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Intracellular Signaling: How do Cells Respond to Single and Multiple Inputs

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Introduction

Photosynthetic organisms have a variety of ways in which they optimize the light intensity and quality in their environment. One way is through a phototactic response in which cells use their cilia to either swim toward or away from a light source. Phototactic algae orient themselves towards, away from, or normal to the light direction known as positive, negative, or dia-phototaxis respectively [Foster and Smyth, 1980].

The single celled organism, *Chlamydomonas* (Fig 1), is ideal for studying phototaxis which is in turn a useful behavior for conducting studies on intracellular signaling at the real time scale. *Chlamydomonas* is a freely swimming eukaryotic microorganism of 10 µm in diameter that has haploid genetics and exhibits rapid and easy growth (in liquid or plate culture), with a short generation time. Importantly, the whole genome sequence is known. It has many quantifiable outputs, e.g., gametogenesis, mating, ciliary excision, and phototaxis. There are many photoreceptors such as blue light, red light receptors, rhodopsin, and chlorophyll (Foster and Saranak, 1989).
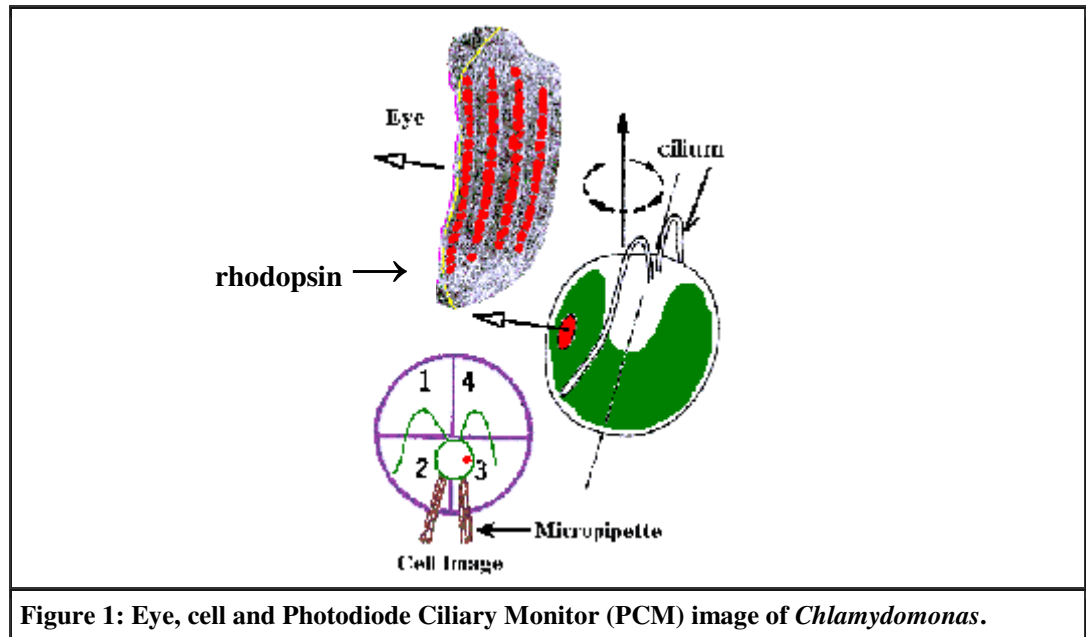
Figure 1: Eye, cell and Photodiode Ciliary Monitor (PCM) image of *Chlamydomonas*.

Phototaxis is the output of an integrated signal processed through a light-sensitive antenna positioned at the equator of the cell body. The plasma membrane overlying the eyespot contains rhodopsin, an integrated membrane protein, which functions as the photoreceptor to track the light source. The two cilia (10 µ long) are the organelles that receive intracellular signals to steer the cell to the appropriate direction. The biological significance of phototaxis is to orient the cell to receive the proper intensity of light to optimize photosynthesis and minimize photodamage [Foster and Smyth, 1980].

The Antenna

Chlamydomonas has a distinguished eye that lies near the cell's equator with rhodopsin photoreceptor facing outwards. The eye (Fig. 1) is composed of layers of carotenoid pigment spaced in a manner that allows light entering through the cell to be reflected back before reaching the pigment, resulting in monopolar directivity of the eye. The monopolar directivity of the eye is extremely important

because it is the source of the temporal modulation that effects the rotation of the cell. If the eye was not monopolar, and it was sensitive to receiving light from all directions, then there would be no temporal modulation to direct the rotation of the cell.

In natural environments light can be characterized by three properties: 1) color 2) polarization and 3) intensity. Evidence shows that algae orient themselves with respect to intensity patterns. In the natural habitat of algae, under water, light is coming from all directions; thus, the organism orients itself with respect to the average light direction [Foster and Smyth, 1980]. The organism accomplishes this task by a conical-scan tracking mechanism to scan its environment. The cell scans its environment by continuously left-handed rotating about its longitudinal axis at 2 Hz as it swims forward by ciliary beating. The antenna is rotated about a single axis resulting in two important angles: the scan angle φ, the angle between the antenna and the axis of rotation, and the phasing angle θ, positive in the direction of rotation. The antenna determines the light intensity pattern from the scan angle and its own directivity, and uses this information to orient towards the mean light direction [Foster and Smyth, 1980].

The Photoreceptor

The rhodopsin, the photoreceptor pigment for phototaxis, forms a patch in the plasma membrane over the eyespot [Foster and Saranak, 1989]. It belongs to a G-protein catalyzing receptor family. It is composed of the seven alpha helix transmembrane protein (opsin) and a chromophore which is retinal bound to

nitrogen of the lysine amino acid in the seventh helix. In visual systems, one

photon is absorbed by one

molecule of photoreceptor

pigment, thereby

producing an activated

molecule that initiates the

remaining steps of the

visual system. In

experiments carried out on

humans, it was found that

five to eight photons had to

Figure 2: Vertebrate rhodopsin
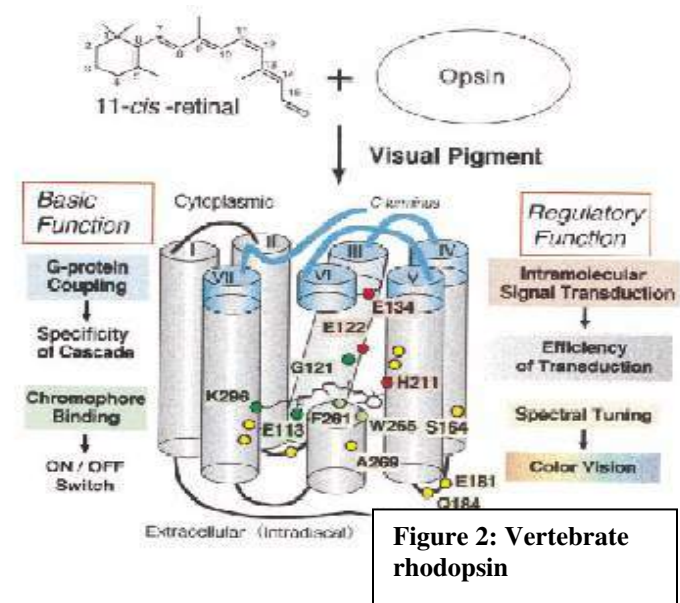
be absorbed by receptor pigments in order for a flash to be seen. This experiment

resulted in two key principles: 1) the probability of absorption of a photon is

proportional to the amount of pigment and 2) the effect of photon excitations must

be additive. The alga can detect a single photon in dim light but the signal might

be insufficient to provide a response. Thus, as in most intracellular signaling

pathways, there must be either a single or multiple amplification stage between

the initial reception of a signal and the final output [Smyth et al, 1988]. The

amplification of rhodopsin signal occurs first by activating the G-protein

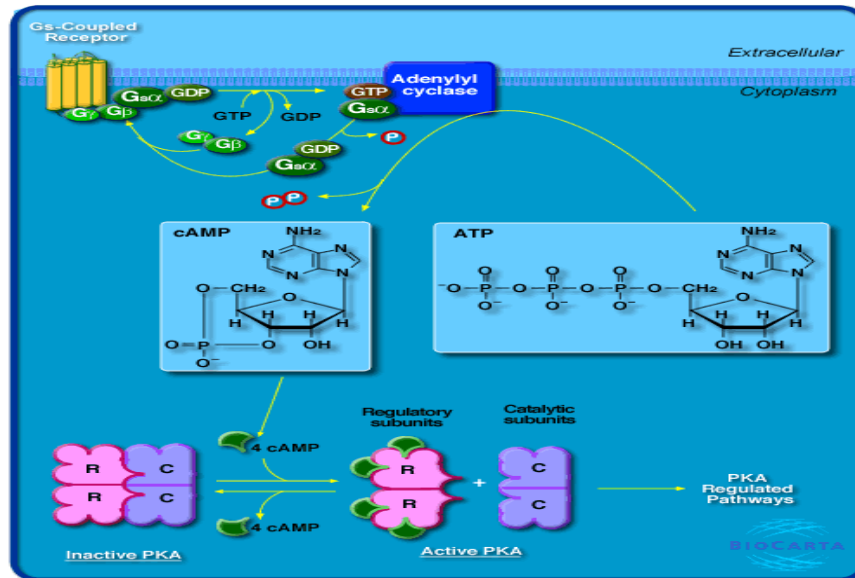(transducin) at the cytoplasmic side of the rhodopsin molecule.

