Screen for Photoreceptor and Other Eyespot Defective Mutants in Chlamydomonas

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Screen for Photoreceptor and Other Eyespot Defective Mutants in Chlamydomonas

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Abstract:

The purpose of this project was to test mutant strains of *Chlamydomonas reinhardtii* for a variation in eyespot location and size. The technique of phototaxis was used to determine if mutant strains have an altered ability of light reception. Cells from 96-well plates were transferred into the respective tubes filled with 2.2 mL of M medium. One row of tubes was placed in a test tube rack and two racks were placed in each phototaxis box. After twenty minutes in each box, where the only amount of light being let in is from a tiny slit directed on the sample, the tubes were removed and then checked for completion of phototaxis. After noting whether the samples completed phototaxis or not, the phototaxis-negative cells were regrown for further observation and ultimately a PCR screen for the mutant gene sequences. Out of roughly 5,000 mutant samples screened using phototaxis, twelve samples were phototaxis-negative and only four of the twelve samples were phototaxis-negative the second trial. Further experimentation would include the observation of the eyespots under a microscope, culturing these samples, harvesting the DNA via electroporation, and screening the samples using PCR.

Introduction:

The focus of this research is directed at the idea of screening for photoreceptor and other eyespot defective mutants in Chlamydomonas. *Chlamydomonas reinhardtii,* biflagellate green algae, has a specialized organelle known as the eyespot that is necessary for sensing light and performing phototaxis, or a response to light stimulus. Figure 1 below depicts the steps leading up to the formation of the eyespot seen in the last image of the two separate cells. As can be seen in the first few images, the D4 rootlet goes through outgrowth and acetylation ultimately leading to the position of the eyespot and photoreceptor patch, all necessary for light sensing/reactions in these cells. By looking into the division of a cell, it may be possible to determine which specific genes are related to the eyespot formation and size and therefore determine the affects of mutations to specific genes necessary for proper eyespot formation.

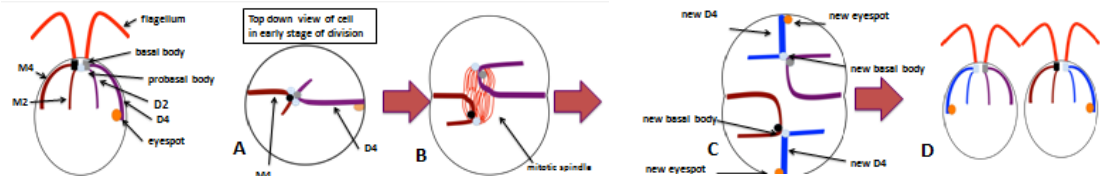

Figure 1: Formation of the eyespot in Chlamydomonas cells. The image at the far left depicts the wildtype Chlamydomonas cell with labels corresponding to important structures that are used during cell division and are correlated to eyespot formation. **A**. Once the cell is ready for cell division, the flagella are reabsorbed, and then the cell makes a 90º rotation within the mother cell wall. The four-membered rootlets of the mother and daughter basal bodies set up the plane of cell division and the eyespot disappears. **B.** The basal body pairs separate and the mitotic spindle forms. **C.** The new mature daughter basal bodies produce their own sets of microtubule rootlets, and the new eyespots are assembled opposite the plane of cell division associated with the new D4 rootlets. **D.** The cell completes division to produce two new daughter cells each with their own eyespots.
Figure courtesy of Mark Thompson; adapted from Holmes, J.A., & Dutcher, S.K. (1989))

What is unknown in regards to the eyespot is which specific gene(s) is involved in the inhibition of light reception by the eyespot if the eyespot is defective in comparison to the eyespots of normal Chlamydomonas cells. There are four genes of interest that will be the focus of the experimentation in order to determine which specific genes are responsible for the eyespot formation and what happens when certain genes are inhibited in Chlamydomonas cells. A large

