intensity. The plant may begin to grow or bend in a certain angle to accommodate the need for more or less sunlight. This phenomenon is known as phototropism. This process can take several days or longer depending on conditions like light intensity, plant species, and other environmental stimuli. However, another usually quicker response can take place at the chloroplast level. Photo-movement, described as chloroplast motility, is the chloroplast response to a light source.

Since chloroplasts are very sensitive to light, a suitable response to light conditions must occur in order to both preserve the integrity of the cell and optimize light absorbance. Two distinct photo-movement responses in chloroplasts are termed photo-accumulation and photo-avoidance (Sato, 2001). Photo-accumulation occurs when chloroplasts collect in a concentrated area to fully optimize light availability. On the contrary, a photo-avoidance response favors the dispersal of chloroplasts in a given area to lessen the harmful effects of too much light (Sato et. al., 2001). Photo-movement responses are central in order to grasp the experimental conditions of this MQP. Setting up a precise amount of red light for the purposes of acquiring unidirectional protenemal growth patterns and uniform chloroplast banding (Sato et. al.,2001), was indeed a challenging task.

1.7 Molecular Movement along the Cytoskeleton and Motor Proteins

The cytoskeleton is a part of cells that's primary role is to provide a framework for the cell. Comprised of a series of proteins, the cytoskeleton provides much more than structural integrity to the cell. Within the cytoskeleton, molecular transportation occurs via membranes and various organelles. The dynamic flux of passive and guided molecular movement makes this area of the cell a multifunctional unit. Importantly, the cytoskeleton is the region believed to contain molecular motors responsible for chloroplast motility. Specifically in chloroplast containing cells, recent studies have suggested an intricate system supporting photo-movement responses in our model moss *P. patens*. Two main components of the cytoskeleton are the microtubules (MT) and the smaller microfilaments (MF). Respectively, these cylindrical and linear shaped molecules serve as multifunctional backbones, which together comprise the greater cytoskeleton (Vale, 2003). Furthermore, both MT's and MF's allow important motor protein movement. These are ATP-dependent reactions, meaning that movement is guided through ATP-motive forces.

1.8 Kinesins

In MT's, the main motor proteins present in plant cells are known as kinesins (Vale, 2003). Kinesins move across the MT's as a transport mechanism of cell cargo. Due to their physical makeup and the interaction with MT's structure, kinesins have a specific direction of motion. Generally, MT's are polar structures drawing to the kinesins affinity to take a path of directionality (Schnitzer, 1997). However, it should be noted that not all kinesins act in anterograde transport. For example, kinesins of the superfamily 1 and 2 in *P.patens* have been observed to move toward the MT's positive end

while kinesin 14 members travel in the opposite direction, towards the negative end (Shen et. al., 2012). Made up of nearly 360 amino acids, the kinesin motor domain is quite variable. The kinesin head is not conserved over all kinesin families in *P.patens* as it can be located at either terminus, the amino-terminus or carboxyl terminus, as well as in the center of the kinesin (Shen et. al., 2012). With so much variation among the kinesin families, much of the specific kinesins functionality in our moss is yet to be understood. Kinesin identity has currently expanded to 72 different known kinesins organized into 15 superfamilies for *P. patens* (Shen et. al., 2012). However, of the 72 known kinesins, only 43 of them are MT-based (Miki. et. al., 2014).

MT-based kinesins serve an integral functionality for the expansion of the moss cells. Specifically, these kinesins regulate cytokinesis by directing and separating the cells chromosomes, which occurs at the mitosis phase (Zhu, 2005). Based on the findings of Miki et. al., stating that only a portion of all 72 known kinesins are MT-based, a significant question as to what the other kinesins are responsible for in terms of processes, concerns itself into the broader scope. Analysis of these kinesins shows that two particular types of kinesins are not involved with any regulation of cytokinesis during mitosis. These two kinesin subfamilies of *P.patens* that may be of particular interest in this MQP are referred to as kinesins 4II and 7I (Miki. et. al., 2014). Since their functionality is non-inclusive of cell growth, a potential purpose for their existence is the regulation of chloroplast motility.

1.9 Phytochrome and Blue Light Receptor

Absorption efficiency, or the action spectrum, for the light absorbing pigments in *P. patens* plays a key role in determining how well the light receptors will respond to various test conditions. . Two particular spectrum ranges that are chief to this MQP fall within the blue and red ranges, respectively. It is the wavelength ranges that predominantly translate to blue and red light, that are at the highest absorption rates in most plants including *P.patens*. Since the mosses receptors help optimize light absorption, light from of these two wavelengths are vital for proper photosynthetic activity. Chlorophyll a and b, the main photosynthetic pigments, are highly sensitive to red and blue light. *Figure 3* below, graphically shows absorption rates of several plant pigments at specific wavelengths. It is most important to note

the absorption wavelength ranges of chlorophyll a and b, as these are the pigments of interest. Depicted are high absorption rates of wavelengths for blue light and red light.