Figure 3: G-protein activation leading to cascade of cell signaling

The activated G-protein then triggers other enzymes down the chain of intracellular signaling. This cascade of reactions amplifies and carries the signal to the responding organelles which, in *Chlamydomonas*, are the two cilia to steer to the appropriate direction. The signal processing network must be well regulated by both electrical and biochemical events so that the output is quick and productive.

Phototaxis Behavior

The eye serves as the antenna for the organism to regulate light absorption by the rhodopsin. Maximum light absorption occurs when the eyespot faces directly towards a light source. This occurs because of constructive interference of light being reflected from the eyespot layers. When the eyespot is not aligned to give maximum illumination, other factors must work together to receive light (i.e. absorption by chloroplast pigments and eyespot pigments, reflection by eyespot layers, or scattering by cell particles).

The cell swims (100µm/s) in a helical path while its body maintains a fixed orientation with respect to the axis of rotation by using its cilia. As the cell swims, it rotates about 2.0Hz around its axis. Due to the direction of the antenna the amount of light, or illumination, increases and decreases as the cell rotates resulting in the error signal. The cell processes the error signal to determine when to make a correction in position to realign with the path to the light. The cilia respond by changing the orientation of the cell, thereby changing the signal during the next scan cycle [Foster and Smyth, 1980]. The change in direction is timed relative to the modulation cycle of the light that either allows the cell to turn towards the light, positive phototaxis, or away from the light, negative phototaxis.

Furthermore, it has been experimentally proven that *Chlamydomonas* is capable of responding to single photons. Hegemann and Marwan (1988) showed that at low light intensities the cells are capable of giving single photon responses. The probability that a single photon will be absorbed by a photoreceptor pigment is modulated by the directional properties of the antenna. Thus, when the antenna is directed towards bright light it absorbs more photons than when it is directed towards dim light [Smyth et. al, 1988].

Thus, *Chlamydomonas* satisfies the three criteria for a visual system: 1) detects single photons 2) detects a pattern in the illumination and 3) produces a behavioral change in response to detecting the pattern, that make it an optimal organism to study.

Visual Transduction

In vertebrates, visual transduction is initiated by the absorption of light by the photoreceptor, rhodopsin, at plasma membrane of the outer segment of the rod cells. Initially, rhodopsin proceeds through a series of conformational changes in the protein part of the molecule. After a series of conformational changes metarhodopsin II, an intermediate of the photochemical cycle of rhodopsin, is produced. Metarhodopsin II interacts with a trimeric GTP-binding protein, transducin.
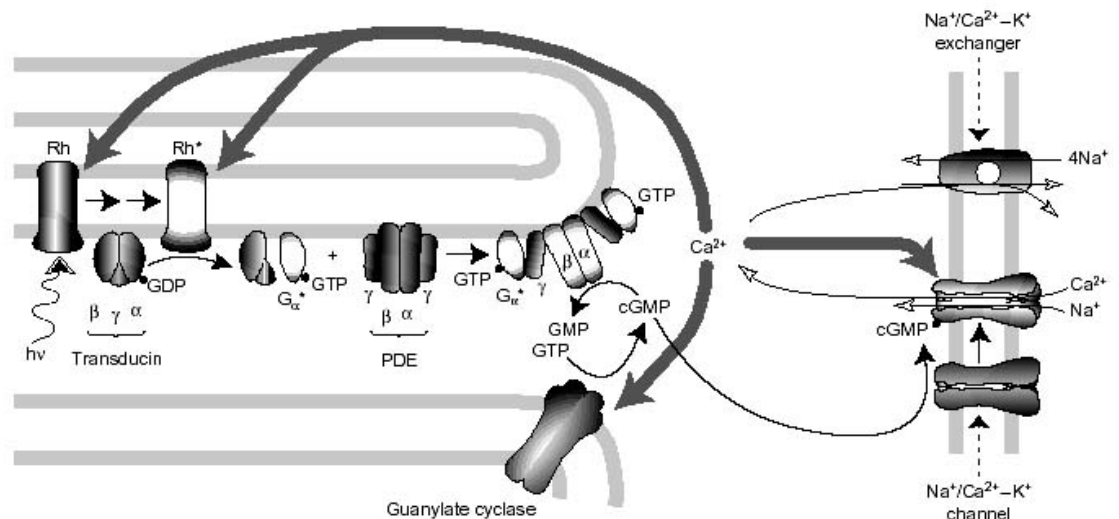

Figure 4: Schematic diagram of vertebrate visual

This interaction promotes the exchange of bound GDP for GTP at the α-subunit of transducin, and results in the dissociation of the α-subunit from the βγ-subunit of transducin. The α-subunit is then free to interact with phosphodiesterase (PDE). Prior to this interaction the subunits of PDE, α and β, are both bound to a small γ-subunit that inhibits PDE from hydrolyzing cGMP during resting conditions. During these resting conditions, or in the dark, cGMP concentration is in abundance and causes cGMP-gated cation channels in the plasma membrane to

remain open. Once the α-subunit of transducin binds to PDE, it removes the inhibitory subunit and allows PDE to hydrolyze the intracellular messenger of photoreception, cGMP to GMP. This results in a decrease in the cGMP concentration and causes cGMP-gated channels in the plasma membrane to close. Two mechanisms, one fast and one slow, work together to deactivate the rhodopsin photoreceptor; 1) the rhodopsin is phosphorylated and 2) the rhodopsin is capped by the protein arrestin. These two steps lead to a significant decrease in catalytic activity. In order to completely deactivate the rhodopsin, the conformational changes that occurred to activate it must be reversed. Thus, the photoisomerized all-*trans* retinal is hydrolyzed from the opsin protein and eventually replaced by the original conformational structure, 11-*cis* retinal (Burns and Lamb, 2003).

Intracellular Signaling and Phototaxis

Sensory transduction along with intracellular signaling, are the processes of communicating within the cell by using electrical or chemical methods (Foster and Saranak, 1989). In the *Chlamydomonas* system the intracellular signaling pathway for phototaxis is not well studied. Like most other photoreceptors, the signal is amplified, light is adapted, signal is compressed, and then transmitted to an output structure (in this case, cilia) [Foster and Saranak, 1989]. Once an initial signal is generated it is usually followed by a series of cascades that eventually result in an integrated response by the organism.

In order to understand intracellular signaling better, some researchers have chosen to focus on one particular contributor, the rhodopsin photoreceptor which

is used in microorganisms such as green alga (Foster et al, 1984), fungus (Saranak and Foster, 1997) for phototaxis, euglenoid (Saranak and Foster, 2005) for changing direction, and in multicellular animals for vision. Activation of rhodopsin in *Chlamydomonas* results in phototaxis, the movement of a cell in relation to light direction (Saranak and Foster, 1997). Phototaxis can be either positive, cells swim towards the light, negative, cells swim away from the light, or orthogonal, 90 degree relative to the light. The signal-processing network for phototaxis can be divided into three parts, the cell body proper and the two cilia. The controlled motion of the pair of cilia that steer the cell is among the principle outputs [Josef et al., 2005]. Although conditions can usually be found where a particular strain is reproducibly positive or negative, the control of the sign of phototaxis is not understood (Smyth et al., 1988).

For this study I focus mainly on intracellular signaling following rhodopsin stimulation by green light using negative/positive phototaxis responses recorded by the computerized population cell tracker as the outputs. Single or multiple inputs (e.g. light of various wavelengths/intensities, chemicals modifying intracellular signaling) are applied to the cells and the responses are compared with controls. I hope to answer certain questions: 1) how cells respond to light of different colors and intensities 2) if there is a change in direction, what are the possible factors leading to it and 3) does the second messenger, cAMP, regulate direction of phototaxis. The data will be used in combination with other studies in the group including ciliary motion, electrical signal, and biochemical studies to

generate an analytical explanation for how cells process information and make decisions.

The understanding of intracellular signaling, and how cells make decisions, is extremely important. Diseases such as Alzheimer or Parkinson's are the result of faulty intracellular signaling that lead to cell death (apoptosis). Cancer results from failure of signaling from membrane receptors to control cell growth (Darnell et al., 1986).

Methods

Phototaxis Measurement

The phototaxis experiments are taken place in a dark room, with constant temperature at 68 degrees F. Cell suspension in a rectangle glass cuvette (2x50 mm, 0.2 mm depth) on a microscope stage was illuminated with near IR (850 nm) to avoid any light effects on the cells.
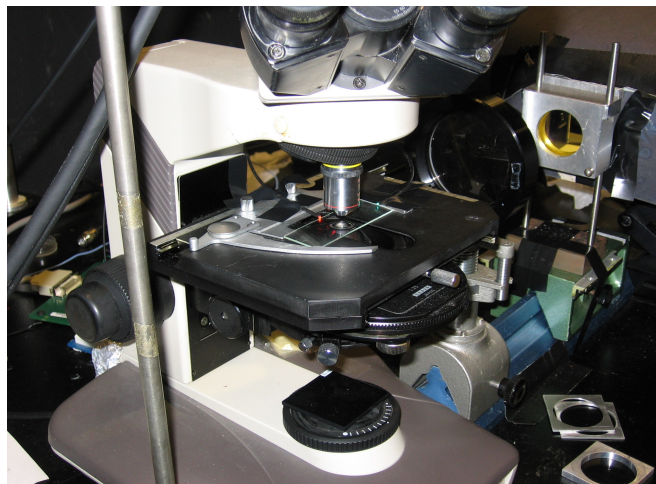

Figure 4: experimental setup

The Nikon microscope is equipped with a bright field condenser and 10X objective. A CCD camera attached to the microscope records the cell motility at the speed of 30 frames per second to the computer which is also used to control

stimulations. The cell tracking software developed in our lab is used to record, view, and collect different parameters of cell behavior such as average speed, net angle, net displacement, number of cells observed, and circular variance. The motion of the cells is then integrated to produce a rose diagram of the cells motion, in different directions (Fig. 6). The net displacement (solid black line) represents the net movement (µ) of the cell population and the net direction that can be classified as positive, negative, or no phototaxis.