phototaxis-negative mutant collection will be created in a ΔCOP3/ARG7-8 mutant strain background in Chlamydomonas, and then mutations in COP4, Soul3, Morn Repeat, and (PAP)-fibrillin domain will be screened for in this study. The reasoning behind these four specific genes is because the research is directed at determining which genes are linked to eyespot formation, and which genes, when mutated, result in a lack of eyespot formation or eyespot function and ultimately a lack of phototaxis. COP4 is also known as a light-gated ion channel, similar to COP3, which is important in recovering a double *cop3 cop4* photoreceptor mutant. Soul3 is thought to affect the eyespot placement and eyespot size and it would be expected that a mutation in this gene would lead to a misplaced eyespot or an eyespot that is not normal in size. Morn Repeat functions in attaching proteins to the membrane of the cell during formation and the (PAP)-fibrillin domain protein may be involved in the stabilization and organization of pigment granule arrays (Thompson PowerPoint). By focusing on all four of these genes, it may be made possible to determine which of these genes have an effect in the overall eyespot formation of Chlamydomonas and thus a mutation in one of these genes would lead up to the inability of Chlamydomonas to phototax.

This research begins with electroporation of Chlamydomonas cells by adding an externally applied electrical field to increase the permeability of the cellular membranes in order to get the desired DNA from the transformants. After creating over 10,000 mutant strains via the electroporation technique hybrid known as the Thompson protocol, it is desired to be able to identify strains of the Chlamydomonas cells that are harboring the marker gene which was selected to be the ARG7-8 gene. This gene was chosen due to it being an auxotrophic selectable marker that transforms into the cell thus making it possible to design primers to insert into the genes of Chlamydomonas cells that can be identified during later screening. This gene, if

present, will be identifiable in the genome of the new cells via a PCR screen and will help determine if a mutation has occurred by observing the changes of an eyespot under a microscope and by performing phototaxis in order to determine if the light receptors of Chlamydomonas are functioning properly. In order to reach the goal of the mutant strains the past semester, approximately eight electroporations were completed per day for a total of 56 in a week, and approximately 70 transformants per electroporation were recovered to obtain 3,920 transformants. The goal was to complete ninety 96-well plates. During the first procedure, there were three PCR cocktails per target gene completed. Then, for four target genes, this meant there would be twelve PCR cocktails per pooling of strains in order to look for mutation in the four aforementioned genes that could have a desirable effect on the eyespot. Instead of pooling the plates of transformants, the transformants that were picked and grown on the plates and transferred to M medium-filled tubes were to be tested using phototaxis. For the cells that did not phototax, they would be observed under a microscope to look for eyespot abnormalities and run through a second round of phototaxis to double check that the cells did not phototax. For the abnormal looking cells, the cells would be grown as fresh cultures, the DNA would be harvested, and then a PCR screen would be completed to screen for the desired PCR product that would result from the primer used. By doing so, this will tell researchers whether a specific gene was involved in the inability of the eyespot to absorb light due to a mutation.

While Mark Thompson was completing the work mentioned in the above paragraph, such as performing the electroporations to create the transformations, the work I was in charge of involved transferring colonies from the transformed Chlamydomonas cells to the 96-well plates to grow for four to five days, complete gel dot transfers of colonies (during the first semester), pool wells of cells (first semester), transfer cells from the 96 well-plates to pre-filled medium test

tubes, make more batches of medium to fill tubes for autoclaving, and running phototaxis on the cells. The purpose of this experiment was to generate a large mutant collection in order to identify specific mutations in the four genes of interest. After the completion of lab this semester, it is desired that the goal of finding these mutants and properly identifying them is reached. Once strains that are phototaxis-negative are determined, those strains will be further observed using a light microscope and PCR screening to determine if the eyespot is in a different location from normal Chlamydomonas cells or if the eyespot is lacking as well. If this is the case, it has been made clear that this chosen mutant using the ARG7-8 gene is the desired mutant strain of Chlamydomonas that has been created in the Dieckmann Laboratory and ultimately leads to the inability of the *Chlamydomonas reinhardtii* cells to perform phototaxis.

Materials and Methods:

Method I: Colony transfers to 96-well plates

For the initial start of the project, the main focus was to transfer colonies that had been growing on TAP medium plates to the 96-well plates. The flame is lit and used throughout this step to ensure contaminants are away from the workspace. Strains of Chlamydomonas had been placed on tap plates and allowed to grow until the colonies were large enough to be transferred. To complete this procedure, each well was filled with 200 µL of TAP medium. Then, autoclaved toothpicks were used to transfer one colony to one single well of the 96-well plate. The colonies were broken up in the medium to ensure maximum growth abilities for the individual cells living in that colony. After one TAP plate was empty of colonies, the empty plate would be thrown away and another plate was taken from the incubator. New colonies were taken from that new plate and used to continue filling the 96-well plates. After growth in the 96-

well plates for a few days, the cells would be transferred to a new 96 well plate to maximize cell growth prior in fresh medium prior to phototaxis.