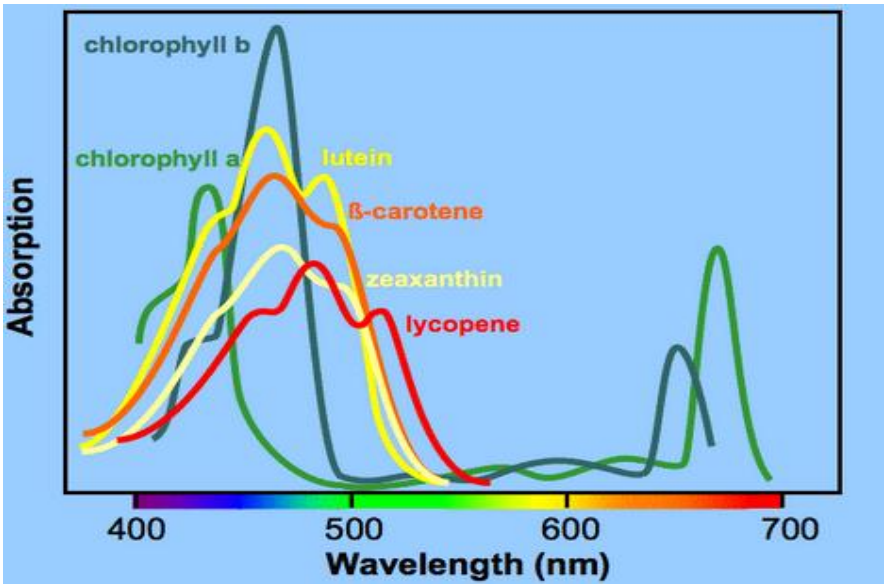


Figure 3: Pigment Absorption Rates vs. Wavelength (nm.) (Koning, 1994)

Due to the tendency of *P.patens* to absorb red and blue light more readily, a certain dynamic relationship takes place between light receptors and chloroplast organelles. Light induced chloroplast movement can be accomplished by isolating red or blue light *in-vitro* and directing growth to either an MT track or an MF track (Sato et al., 2001). Both blue and red wavelengths have corresponding light receptors in *P. patens* responsible for initiating necessary biochemical responses. Blue light is mainly processed by the phototropin and red light by phytochrome receptors(Sato et al., 2001; Kasahara et al., 2004). It has been observed however, that phototropin light receptors in *P.patens* can mediate both blue and red light-induced chloroplast movement (Kasahara et al., 2004). Therefore, light-induced chloroplast movement is not exclusively driven by the corresponding light receptor for phototropin dominated reactions. Moreover, *P. patens* chloroplast photo-accumulation and photo-avoidance movement has been determined to be mediated by both the blue light receptor as well as phytochrome (Sato et al., 2001). This study also found that an MF-based photorelocation track took place in the moss. Additionally, results showed a differentially selected MT-based photorelocation track (Sato et al., 2001). Using immunofluorescence labeling of MT's and MF's, chloroplast photo-accumulation and photo-avoidance response factors were induced with blue light and red light microbeam irradiation. Further, the effects of inhibitors Cremart and cytochalisin B, on MT's and MF's respectively, were analyzed (Sato et al., 2001). Conclusively, the authors

observed that chloroplasts can use both MT's and MF's as movement tracks, and that these distinct motility systems are regulated differently by blue light receptor and phytochrome. (Sato et al., 2001).

2.0 Project Hypothesis and Expected Results

Chloroplast response mechanisms were closely followed throughout the series of experiments described in this MQP by using specific conditions to isolate this behavior. Chloroplast photo-response alignments demonstrate a characteristic banding pattern in which chloroplasts orient linearly along cellular cross walls. Upon a 90° light rotation, chloroplast responses were expected to follow re-orientation behavior-gathering at cellular flanks facing the plane of light. The specific chloroplast responses after the 90° rotation are not well documented. Hence, it is our goal to partially recreate results described by Kadota et al. in which chloroplasts were observed to realign under the 90° light rotation. Additionally, certain molecular motors (possibly kinesin 4II and 7I) are believed to be responsible for chloroplast motility under low unilateral red light conditions, in the model moss *Physcomitrella patens*. Initiated through the phytochrome phototransduction pathway, chloroplast photo-movement is thought to be dependent on kinesin activation through this pathway. Therefore, by using gene knock-downs of target genes expressing kinesins responsible for chloroplast motility, future work should focus on generating a loss of function response in these genes of interest.