Cell Preparation

There are three strains of *Chlamydomonas reinhardtii* used in this study. They are 1117 (a wild type positive phototaxis), 806 (1117 with defective *agg* gene, negative phototaxis) and krc1-4A (a photosynthetic wild type, positive phototaxis at low light intensity and negative at high light intensity). All cells were grown in   high salt media agar (HSM) in Petri dishes, or HSM liquid in test tubes kept in the
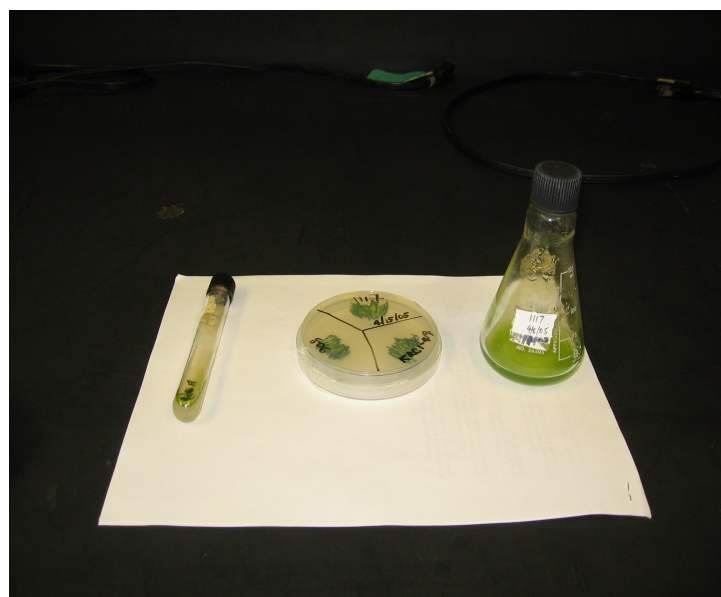

Figure 5: Various growth

clean room under fluorescent light (10 W/m^2) and a constant temperature at $64°$ F. We always use the cells that are 3-7 days old. Before the experiment, we aseptically transfer cells from plate to no-nitrogen minimum media (NMM) in a test tube; cover and vortex briefly. The NMM does not support growth so the cells stop dividing (mitosis) and will transform from vegetative to gamete. The cell suspension is shaken for two hours at 140 rpm under fluorescent light. This two hour duration in NMM was the best time for optimum phototaxis for the 806 and 1117 strains (Peter Clark's unpublished data). It was found for the krc1-4A strain that the duration in NMM for the optimum phototaxis was approximately one day, as the cells are not active before that time.

Preparation of Cells for Light Stimulation

Cells are checked under microscope for at least 70% motility. Under red light, the cell suspension (approximately 10^5 cells/ml) is loaded into a rectangle glass cuvette using capillary effect. The cuvette is placed on a glass microscopic slide on the microscope stage; the position and focus are adjusted for the optimum tracking of the active cells on the monitor in the dark room connected to the CCD camera. The experiment is controlled and the results are recorded by the computer outside of the dark room. For each light intensity, the cells are tracked and recorded for 10s at the delay of up to 10s after light stimulation is on.

Preparation of Cells for Chemical Incorporation

Aliquots of 0.5 or 1 ml of cell suspension in NMM (for 2 hours) were incorporated with different concentrations of the pharmacological compounds affecting biosynthesis and metabolism of cAMP. The cell suspension was

returned to the shaker in front of fluorescent light after chemical incorporation. The cells were checked under the microscope for motility after chemical incorporation. The phototaxis test was done at the time when cells regained the same motility as the control.

3-Isobutyl-1-methylxanthine (*IBMX, Sigma*): IBMX is a phosphodiesterase inhibitor shown to specifically inhibit cAMP phosphodiesterase in *Chlamydomonas*. The IBMX stock solution of 240mM in dimethylsulfoxide (DMSO) is kept at -20° C for about one month. Before each experiment, the stock solution is diluted down to 5mM with distilled water for incorporation to the cell suspension to the final concentrations of 10, 32, and 100 µM with 806 and 2.5, 10, 32, and 100 µM for 1117. When incorporating IBMX in 806, cells can be tested for phototaxis approximately 10 minutes after. However, when using IBMX with 1117, it takes approximately 1 hour for the cells to regain mobility to carryout experiment. This is because IBMX causes ciliary resorption (shorten cilia) which is reversible with time.

2'5'-dideoxyadenosine (Sigma): 2'5'-dideoxyadenosine is a membrane permeable adenylate cyclase inhibitor. It acts inside the cells by blocking adenylate cyclase and thus resulting in a decrease in intracellular cAMP levels. A stock solution of 100mM in DMSO (1 mg/42.52 µl DMSO) is kept in the freezer at -20° C (solution stable for up to 3 months at this temperature). Before each experiment the stock solution was diluted down to 10mM by distilled H_2O. This solution was then used to incorporate to the cell suspension to make 2, 5, 12, 25,

and 75 µM concentrations. It was found that cells should remain on the shaker for approximately 40 minutes to become as active as control.

8-Br-cAMP-Na (Sigma): This compound is a membrane permeable cAMP analog that has a greater resistance to hydrolysis by phosphodiesterase than cAMP. Thus, it would get to the cytoplasm and should affect the cells similarly to increase cAMP. The stock solution of 5mM in distilled water was used for incorporation to the 806 cell suspension to the final concentrations of 50, 100, 200 µM. It is necessary to leave cells on the shaker for a minimum of 10 minutes.

Dibutyryl cAMP (Sigma): It is a cell permeable cAMP analog activating cAMP-dependent protein kinase A. Dibutyryl cAMP 1mM solution in distilled water was incorporated into the cell suspension to the final concentrations of 100 and 200 µM. When using the compound with the 806 strain cells must remain in the shaker for approximately 30 minutes. In addition, testing was also done in the presence of IBMX to inhibit the hydrolysis of the compound. IBMX was incorporated first as described previously and then proceeding to add the dibutyryl cAMP solution after 5 minutes.

Light Stimulation

There are two sources of light stimulations, the LED's (525nm, 505nm, or 640nm) of varied intensities controlled by computer and the tungsten light source with 3-cavity interference filters (500 nm) for higher intensities controlled manually. The light is brought to 25mm away from the objective lens (where the sample is recorded) by 1 mm diameter jacketed fiber optic. When the wavelengths or intensities are not obtained by available LED's, a tungsten light

source equipped with interference and OD filters are used. In addition, a laser producing red light (670nm) of $800\mu W/0.38 cm^2$ is used to add high intensity red light stimulation. Stimulation is usually for 20s (10s initial delay and 10s of image capturing). Cells are dark adapted for few minutes while dark control data is collected. Stimulation is from low intensity (near threshold) and increased (2x or 3x) until maximum intensity or maximum response. The relative light intensity was measured by a photometer model 88XLA (Photodyne Inc.) expressed in $\mu W/0.38 \ cm^2$ ($23.9 \ \mu W/0.38 \ cm^2 = 10^{18} \ photons/m^2 \ s$).

Variables

Either one or more simultaneous wavelengths (nm) can be used for stimulation; the light intensity ($\mu W/0.38 cm^2$) is varied to get a complete intensity response curve. Direction of light stimulation can be changed or alternated to fit with the aim of each experiment. Other input variables are various chemicals; the concentration levels (μM) incorporated into the cells. In order to observe the effects of the input variables, I quantify phototaxis (movement towards or away from light), cell motility, swimming velocity, and the total number of cells recorded.

Data Analysis

To analyze the data collected we use either the PSI or excel program to plot the net phototaxis distance versus light intensity (in log scale) in order to identify thresholds (the lowest intensity that produces a response) maximum response, slope, and shape of the intensity-response curve. The positive and negative phototaxis are confined within ±45°. Positive and negative phototaxis are

plotted as the positive and negative sign respectively on the Y-axis (see Fig. 8). The data from different strains are compared to wild-type *Chlamydomonas* tested the same way. The data of different treatments are compared to control of the same batch of cells tested on the same day.