This current semester, a slight change was made to this procedure. Instead of transferring the cells in the original 96-well plate to a new plate with fresh medium, the cells would be left to grow in the first 96-well plate for four to five days and then would be used straight from this plate.

Method II: Dot gel transfer of colonies

The flame is lit and used throughout this step to ensure contaminants are away from the workspace. As a separate test from using toothpicks to place colonies in prefilled wells in the 96-well plate, a new procedure known as the gel dot transfer of colonies was implemented during the first semester. Instead of transferring colonies to a well, the colonies would be transferred to a new TAP medium gel plate. Under the plate was a grid of squares with a black dot in the center. Each colony would be rubbed on the center of the dot and allowed to grow. After growth on the gel for a few days, the cells would then be transferred to a 96-well plate using a multi-tipped pipetter for further growth before other procedures were utilized. This process was only used during the experiments of the August to December semester.

Method III: Making M medium

When fresh tubes of medium would run out, it was necessary to refill them and then have them autoclaved. To make the medium, the following materials were added together to make a 1 L batch: 5.0 mL 10% $Na_3C_6H_5O_7$, 1.0 mL 1% $FeCl_3*6H_2O$, 1.0 mL 4% $CaCl_2*2H_2O$, 3.0 mL 10% $MgSO_4*7H_2O$, 1.7 mL 13.1% $K_2HPO_4*3H_20$, 1.0 mL 10% KH_2PO_4, 3.0 mL 10% NH_4NO_3,

1.0 mL trace metals, and 983 mL distilled water. After the 1 L batch of medium was made, a repeat pipettor was used to fill test tubes with 2.2 mL of medium per tube. The tubes were then capped, placed into test tube racks, and autoclaved for sterilization.

Method IV: Pooling cells for PCR screening

The flame is lit and used throughout this step to ensure contaminants are away from the workspace. During the first semester, Mark Thompson needed the cells to be pooled in pre-filled medium jars in order for him to complete PCR screens for the desired mutants. To do so, 40 µL of each well was added into the pre-filled medium jar. If a well was dried or almost empty, more TAP medium was added to bring the cells back to life in order to add them to the pool. After adding cells from all ninety-six wells, more TAP was added to each of the low wells in order to allow the cells in those wells to continue to grow for future tests. This pooling technique was only used during the first semester.

Method V: Transferring cells to medium tubes

The flame is lit and used throughout this step to ensure contaminants are away from the workspace. Prior to phototaxis, cells needed to be transferred from the 96-well plates to the autoclaved tubes of M medium. To do so, a test tube rack was set up with the same number of tubes corresponding to the number of wells. The first and last tube of each row was labeled with the 96-well plate number to identify which set of samples was being used, and then the row name and number to match the labels on the 96-well plate (i.e. 4A1 for plate 4, well A1). Then, 150 µL of each well was added to the corresponding test tube. Once the test tubes were filled, the

test tube rack was placed in the incubator to allow the cells time to grow in the medium prior to phototaxis.

Method VI: Phototaxis

After about two days of growth in the incubator, the tubes were removed in order to start phototaxis. The amount of days for growth is dependent upon how green the cells in medium look. For this method, it is desired for the cells to be a darker green to aid in the determining if phototaxis was completed. Four trays are tested at a time by placing one row of tubes at the front of each tray. Then, two trays are placed in each of the two boxes that are in front of the light source. The boxes are then closed off to all light except for the slit in the front that is aligned with the bottom of the test tubes that will let the light in. After twenty minutes in the dark boxes, the tubes are removed and it is determined if the cells phototaxed. Data is recorded for each well as to whether the cells were phototaxis-negative or positive. After a little time, it is then noted whether or not the cells began to swim again. This process is repeated until all tubes have had phototaxis completed on them. After phototaxis, the tubes are rinsed out and added to the bin of dirty lab items.