Chapter 2: Methods

Consistently throughout the span of seven months, moss lines of *P. patens* were cultured using sterile cell culture technique. Usually conducted every 3-4 days, new lines were passed in order to keep fresh reserves of cells for experimental analysis. Approximately 10 days after cell lines were passed, cell proliferation became too dense throughout the plate for quality microscopy imaging. Additionally, unidirectional protonemal growth patterns as well as chloroplast banding was clouded by cell density. Hence, there was a constant need for fresh cell lines. Initially acquired from several master lines (courtesy of Professor Vidali's lab at Gateway Park), daughter cells were grown with pre-packaged moss specific cell culture media containing $PpNO_3$, manufactured by Caisson Labs Inc. Although, early colonies used a different moss media, $PpNH_4$. Due to the partial insolubility of $PpNH_4$, this medium left behind a precipitate. All experimental results were obtained using $PpNO_3$ instead. Other constituents in the moss media included high gel strength moss agar produced by Sigma Life Science, 800 mL of deionized water, and 4 grams (.005%) of sucrose. Sucrose addition to the media was not consistent in the early stages of the experimental results, but was included for the majority of plates analyzed for data. The use of carbohydrates as a supplemental energy source was adopted from Kadota et al. in which they describe the use of glucose to grow protonemata of *P. patens* under red light conditions (Kadota et al., 2000).

Cell transfer was entirely conducted under a Nuaire class II fume hood while adhering to all sterile cell culture protocols (see appendix). Surface sterilization was treated with 70% ethanol. Daughter cell lines were transferred sterilely to individual 100 mm. by 15 mm. Celltreat Petri dishes containing PpNO3 media covered by a thin layer of cellophane. New plates were cultured using low density moss cells; about one-third of the master cells were obtained for each new set of cells using a sterile metal spatula and transferred to sterile test tubes containing 4 mL of water. After initial cell transfer to each test tube, a cell grinding process took place using the Omni TH Tissue Homogenizer. For each sample, a separate sterile plastic grinding tip was replaced into the grinder head. The grinding process was mainly

conducted at low speeds for 10 to 15 seconds to ensure a thick mixture with no cell clumps. Next, 1.5 mL from each mixture was evenly transferred using a sterile pipette into both of the corresponding $PpNO_3$ Petri dishes. Additionally, 1 mL was transferred into an LB agar dish and stored at room temperature which provided a control for contamination. PpNO3 plates were incubated at 25°C for under artificial light for 18 hours each day and no light for the other six hours per day.

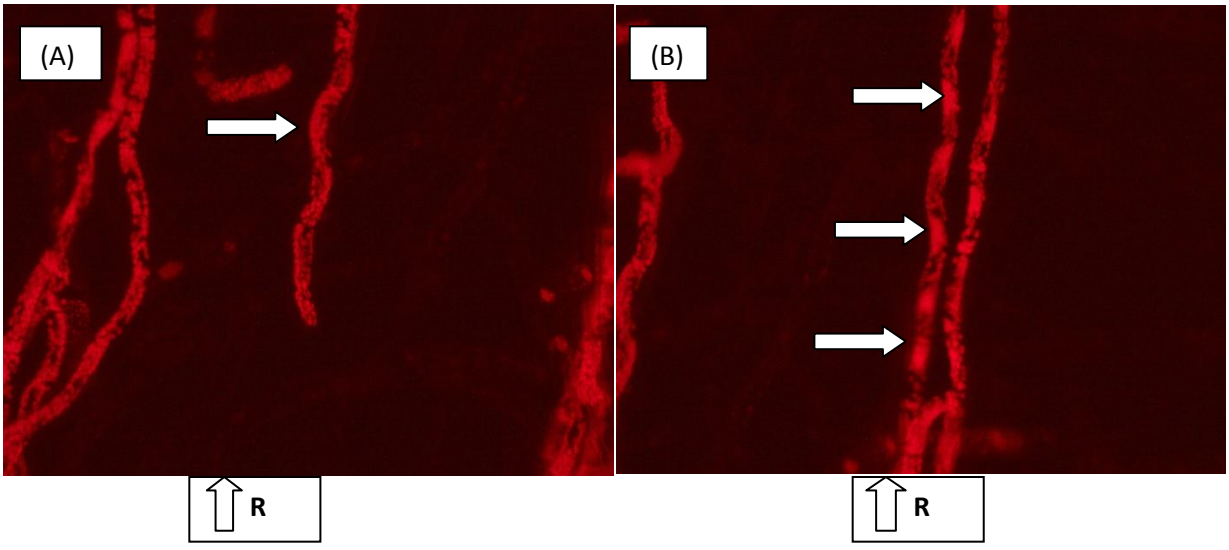
Early in experimentation, a one week growth phase was applied under LED red/blue light before moving samples to unilateral LED red light conditions for 1-3 days. A conditional alteration was made in which samples were immediately placed in red LED light conditions for 7-10 days. This change attempted to more effectively isolate the chloroplast photo-movement response. Three 22.5 mm. multi-celled vertical column plastic holding containers were integrated into red light conditions for the purpose of facilitating unidirectional growth patterns. The entire vertical shelving system was painted black in order to absorb the light surrounding the samples. This method ensured that each sample would receive an equal concentration of light by removing the possibility of light dispersion and reflection. The specific amount of light that the samples received under red light conditions was measured using an Extech footcandle (Fc) light meter. This converts the light emmitance from any light source into foot-candles. Finding the optimal low light levels under red light conditions was a challenging task. After varied results, the desired dim light condition was determined to be only 6 footcandles. In order to acquire such a low level of light, standard white parchment was folded twice to form a double layered barrier which allowed for low light transmittance.