Results

The cell tracker program represents the rose diagram at the end of each trial. We are able to classify each trial as positive or negative phototaxis, as shown in Figure 6.
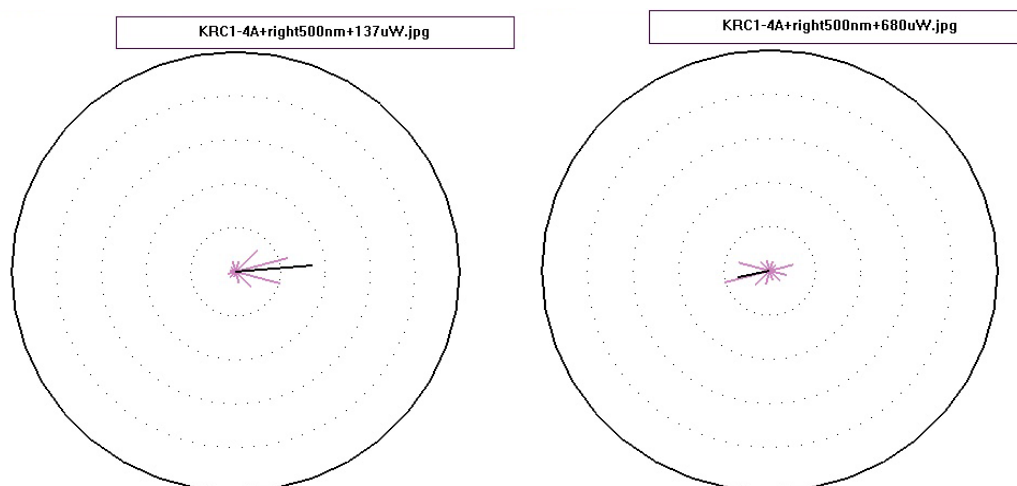

Figure 6: krc1-4A with 500nm (on the right) shows positive phototaxis at an intensity of 137 µW/0.38cm^2 and negative phototaxis at 680 µW/0.38cm^2

From the remaining data compiled by the computer program, we are able to plot different parameters of the cell behavior.

The 806 strain consistently shows negative phototaxis in respond to either green light only or green and red light at the green intensities tested (Fig. 7). However, the red light (orthogonal to green) increases the threshold for the

negative phototaxis (see the linear extrapolation to X-axis). Apparently, the intensity-response curves are at the maximum response and the reversal of slope indicates that some cells might have changed from negative to positive phototaxis.
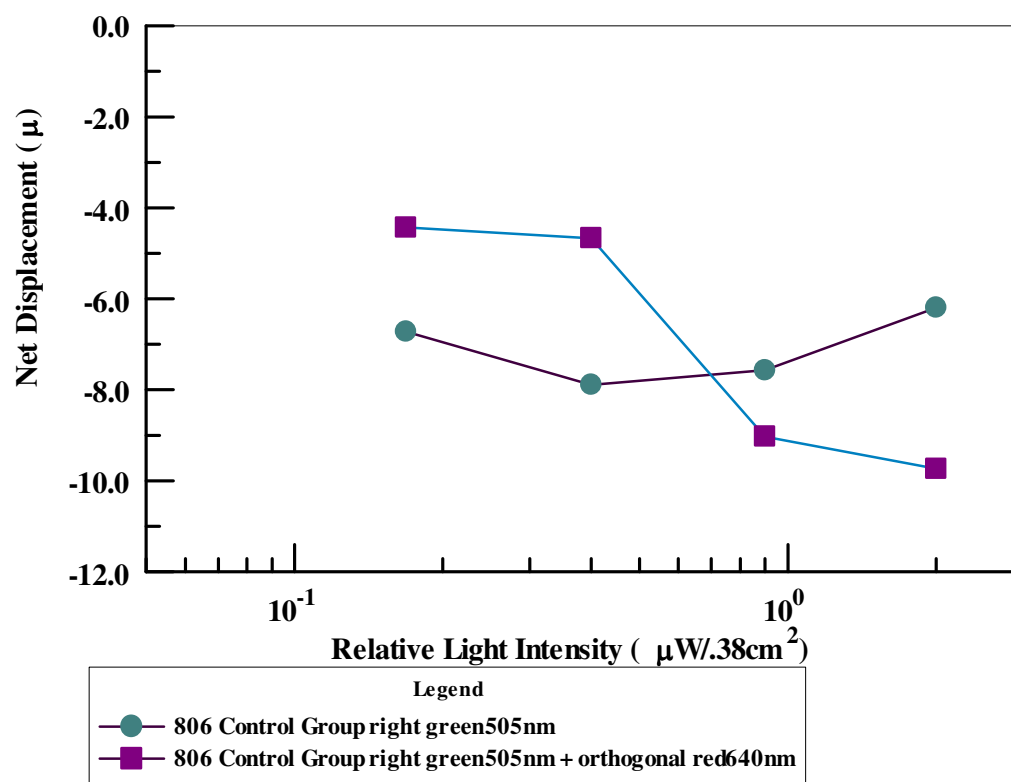

Figure 7: Red light slightly increases maximum response of 806

Strain 806 has significantly less intracellular cAMP concentration (measured as dark basal level) than 1117 (Table 1). To determine if a higher cAMP level corresponds to positive phototaxis we incorporated IBMX (a PDE inhibitor) to increase the cAMP levels of 806. Figure 8 shows that IBMX 32 and 100 µM reverses direction of phototaxis of 806 from negative to positive at the green light intensities that only cause negative phototaxis in control group. As

shown in Table 1, IBMX from 10 to 100 µM raises concentrations of cAMP in

806 to 2 fold higher than basal control. The level is not as high as the basal level

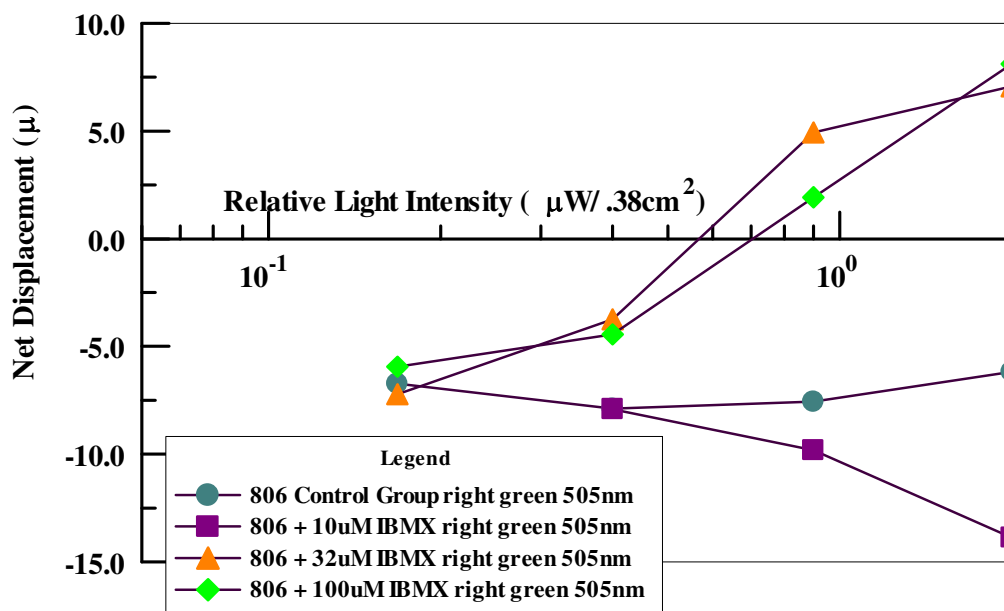of 1117 (5 fold higher than 806) but enough to induce positive phototaxis in 806.

Figure 8: Reversal of negative phototaxis of strain 806 by IBMX

Next, we wish to see if the presence of red light, normal to the green light, has any

effect on 806 incorporated with IBMX; the results are shown in Figure 9. The 806

cells again reverses phototaxis direction at 32 and 100 µM of IBMX. The

intensity at which the cells change direction and the amplitude of the positive

phototaxis are similar to Fig. 8 with green light only. Although red light increases

the threshold of negative phototaxis (Fig. 7), it seems to have no effect on the

IBMX reversal. Thus, under this experimental condition, IBMX in 806 clearly

reverses the negative to positive phototaxis likely because of raising intracellular cAMP levels. The reversal is not influenced by red light.
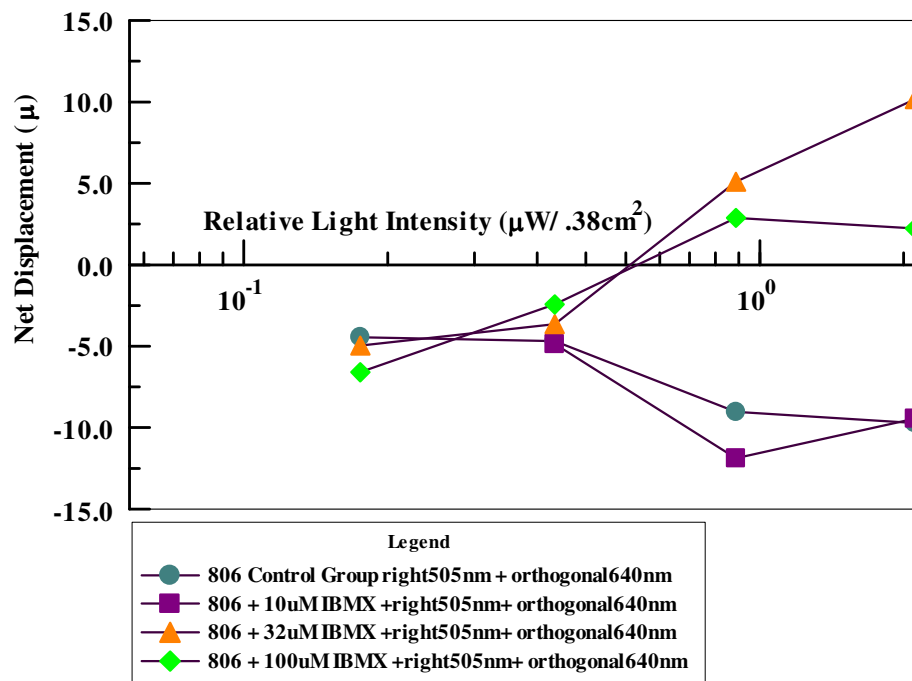