Method VII: Pulling zygotes

For this step, the flame must be on to prevent contamination. Once the plate with zygotes is unwrapped, a sterile razor blade is used to scrape off all green colony matter to leave behind the desired zygotes. Using the square-headed metal tool which is sterilized using the flame, the zygotes are scraped into piles and then that section of the gel is cut out. Using the razor blade, straight, indented cuts are made into a D plate to form lanes. The zygotes on the cut out gel

piece are then slid across the top of the cut marks to spread the zygotes across the D plate. The sterile glass tool is then used to move one zygote into each of the lanes. The zygote is dragged in the pool of water that is formed when light pressure is applied to the gel plate in order to aid in the moving of the zygote into each of the lanes. The zygote only needs to be a short distance down the lane (< 0.5 inches down the lane). Once all lanes have one zygote, the D plate is placed in the incubator to allow further growth of the zygotes for future experimentation.

Results:

Table 1: Phototaxis Results for 1st Semester

Number of Mutants Screened	Number of Phototaxis-Negative	Number of Swimming-Negative	Percent of Phototaxis-Negative	Percent of Swimming-Negative
1424	20	192	1.4%	13.5%

Table 2: Total Number of Cells Collected, Screened, and Phototax Negative

Total Number of Mutants Collected	Total Number of Mutants Screened	Number of 96-well plates Used	Number of Phototaxis-Negative
10,080	2,016 in ΔCOP3/ARG7-8 2,304 in ARG7-8 only	45	12

Table 1 is important because it informs you of how many samples have been phototaxis-negative and swimming-negative. It was expected to find about 1 in 100 samples that do not phototax and about 1 in every 10 that do not swim. From the table, it can be seen that so far, there have been 20 samples that are phototaxis-negative and 192 that are swimming-negative out of a total of 1,424 samples screened. Out of the total mutants screened, only 1.4% has been phototaxis-negative and 13.5% has been negative for swimming.

Table 2 depicts the most current data from the second semester that was collected. It gives numerical values for the total number of plates of mutants that were screened using phototaxis.

The total number of mutants collected depicts the total for this year. The total number of mutants screened represents how many mutants were screened using the phototaxis technique. Out of the mutants screened, only twelve mutants were phototaxis-negative.

It was found that when only 40 µL of each well was added to the tubes for phototaxis, the cells did not grow well enough, to tell whether or not they were swimming after phototaxis or whether or not they completed phototaxis due to how light the culture was in the tube.

During the zygote method, it was determined that if too much time had passed before transferring zygotes from the agar plates to the D plates, then after the green cell culture was scraped away with the razor blade, the resulting cells were not all nicely intact zygotes. Under the microscope, zygotes were visible but were placed among a bed of what appeared to be broken cell pieces. Zygotes were still able to be transferred from the pile of pieces it was just difficult getting just a zygote into a lane without bringing along other cell parts.

Discussion:

The primary goal of this experiment was to find mutant strains of Chlamydomonas that were phototaxis-negative and thus had a mutation in the eyespot location and/or size. By finding these strains, if any, further experimentation would be completed in order to determine if the location of the eyespot on these strains varied from the eyespot location of normal Chlamydomonas cells and/or if the size of the eyespot varied. It was expected that out of all the cells that were tested for phototaxis and swimming after phototaxis, about 1 in every 100 would be negative for phototaxis and about 1 in every 10 cell samples would be negative for swimming. For the main purpose behind this experiment, the mutants that were negative for swimming did not hold significant importance during the last semester but it was common to see that the mutant cells

that were phototaxis-negative were also swimming-negative. Overall, around 10,000 samples were collected last semester with only about 5,000 being screened using phototaxis. This semester, 2,016 mutants were screened using the ΔCOP3/ARG7-8 gene and 2,304 were screened using the ARG gene. Out of the total number of samples screened this semester during the current experimentation, only twelve samples were phototaxis-negative. After plating these samples and regrowing them in M medium tubes, a second round of phototaxis was run which resulted in only four of the original twelve samples being phototaxis-negative. Due to these results, it can be said that the next step for the four phototaxis-negative cells would be to observe them under the microscope, take pictures of the eyespots in comparison to a control, ΔCOP3/ARG7-8, and measuring the eyespots to determine if there is any noticeable size difference. Thus far, pictures have been taken and it appears that some of the cells contain eyespots that are larger in size, with some samples containing multi-eyed Chlamydomonas cells. The approach that would need to be taken for these four phototaxis-negative cells would be to further determine what specific genes were affecting the eyespot abnormality. If time allows it, the cells from these four samples will be cultured to harvest the DNA from these mutant strains, and then a PCR screen will be utilized to further delve into the DNA sequences of the specific genes thought of to be involved in eyespot size and location specification.