For microscopy, samples were observed using an inverted Zeiss epi-fluorescent microscope equipped with a Semrock BrightLine fluorescent filter cube. Prior to analysis of chloroplast movement, some preparatory work was necessary. Less than 10 mL of moss media was melted and poured into a Petri dish cover and allowed to dry. Before microscopic examination, the cellophane containing moss cells was carefully taken out of its original dish using forceps, and placed in the new dish cover. The purpose for conducting a pre-microscopy cellophane transfer is because the Zeiss viewing lens is inverted, creating an inverted view frame for imaging. By also inverting the cellophane strip onto a new plate cover, the view becomes inverted. Moss samples that were taken out of their respective growth chambers for analysis, were stored in a dark box during relocation between Goddard MQP labs (GH 006) and the Goddard microscope

lab room (GH 210). Light levels in this room were kept very low in order to minimize any light contamination to our chloroplast photo-response. Sample exposure to the unilateral light angle was maintained by drawing an arrow at the bottom of each Petri dish, indicating the open slots facing the light source. Under microscopic analysis, protonemata growing on the periphery of the plates tended to grow under much less cellular density than protonemata in the middle of the plates. Therefore, the majority of the results were acquired from these protonemata. Additionally, observed moss samples containing protonamata with chloroplasts responding in a banding pattern, were chosen for further incubation. These samples were rotated 90° and exposed to the same unilateral red light as before. This additional incubation time was only for 24 hours at which point the samples were again analyzed using fluorescent microscopy.

Chapter 3: Results

In these experiments, cultures of *P. Patens* were grown under low intensity unilateral LED red light for 7-10 days. The results were representative of chloroplast motility which was observed and recorded through microscopy imaging. The selections of figures for the results were chosen from over forty acquired pictures to best demonstrate the chloroplast movement. In order to observe chloroplast photo-movement in relation to the light plane, light direction is represented by the "R" labeled arrows in figures 4 and 5 below. All photographs below were taken with a fluorescent filter cube to capture the chloroplast alignment. Figure 4 shows chloroplast banding in several protonemal cells. The protonemal cells developed a long cylindrical morphology with little to no branching of filaments. Protonemata grew phototropically toward the unidirectional light. Chloroplasts tended to gather at the cellular cross-wall regions separating adjacent cells as seen in panel (C). This is depicted by areas of intense red at the division of each cell. Chloroplasts which responded in this manner demonstrated photo-accumulation. However, this observation is inconsistent through all protonemata. While some chloroplasts do demonstrate the banding patterns as expected, others do not have any uniform orientation. The overabundance or saturation of chloroplasts in one area is also evident in figure 4 panels (A, B, and C). Some protonemal cells have a majority of chloroplast banding, while others in the same picture are variable.