Figure 9: Red light has little effect on cell behavior of 806 with IBMX

As we look at 1117, like 806, without any chemical substances added, the strain displays one direction of phototaxis. In Figure 10 IBMX has been added to 1117, thus increasing the levels of cAMP (2.7 fold at 10 µM, Table 1). Unlike 806, the addition of IBMX does not change phototaxis direction of 1117, reduces the magnitude of positive phototaxis. However, this reduction is likely due to the effects on cell motility since the number of swimming cells are significantly less than control. Note that IBMX causes ciliary resorption which is more apparent in 1117 than 806. So the IBMX doses used with 1117 is less than with 806. The presence of red light normal to green light has a slight effect on the behavior of 1117 treated with IBMX (Figure 11).
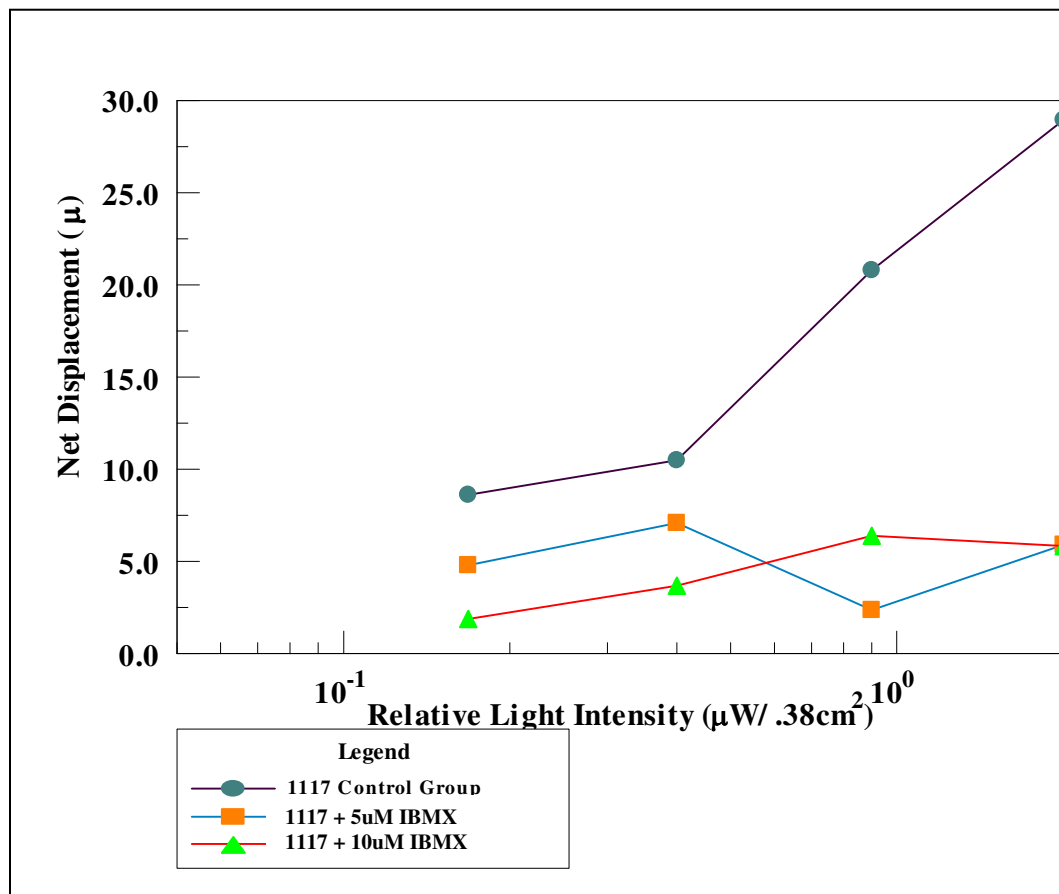
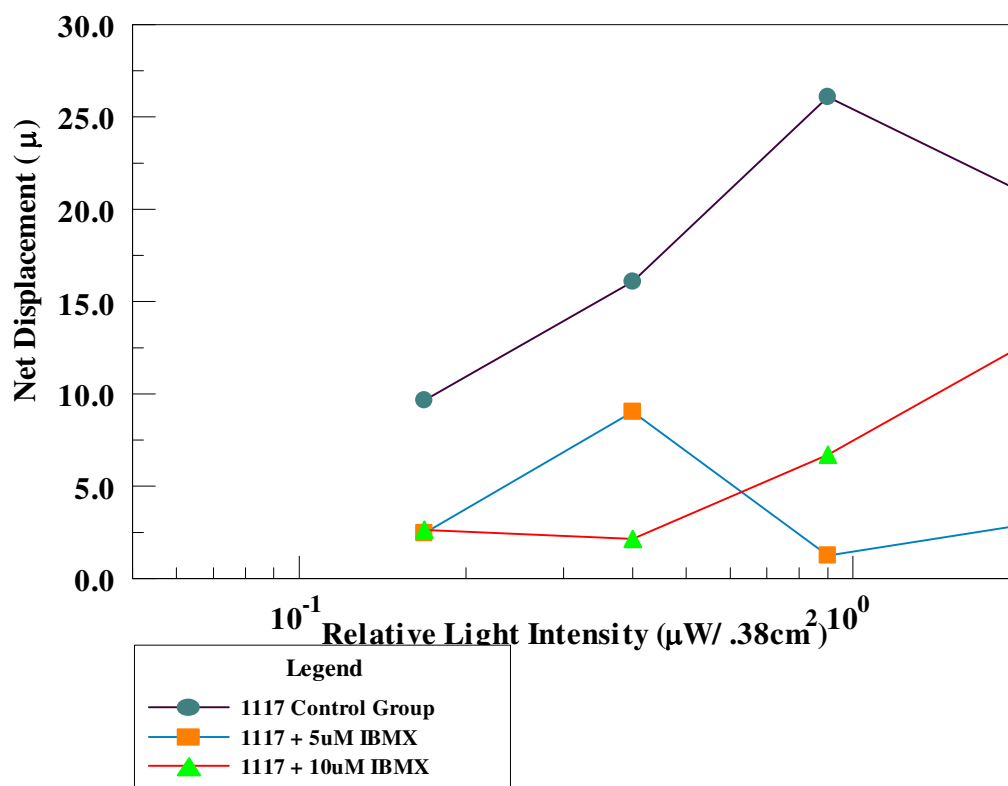**Figure 10: IBMX decreases maximum response of 1117 with green 505nm**

Figure 11: Red (640nm) light, orthogonal to green 505nm, has little effect on 1117 incorporated with IBMX

To further investigate the role of cAMP on the direction of phototaxis, some cAMP analogs which can get to the cytoplasm and resist to PDE were incorporated into the 806 strain to mimic directly raising the intracellular cAMP level. Since red light does not show any significant effects on the cAMP studies as described above, only green light stimulation would be used for most of the rest of this project. Effects of 8-Br-cAMP-Na in 806 stimulated with green light only are shown in Figure 12. The compound does not cause the cells to change direction but, instead increases in the magnitude of negative phototaxis. There is a slight reduction of negative phototaxis by 50 µM 8-Br-cAMP.
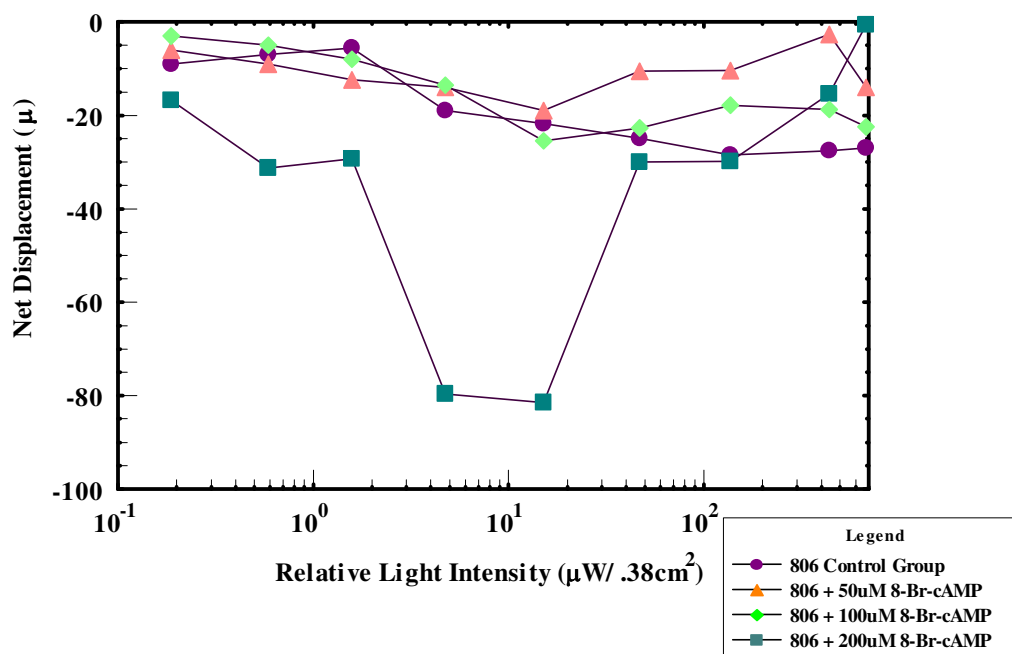
Figure 12: Effect of 8-Br-cAMP-Na in 806 with green light (500nm) stimulation.