As previously mentioned, the amount of sample used for phototaxis was increased from 40 µL to 150 µL. This was due to the fact that when only 40 µL was used, the cells did not grow green enough to effectively do phototaxis. With that small amount, it was difficult to determine if the cells were negative or positive for phototaxis and it was also hard determining if the cells began to swim afterwards. Due to this, the amount of sample was increased in order to allow the cells to grow more and make it easier to tell if the cells were completing phototaxis. By adding

more sample to the M medium test tubes, the result was a darker green solution meaning that there was more cell growth than had been seen with just the 40 µL solution. With a darker solution, it was possible to further determine whether or not the cells phototaxed because there would be a clear distinction between M medium at the top of the tube and a cluster of cells towards the bottom of the tube that swam towards the light source.

As far as the actual phototaxis results, many things need to be brought up before deciding how well the technique worked. One issue with using the 96-well plates each filled with 200 µL TAP medium and the transferred culture was that after the few days of growth, the well plates typically had liquid at the top of the lid facing the wells and at times there was green cell growth in medium in the spaces between the wells. What this means is that it is possible that cells from one well were transferred into the well of a different culture which would ultimately contaminate that well and could lead to false phototaxis results. Due to the amount of liquid that was loose in the well plates, it would be fair to say that some of the wells did get contaminated from other wells since there was an excess of loose liquid on the well plate. This could be one explanation behind why, at times, there would be samples that would originally be phototaxis-negative during the first run but then be phototaxis positive and look normal under a microscope during a second run. Another source of error or a source of false results can be accounted to the fact that some of the samples were dried out from sitting in the incubator for so long. This could have resulted in a cell sample that was not flourishing as it should be and thus cells that were either dead or lethargic when it came to properly doing phototaxis. All in all, if cells did not phototax during the first trial, the mutant strain would be regrown and would be phototaxed a second time in order to determine whether or not these cells were actually phototaxis-negative or if it was a false negative the first time around.

With no samples positively containing the desired mutant strains, there is a future direction for this experimentation that was briefly touched upon in previous paragraphs. Once samples have been determined to be phototaxis-negative, these samples would be regrown in order to perform a second trial of phototaxis to double check the accuracy of the first trial. If the samples are phototaxis-negative a second time around, these samples will be spun down in an incubator to remove a majority of the medium. Afterwards, the concentrated cell sample will be observed under a microscope in order to note observations such as eyespot location, eyespot quantity or presence, and overall eyespot size. For each of the samples, about twenty pictures will be taken of the mutant strain containing cells along with twenty photos of the control sample. The purpose behind this is to make eyespot measurements of the mutant strains to be compared to the size of the control eyespots. If no apparent difference is noted, then it is clear that there is more going on in the cell that makes it phototaxis-negative than just the size of the eyespot or the location of the eyespot. Afterwards, it would be important to take the mutant strains and grow them in cultures in order to harvest the DNA via electroporation and the harvested DNA will then have a PCR screen run on them to obtain more definite results on the gene sequences in these mutant strains.

All in all, this research was important in the development of mutant strains in *Chlamydomonas reinhardtii* in order to reach the end goal of determining which genes were involved in the eyespot location and formation which is important for light reception in the cell. Through this research, an experiment has been implemented making it possible to test mutant strains effectively and efficiently via the use of phototaxis as a preliminary test for an inability to sense light. After phototaxis, the DNA of these cells will be harvested in order to run a PCR screen in hopes of making it possible to make your own mutants and find that mutant in only a

few weeks. This is important because it would allow scientists to design their own primer and ultimately have the ability to make mutant strains of the cells they are experimenting on, thus making it possible to study multiple aspects of that cell in a small amount of time.

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