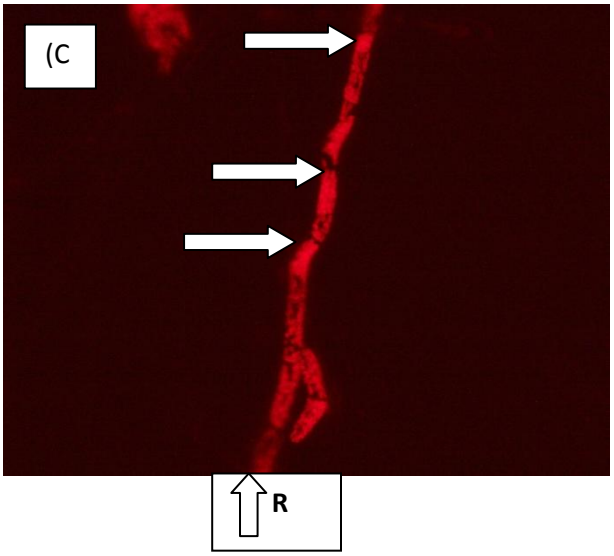
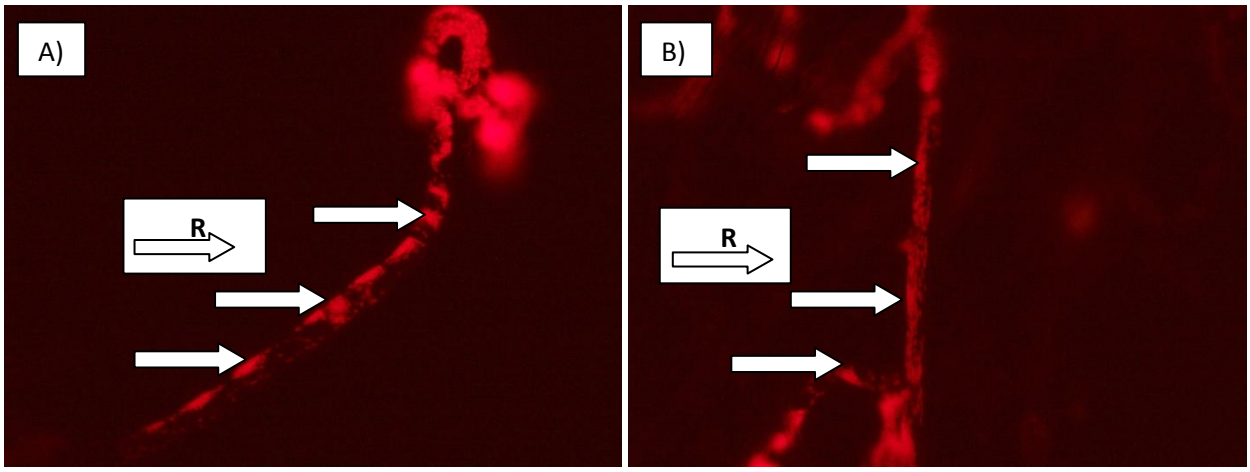


Figure 4: Chloroplast Banding along Cross-Walls

To try to establish a more reproducible assay for chloroplast accumulation after allowing the protonemal cells of *P. patens* to elongate under unidirectional red light, an additional 24 hour incubation period was applied. Cells in this additional treatment were rotated 90° and exposed again to unilateral red light. Figure 5 shows chloroplast response to 90° light rotation after conditions where some chloroplast banding in the protonemata was previously observed. We see clear photo-movement of chloroplasts under this condition. Instead of a uniform banding pattern along cellular cross-walls however, chloroplasts in figure 5 accumulate along the longitudinal sides of the cells, facing the light plane. Protonemata are still growing in the initial direction of the light plane from figure 4, but the chloroplasts accumulate according to the 90° rotation. Little filament branching is observed in figure 5, but more than observed in figure 4.



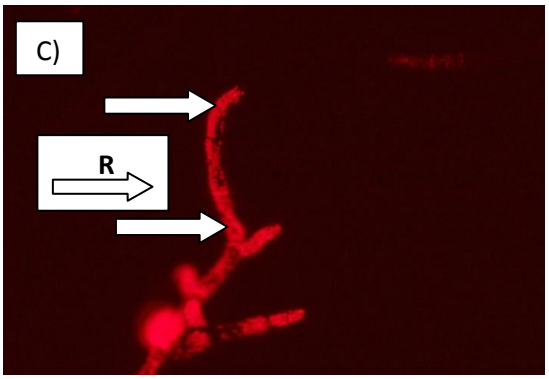


Figure 5: Chloroplast Accumulation along Cellular Flanks facing the Light Plane after 90° Rotation

A close up of this phenomenon is showed in figure 6 below. Note that chloroplasts are mainly absent on one side of the cell periphery. This indicates the opposite side of the cell that is not directly facing the light plane. Chloroplasts reorient themselves in figure 6 in accordance with a southern light plane light.