In addition to 8-Br-cAMP-Na, another cAMP analog, dibutyryl cAMP, was used (Figure 13). Similar to 8-Br-cAMP effect, the cells do not change direction; the maximum response is slightly increased, and the threshold for negative phototaxis is reduced.

The addition of red light normal to green light has a slight effect on the phototaxis of 806 cells treated with dibutyryl cAMP (Figure 14). Both cAMP analogs at 100 and 200 µM increase the negative phototaxis in 806 cells. However, 8Br-cAMP at 50 µM decreases the negative phototaxis which, according to cAMP hypothesis, might lead to reversing direction to positive phototaxis at higher green intensities. This low dose effect agrees with the results of IBMX that raising intracellular cAMP reverses negative to positive phototaxis. This result suggests that lower doses (<50 uM) for both cAMP analogs should be

tested. Too high doses might be less specific leading to activation of cGMP or trigger the feed back regulation, which complicate the interpretation of the results.
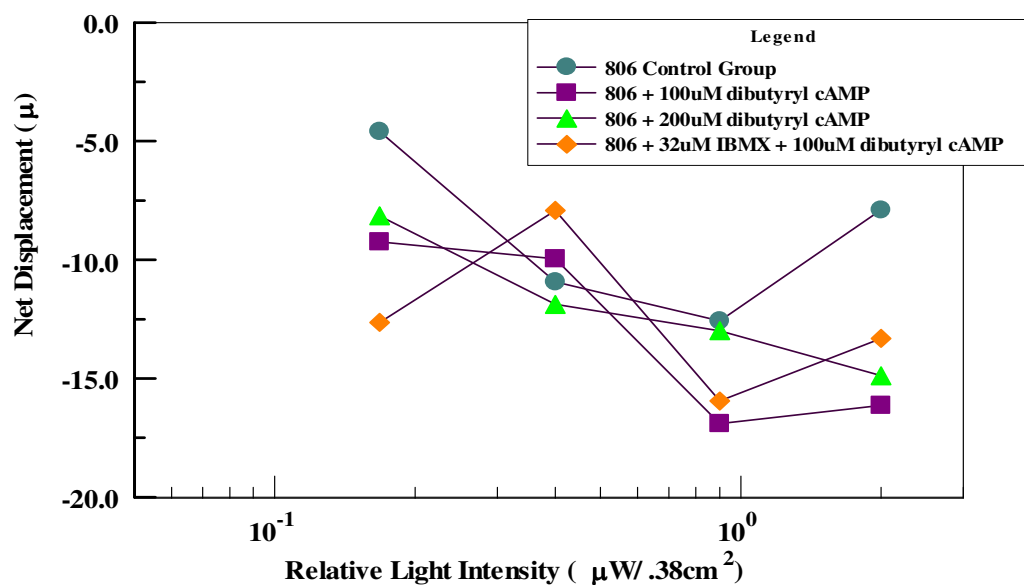

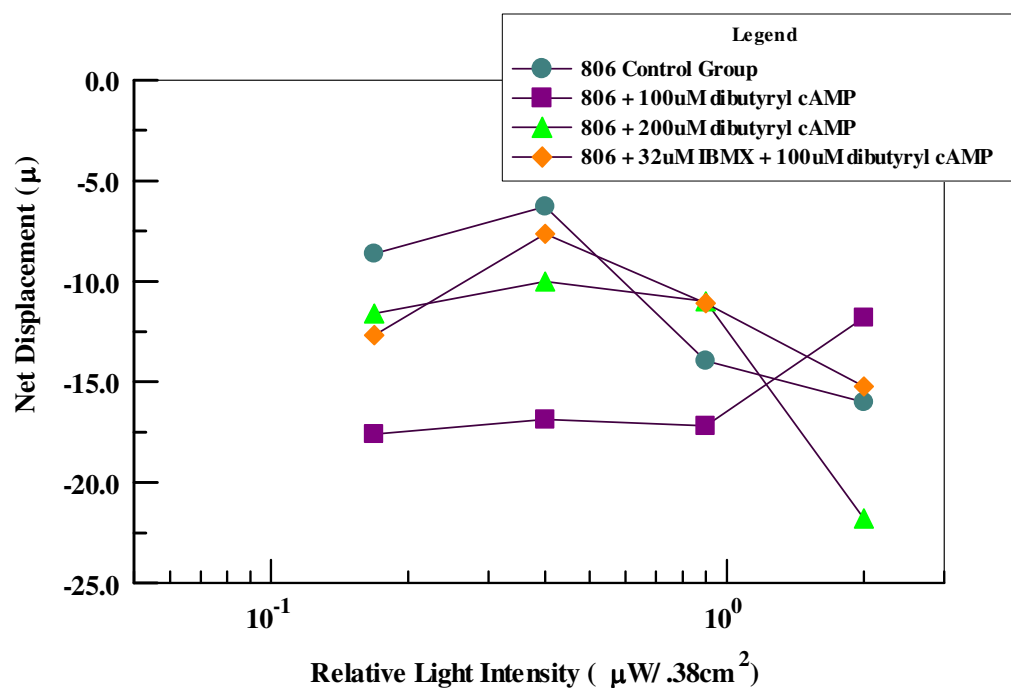**Figure 13: Effects of IBMX and dibutyryl cAMP on phototaxis of 806 stimulated with green 505nm**

Figure 14: Effects of IBMX and dibutyryl cAMP on phototaxis of 806 stimulated with green 505nm and red 640nm orthogonal to green

Experiments were conducted with the 806 strain, with IBMX (a phosphodiesterase inhibitor to prevent the degradation of dibutyryl cAMP) and then adding dibutyryl cAMP 5 minutes later. In Figures 13 and 14, at low intensities with both compounds the negative phototaxis behavior appears to decrease and then increase at high intensities. In comparison with dibutyryl cAMP alone, the maximum response appears to increase. More experiments need to be run in order to identify the threshold so that the results can be compared.

We incorporated 2'5'-dideoxyadenosine, an adenylate cyclase inhibitor, into 1117 in attempt to decrease the cAMP level in the cell to see if the cells would reverse direction. Although positive phototaxis remained the sole behavior, the magnitude in which the cells moved toward light is reduced by the high dose (75 µM) of the cyclase inhibitor (Figure 15). At lower doses, however, there is no clear difference from the 2 controls (done at beginning and end of the experiment, 4-6 hours apart).
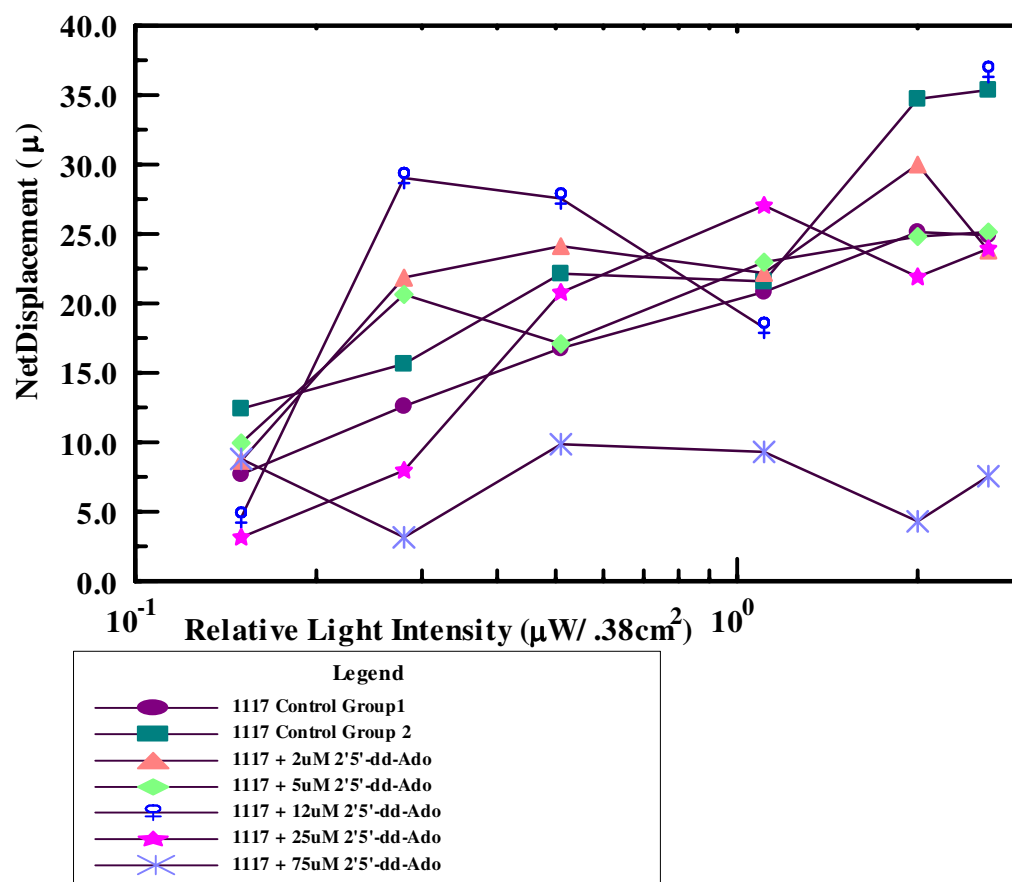
Figure 15: Effects of 2'5'-dideoxyadenosine on 1117 stimulated with green light 505nm
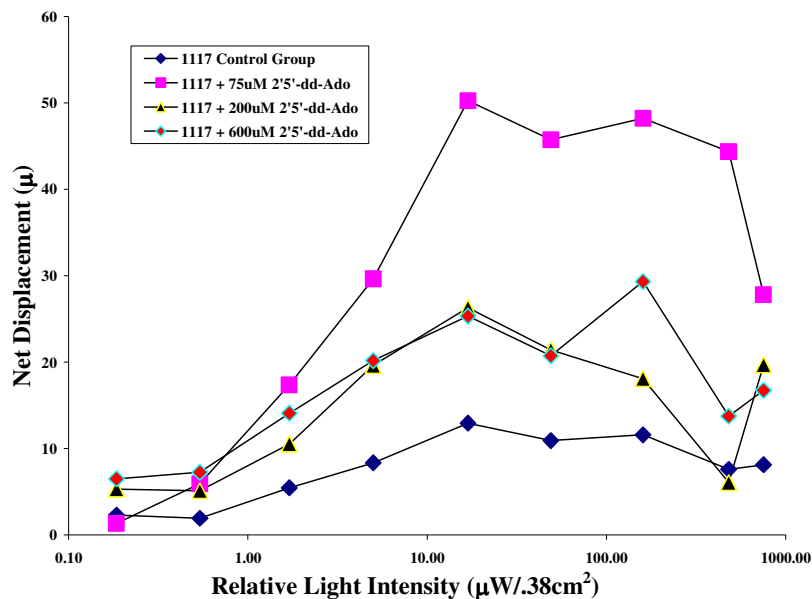
Figure 16: Effects of high doses of 2'5'-dideoxy adenosine on 1117 stimulated with 500nm