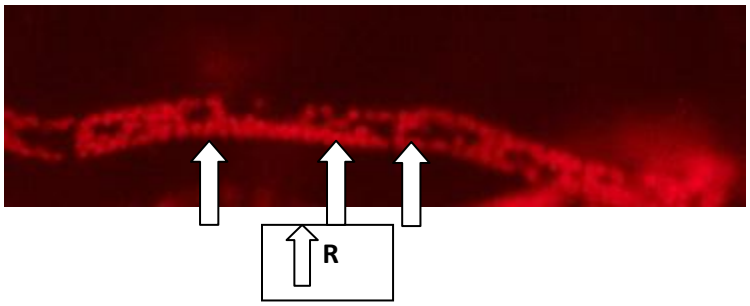


Figure 6: Close up of Chloroplast Photo-Relocation

Chapter 4: Discussion

Through development of the series of experiments, we analyzed the specific chloroplast photo-movement and orientation effects under exposure to low intensity red light. It is reported here that chloroplasts exposed to unilateral red light for approximately 1 week demonstrated a characteristic banding response along the cellular cross-wall sections in the protonemal cells. High groupings of chloroplasts along these cellular separation regions were qualitatively seen in figure 4as intense red output sections. This response was in fact expected as previously described by Kadota et al, 2000. However, a major inconsistency has been observed in figure 4as many individual protonemta do not demonstrate chloroplast banding which can be observed in adjacent cells. Rational for this variable behavioral may be due to the over saturation of light. While the light output was measured at approximately 6 footcandles, lower light intensity with the use of a filter may be required for future experiments.

Additionally, we observed a chloroplast photo-movement response that differed from the banding alignment. With the application of a 90° rotation of the unilateral light source and an additional 24-hour incubation period of samples demonstrating chloroplast banding, we observed chloroplast accumulation in response to the electrical vector. When the light source was parallel to the cellular cross-walls, chloroplast distribution along these cross-walls followed. This phenomenon was described previously as the banding response. However, when the electrical vector was rotated 90° (ie. the light source being perpendicular to the cross-walls), no chloroplast banding was observed. Instead, accumulation of chloroplasts along cell flanks as well some random distribution occurred. Since we see chloroplasts capable of movement and distribution along areas of the cell that would maximize light exposure in only 24 hours, it is suggested that the chloroplast photo-movement is controlled differently controlled differently than the protonemal phototropic response.

It is suggested that protonemal tropic responses are regulated by phytochrome molecules found at the cell periphery on the plasma membrane (Etzold 1965; Kadota et al., 1989). Chloroplast photo-relocation responses have been observed to be differentially regulated by phytochrome as well as the blue light receptor (Sato et al. 2001). The molecular motors that are thought to be responsible for the chloroplast relocation phenomenon are kinesins.

Specifically, kinesins of the family 4II and 7I are not involved in cytokinesis but may be responsible for regulation chloroplast photo-movement (Miki et al. 2001). In order to accurately determine which of these kinesins are molecular motors driving chloroplast photo-relocation, both genes would have to be knocked down separately. Through a loss of function analysis, future work could therefore direct research to the exact genes responsible.

Improvements to the experiments presented here may be made in order to better demonstrate chloroplast photo-movement. It was determined that a low intensity of red light was required during the incubation phase of the moss cells. However, we observed chloroplasts that were largely saturated in areas that were inconsistent with our hypothesis. By reducing the light intensity to around 3 footcandles, it is assumed that less chloroplasts would respond in this inconsistent manner. Additionally, another design change may have to be considered which substitutes glucose for sucrose dissolved in the moss media. It was demonstrated by Kadota et al. that the use of 0. 5% glucose as a supplemental nutrient source yielded consistent results. Finally, it should be noted that many results failed in this experiment due to mold contamination. Creating optimal grow environments and successful protonamata responses was an extremely difficult task. Successful results discussed in figures 4-6 should be repeated and quantified in order to measure the reproducibility of the established assay.

Chapter 5: References

In APA form:

"Color Basics." Beer Color Laboratories, n.d. Web. 10 Aug. 2015.

Cecie Starr (2005). *Biology: Concepts and Applications*. Thomson Brooks/Cole.ISBN 0-534-46226-X.

Cole, R. A., & Fowler, J. E. (2006). Polarized growth: maintaining focus on the tip. *Current opinion in plant biology*, *9*(6), 579-588. "Color Basics." Beer Color Laboratories, n.d. Web. 10 Aug. 2015.

Cove, D., Bezanilla, M., Harries, P., & Quatrano, R. (2006). Mosses as model systems for the study of metabolism and development. *Annu. Rev. Plant Biol.*,*57*, 497-520.

Cove, D. J., Perroud, P. F., Charron, A. J., McDaniel, S. F., Khandelwal, A., & Quatrano, R. S. (2009). The moss *Physcomitrella patens*: a novel model system for plant development and genomic studies. *Cold Spring Harbor Protocols*, *2009*(2), pdb-emo115.

Cove, D. J., Knight, C. D., & Lamparter, T. (1997). Mosses as model systems.*Trends in Plant Science*, *2*(3), 99-105.

Daneholt, B. (2007). Advanced information: RNA interference. *The Nobel Prize in Physiology or Medicine 2006*.

Doonan, J. H., Cove, D. J., & Lloyd, C. W. (1988). Microtubules and microfilaments in tip growth: evidence that microtubules impose polarity on protonemal growth in *Physcomitrella patens*. *Journal of Cell Science*, *89*(4), 533-540.

Jaffe, L., & Etzold, H. (1965). Tropic responses of Funaria spores to red light. *Biophysical journal*, *5*(5), 715.

Harries, P. A., Pan, A., & Quatrano, R. S. (2005). Actin-related protein2/3 complex component ARPC1 is required for proper cell morphogenesis and polarized cell growth in *Physcomitrella patens*. *The Plant Cell*, *17*(8), 2327-2339.

Hepler, P. K., Vidali, L., & Cheung, A. Y. (2001). Polarized cell growth in higher plants. *Annual review of cell and developmental biology*, *17*(1), 159-187.

Kadota, A., Sato, Y., & Wada, M. (2000). Intracellular chloroplast photorelocation in the moss *Physcomitrella patens* is mediated by phytochrome as well as by a blue-light receptor. *Planta*, *210*(6), 932-937.

Kadota, A., & Wada, M. (1989). Photoinduction of circular F-actin on chloroplast in a fern protonemal cell. *Protoplasma*, *151*(2-3), 171-174.

Kagawa, T., & Wada, M. (2002). Blue light-induced chloroplast relocation. *Plant and cell physiology*, *43*(4), 367-371.

Kasahara, M., Kagawa, T., Sato, Y., Kiyosue, T., & Wada, M. (2004). Phototropins mediate blue and red light-induced chloroplast movements in *Physcomitrella patens*. *Plant Physiology*, *135*(3), 1388-1397.

Koning, Ross E. 1994. Light. Plant Physiology Information Website. http://plantphys.info/plant_physiology/light.shtml.(8-2-2015)

Miki, T., Naito, H., Nishina, M., & Goshima, G. (2014). Endogenous localizome identifies 43 mitotic kinesins in a plant cell. *Proceedings of the National Academy of Sciences*, *111*(11), E1053-E1061.

Miki, T., Nishina, M., & Goshima, G. (2015). RNAi screening identifies the armadillo repeat-containing kinesins responsible for microtubule-dependent nuclear positioning in *Physcomitrella patens*. *Plant and Cell Physiology*, *56*(4), 737-749.

Pecora, R. (Ed.). (2013). *Dynamic light scattering: applications of photon correlation spectroscopy*. Springer Science & Business Media.

Rensing, S. A., Lang, D., Zimmer, A. D., Terry, A., Salamov, A., Shapiro, H., ... & Rushton, P. J. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science*, *319*(5859), 64-69.

Sato, Y., Wada, M., & Kadota, A. (2001). Choice of tracks, microtubules and/or actin filaments for chloroplast photo-movement is differentially controlled by phytochrome and a blue light receptor. *Journal of Cell Science*, *114*(2), 269-279.

Sato, Y., Kadota, A., & Wada, M. (2003). Chloroplast movement: dissection of events downstream of photo-and mechano-perception. *Journal of plant research*, *116*(1), 1-5.

Sato, Y., Wada, M., & Kadota, A. (2003). Accumulation response of chloroplasts induced by mechanical stimulation in bryophyte cells. *Planta*,*216*(5), 772-777.

Schaefer, D. G., & Zrÿd, J. P. (1997). Efficient gene targeting in the moss *Physcomitrella patens*. *The Plant Journal*, *11*(6), 1195-1206.

Schnitzer, M. J., & Block, S. M. (1997). Kinesin hydrolyses one ATP per 8-nm step. *Nature*, *388*(6640), 386-390.

Shen, Z., Collatos, A. R., Bibeau, J. P., Furt, F., & Vidali, L. (2012). Phylogenetic analysis of the kinesin superfamily from *Physcomitrella*. *Frontiers in plant science*, *3*.

Smith, L. G., & Oppenheimer, D. G. (2005). Spatial control of cell expansion by the plant cytoskeleton. *Annu. Rev. Cell Dev. Biol.*, *21*, 271-295.

Takagi, S. (2003). Actin-based photo-orientation movement of chloroplasts in plant cells. *Journal of Experimental Biology*, *206*(12), 1963-1969.

Tyska, M. J., & Warshaw, D. M. (2002). The myosin power stroke. *Cell motility and the cytoskeleton*, *51*(1), 1-15.