In Figure 16, experiments were run approximately one year after the initial experiments in Figure 15 using the same stock solution in DMSO stored at -70ºC. High concentrations of 2'5'-dideoxyadenosine increased the positive phototaxis of 1117, which is inconsistent with the results represented in Figure 15. The response does not correlate with the doses of the inhibitor (higher doses show less effect). The results cannot be interpreted based on the pharmacology of the compounds. In addition to day to day variation with experimental conditions (i.e. temperature, light source, culture), in this case compound shelf life should be taken into consideration. If all the conditions were well controlled and corrected then the inhibitor might have degraded and exerted different pharmacological effects. Thus further testing with 1117 using fresh adenylate cyclase inhibitor is required.

Krc1-4A is a wildtype strain for photosynthesis and exhibits a unique phototaxis behavior: positive at low and negative at high light intensities (Figure 17). This is what we anticipate the cells would do in nature. When light in the environment is low, cells would have positive phototaxis to get enough light for photosynthesis. However, when the light is too high, cells would reverse to negative phototaxis to avoid photodamage. In this particular case, the negative phototaxis magnitude is about 3 times higher than the positive one. Red light normal to green light slightly reduces the reversal intensity. It decreases the magnitude of both positive and negative phototaxis with drastically more on the negative phototaxis. Thus, the magnitudes of both directions of phototaxis of krc1-4A in the presence of red light are approximately the same. This is an interesting strain for studying how the cells switch direction.
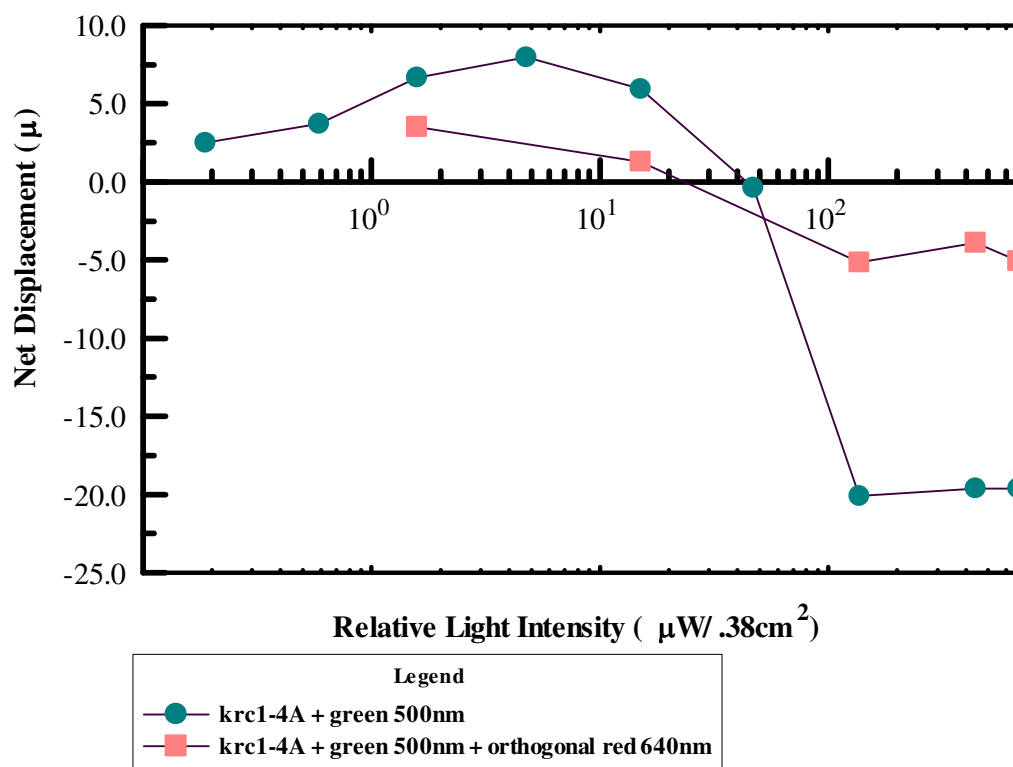
Figure 17: Phototaxis behavior of *Chalmydomonas* strain krc1-4A. The cells were grown on HSM plates for 3-7 days and then suspended in NMM for 27 hours before experiment.

Thus from all of the experiments, intracellular cAMP, concentrations of the chemicals, wavelength and intensity of the light, cell strains and conditions play significant roles in determining the extent and direction of a cell's phototaxis behavior. Under this experimental condition, red light has a slight effect on the phototaxis of strain 806 and 1117. It raises the threshold for negative phototaxis in 806 without or with IBMX. There is no interaction between cAMP regulation and red light. However, red light shows strong effect in reducing negative phototaxis in krc1-4A.

Experiments completed on the time course of gametogenesis on spontaneous reversal of phototaxis in krc1-4A produced conclusive results. In experiments done with krc1-4A and green light (including 500 nm, produced by tungsten lamp, and 525 nm produced from a LED), at low intensities the cells showed positive phototaxis but at high intensities the cells then displayed negative phototaxis, Figure 19. However, it should be noted that while using the 500 nm light cells switched to negative phototaxis at a much higher intensity, approximately 480 $\mu W/0.38cm^2$; while using the 525 nm light the cells switched to negative phototaxis at an intensity of 6.86 $\mu W/0.38cm^2$, Figure 20. Similar differences were observed with using different wavelengths with 806, which suggest that the intensity for reversal is wavelength specific. When the wavelengths are close to the absorption peak of chlorophyll then cells have the tendency to move toward the light.

Red light (630-640nm) of two different intensity levels (LED 17.20 $\mu W/0.38cm^2$ and laser 800 $\mu W/0.38cm^2$), orthogonal to green light, were used as the conditioning background. In experiments with 500 nm plus 640nm of 17.20 $\mu W/0.38cm^2$, the reversal of krc1-4A cells was at a lower intensity (137 $\mu W/0.38cm^2$) than with green light alone; the reversal occurred around (440 $\mu W/0.38cm^2$) with green light alone. Similar trends can be seen while using the 525 nm green light source and the same red light intensity. However, when using the 500 nm green light source with a red light intensity of 800 $\mu W/0.38cm^2$, there is no observable reversal of phototaxis.

Discussion

The single-celled organism, *Chlamydomonas,* occupies the same 'small corner of the evolutionary tree' as humans (Foster and Saranak, 1989). The genome of *Chlamydomonas* gives us useful information. Various mutant strains of known defects are readily available for researchers to use. The cell has haploid genetics, plate colonies, rapid and easy growth, short generation time, and is cost efficient. More importantly, we are able to precisely control the stimulations and the environment of the *Chlamydomonas* cell and can record the responses e.g. phototaxis, ciliary motion, electrical activity in millisecond time scale.

For the purposes of my research I used three different strains of *Chlamydomonas*: 806, 1117, and krc1-4A. The 806 strain displays negative phototaxis at both low and high intensities, while the 1117 strain displays positive phototaxis at both low and high intensities. Genetically, 806 is 1117 with a defective *agg* gene. Thus, by studying these two strains with known genotype and behavior differences we hope to be able to learn the regulatory factors of phototaxis intracellular signaling. In addition, the two strains have differing levels of cyclic adenosine monophosphate (cAMP), an important second messenger; 0.85 and 0.17 pMoles/ 10^6 cells in 1117 and 806 respectively (Table 1). Note that green light (514nm) increased cAMP levels in both strains, red light (669 nm) did not increase cAMP level in 806 (not tested in 1117) (Boonyareth's Ph.D.thesis 2005).