Vale, R. D. (2003). The molecular motor toolbox for intracellular transport. *Cell*,*112*(4), 467-480.

Vidali, L., Augustine, R. C., Kleinman, K. P., & Bezanilla, M. (2007). Profilin is essential for tip growth in the moss *Physcomitrella patens*. *The Plant Cell*,*19*(11), 3705-3722.

Vidali, L., & Bezanilla, M. (2012). *Physcomitrella patens*: a model for tip cell growth and differentiation. Current opinion in plant biology, 15(6), 625-631.

Wada, M., Kagawa, T., & Sato, Y. (2003). Chloroplast movement. *Annual Review of Plant Biology*, *54*(1), 455-468.

Welte, M. A. (2004). Bidirectional transport along microtubules. *Current Biology*,*14*(13), R525-R537.

Yoo, C. M., Quan, L., Cannon, A. E., Wen, J., & Blancaflor, E. B. (2012). AGD1, a class 1 ARF-GAP, acts in common signaling pathways with phosphoinositide metabolism and the actin cytoskeleton in controlling Arabidopsis root hair polarity. *The Plant Journal*, *69*(6), 1064-1076.

Zamore, P. D., Tuschl, T., Sharp, P. A., & Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *cell*, *101*(1), 25-33.

Zhu, C., Zhao, J., Bibikova, M., Leverson, J. D., Bossy-Wetzel, E., Fan, J. B., ... & Jiang, W. (2005). Functional analysis of human microtubule-based motor proteins, the kinesins and dyneins, in mitosis/cytokinesis using RNA interference. Molecular biology of the cell, 16(7), 3187-3199.

Appendix I.

Instructions to make PpNO3

- Pouch into ~800 ml of water
- Add water to pouch 3 times to get all the stuff
- Stir 30-60 min
- Add 8 grams of Plant tissue culture agar
- Autoclave at 120C for 20 min
- Cool for ~30-60 min
- Pour 30 ml/plate (10 cm)
- Dry at room temp for 24 hr
- Pack on the same plastic bag
- Store at room temp. with lids on top

Appendix II.

Passing Lines Protocol

Preparing Plates

- Using 70% ethanol, spray down the following:
  - inside of hood
  - sleeves of plates (both the media that you're using – generally $PpNO_3$ or $PpNH_4$ – and LB)
  - package of large cellophanes
  - ethanol burner (filled with 99% ethanol)
  - bottle of 99% ethanol
  - tweezers
  - marker
  - (gloved) hands
- Take out plates – for each line you're passing, you'll need 2 big media plates and 1 small LB plate.
- Label with line, initials, and date.
- Light burner and flame tweezers before proceeding.
- Open package of cellophane and place one cellophane onto one media plate. Use tweezers to push out air bubbles. Flame tweezers and repeat for each plate.

Harvesting

- Using 70% ethanol, spray down the following:
  - purple tube holder
  - bag of culture tubes
  - 10 mL pipet
  - bottle of dH_2O
  - small metal spatula
  - (gloved) hands
- Take out one culture tube for each line you are passing (put tube in purple holder), and label with line name.
- Fill tube with the proper amount of dH_2O. For 1 line you will need 2 mL. Otherwise you will need 700 µL for each *plate* you are passing onto and ~400 µL for the LB plate.
- Flame spatula well and allow to cool on top of ethanol bottle before proceeding.

- Un-tape the line you are passing and touch spatula to media to cool. Then use spatula to harvest proper amount of moss (generally ~1/4 of the plate). Place moss in the proper tube of water. (Repeat for any more lines that you may have.)

Grinding and Pipetting

- Using 70% ethanol, spray down the following:
  - grinding machine
  - 1 grinder for each line you're passing
  - 1000 µL micropipette
  - Micropipette tips
- Place grinder into grinding machine. Place grinder into culture tube, ensuring that the tip of the grinder is submerged in the water but does not touch the side of the tube.
- Turn grinder on. Start at 1 and move up to 6. Grind at 6 for a moment and move down to 1 again before turning the grinder off. Ensure that no large chunks of moss remain in the water. (Repeat for each line.)
- Use micropipette to put 700 µL of ground moss onto each media plate. Put ~400 µL on the LB plate. Shake plates to distribute the liquid.

Taping/Finishing Up

- Using 70% ethanol, spray down your hands. Then add a little bit more to your hands and use this to disinfect a role of micropore tape.
- Put micropore tape around sides of each plate (both regular media and LB). Press down on tape to ensure no gaps remain that could let in contamination.
- Once tape is sealed, you are done. Take everything out of the hood and spray the hood down with 70% ethanol again.