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Intracellular cAMP Levels (pMoles/ 10 ^{6} cells$^)$

	806 (- phototaxis)	1117 (+ phototaxis)
	cAMP	cAMP
Dark	0.17± 0.01	0.85 ± 0.05
10 µM IBMX in the Dark	0.39 ± 0.05	2.29 ± 0.27
32 µM IBMX in the Dark	0.33 ± 0.01	3.08 ± 0.13
100 µM IBMX in the Dark	0.35 ± 0.02	2.87 ± 0.18
514 nm Light	0.51 ± 0.10	0.92 ± 0.14
699 nm Light	0.23 ± 0.03	-

Table 1 Maskiet Boonyareth's Ph.D. thesis (2005), Pharmacology Department, Faculty of Science, Mahidol University, Bangkok, Thailand.

Krc1-4A, unlike the previous two strains, displays positive phototaxis at low and negative phototaxis at high light intensities. Unfortunately, cAMP levels for krc1-4A have not yet been measured. Thus, these three strains are useful for the study of how the cells switch the direction.

In various tests with the 806 strain of *Chlamydomonas*, I found that without any manipulation of the levels of cAMP in the cells, or of the ion concentrations in the test media, the cells phototaxis direction is not reversed, However, there are differences in the threshold for phototaxis when using green light only versus using green light and red light (normal to green), (Figure 7). The red light increases the negative phototaxis threshold of green light. In other words, red light decreases the negative phototaxis sensitivity (1/threshold) of 806 to green light. The results are the same with or without IBMX (a phosphodiesterase

inhibitor) suggesting that red light effect in raising the phototaxis threshold is independent of intracellular cAMP level.

In order to increase or decrease the level, or activity, of cAMP in the cells I used various pharmacological manipulations of the biosynthesis and metabolism pathway of cAMP (Figure 29).
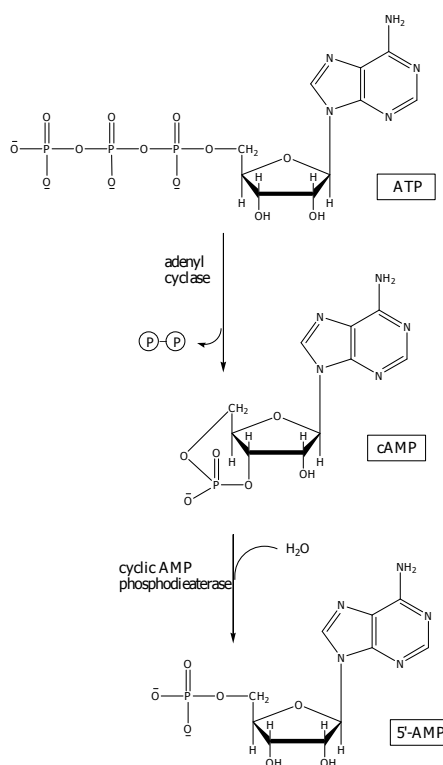
Figure 19: The synthesis and degradation of cAMP

The enzyme adenylate cyclase is responsible for catalyzing ATP to form cAMP. Thus I used 2'5'-dideoxyadenosine to block the enzyme adenyl cyclase to reduce cAMP synthesis and thus decreased the level of cAMP in cells. To increase the level of cAMP in cells I used IBMX to block the phosphodiesterase, an enzyme that breaks cAMP to 5'AMP. The addition of IBMX in 806 increases the level of cAMP (Table 1), and as can be seen in Figure 8, the cells reverse direction at high concentrations of IBMX and high intensities of green light. Red light orthogonal

to green light stimulation raises the threshold of negative phototaxis of 806 without IBMX but has little effect on the reversal intensity and maximum response of 806 with IBMX.

It should be noted that experiments completed with 806 using 500 nm and 505 nm with IBMX both resulted in a reversal from negative to positive phototaxis. The intensity at which the cells showed a clear sign of reversal was distinct for each wavelength. When using 500 nm cells reversed at an intensity of approximately 15.1 μW/0.38cm^2 and 505 nm cells reversed at approximately 0.9 μW/0.38cm^2. This is probably due to different transmission curves of the 2 kinds of light. The 505 nm LED has a wider transmission spectrum compared to the 500 nm narrow band interference filter. Activation of the cells with wavelength in the red usually make cells bias toward the light. In this case 806 cells reverse at lower intensity of LED 505 nm than of narrow band interference filter 500 nm.

Another means used to raise cAMP activity in the cells, was to use a cAMP analog such as 8-Br-cAMP-Na or dibutyryl cAMP. These cAMP analogs are membrane permeable and not readily metabolized by phosphodiesterase. Therefore, they should mimic the effect of increasing intracellular cAMP. In Figure 12, only the lowest concentration (50 μM) of 8-Br cAMP causes a decrease in the magnitude of negative phototaxis, while higher doses (100μM and 200μM) of both analogs increase the negative phototaxis behavior. It is complicated to interpret the data of the high dose since it may be a nonspecific effect on different cyclic nucleotide system or the feedback system of the response and biochemical pathway(s). It has been shown in *Chlamydomonas* that

cAMP and cGMP show opposite effects on the induced phototaxis of a carotenoidless mutant (Saranak, unpublished). The result of IBMX 32 µM and dibutyryl cAMP 100 µM in 806 seems to support the previous notion that IBMX inhibits degradation of the cAMP thus brings the effect on phototaxis close to that of 200 µM dibutyryl cAMP.

Experiments done with 1117 with IBMX caused the magnitude of positive phototaxis to be decreased. As can be seen in Figures 10 and 11 the incorporation of IBMX in 1117, which already has a high cAMP level, causes the level of cAMP to increase and thus become toxic. Evidently, IBMX causes ciliary resorption in 1117 and the cells cannot swim normally. When looking at other parameters measured during the experiment such as the number of cells observed during each individual observation, the number of cells significantly drops from the control group. In the 10 µM concentration, there are approximately one-half the amount of cells observed compared to the control; in the 5 µM concentration, there are approximately one-quarter the amount of cells observed. The number of cells observed during each condition the control, 5 µM, and 10 µM is consistent for the duration of each experiment. The decrease in the number of cells observed means less cell movement is observed, thus the results may be due to the decrease in movement rather than a change in the phototaxis behavior. Further testing of 1117 with IBMX should be done at the time that the cell motility is back to control to draw a conclusion on effect of IBMX on the positive phototaxis.

The adenylate cyclase inhibitor, 2'5'-dideoxyadenosine, should decrease cAMP levels in 1117 and according to the cAMP hypothesis should cause the

phototaxis behavior to lean towards the 806 strain (i.e. less positive or reverse to negative phototaxis). Figure 15 shows the results of freshly obtained data from 2 to 75 µM incorporated in the same batch of 1117, and tested on the same day with two controls (one before and after testing of the treated samples). 2'5'-dideoxyadenosine 75 µM, clearly reduces positive phototaxis of 1117. The reduction is also apparent for 25 µM with low intensities of green light stimulation. There is no clear effect of 2'5'-dideoxyadenosine at 2 -12 µM. While there is no biochemical data of cAMP levels in 1117 after these concentrations of 2'5'-dideoxyadenosine, the reduction of positive phototaxis by 25 and 75 µM suggests that the lower doses might not be enough to decrease the biosynthesis of cAMP and consequently do not change the positive phototaxis of 1117. The concentrations of 25 and 75 µM might be high enough to decrease cAMP biosynthesis and thus cause the reduction of positive phototaxis. This piece of evidence supports the cAMP hypothesis on phototaxis direction and prompts further experiments with higher doses of 2'5'-dideoxyadenosine. I have indeed tried the higher doses of the same batch of 2'5'-dideoxyadenosine stock solution in DMSO stored at -70º C. The results shown in Fig. 16 contradict the previous results (Fig 15). Degradation of the compound over time is one explanation for the contradiction. Also the wavelength and intensities of green light were not the same for the two sets of experiments. Thus, further experiments need to be conducted with the fresh compound, in addition to following (strictly) the same experimental conditions.

Problems and Limitations

Several factors should be considered in planning and conducting experiments such as: the cells are not transparent, stimulation of different photoreceptors can occur, and limitations of light intensities from LED's. Transmission curve of some LED's is not narrow enough. Certain wavelengths are not yet available. However, different kinds of light sources (e.g. laser, tungsten) are available in the lab. The age of cells and different time duration in NMM give variation in the response. To avoid the day to day variation, we always have to compare data taken on the same day, same duration in NMM. No known data of how much of the chemical gets into the cell, how much is metabolized, and importantly no data of how much intracellular cAMP is present after each pharmacological treatment.

Conclusions

From my research, I conclude that green algae respond to changing of light in their environment by moving toward or away to get optimal light levels for photosynthesis and to avoid photodamage. Factors that regulate phototaxis behavior include red light, wavelength and intensity, photosynthesis, and cAMP. From experiments conducted with the cAMP phosphodiesterase inhibitor IBMX, it is clear that increased levels of cAMP cause a change in the phototaxis behavior of 806. In 1117, the presence of IBMX results in ciliary resorption that causes the cell's motility to be limited and thus decrease the maximum response of its phototaxis behavior. Initial results with the adenylate cyclase inhibitor, at high concentrations, suggest that the decrease in cAMP levels causes a decrease in the

maximum response of 1117. However, further experiments need to be conducted to verify that this is reproducible. Further experiments must be completed with the cAMP analogs to understand the effects of both analogs at low and high intensities, as the data suggests that the behavior is different depending on the concentration level of the compound. Krc1-4A, clearly exhibits both positive and negative phototaxis. More experiments will be done to understand factors that regulate the direction of phototaxis, including measurement of membrane potential, ion flux, intracellular pH, pCa.

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