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A Study of Ciliary Beating in Chlamydomonas

Xin Li

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A Study of Ciliary Beating in *Chlamydomonas*

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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and Renée Crown University Honors
May 2014
Honors Capstone Project in Bio-Engineering

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Acknowledgements

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Abstract

The goal of this research is to understand how a unicellular green alga, *Chlamydomonas-reinhardtii*, responds to red light (670nm) and sound waves, by monitoring the beating frequency of its two cilia, which control cell motility. Ciliary beating frequency (BF) is proportional to the production of ATP, mainly generated by photosynthesis. A photosynthesis inhibitor, 3-(3-, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) decreases ATP production by photosynthesis. I used DCMU as a tool to analyze the red light effect on BF.

In the process of studying the effect of red light, I unexpectedly discovered experimental conditions for unstable ciliary beating (as shown in the video). I did a computational analysis of the relationship between ciliary stiffness and BF, to help determine the mechanism of ciliary beating. I studied the initiation of a ciliary wave near the base of a cilium and simulated the effects of changes in the prestress (the static curvature of a cilium) on the wave shape of the ciliary beat.

The fact that the cilia of many organisms respond to mechanical stimuli led to the study of an effect of sound waves on the BF of *C. reinhardtii*. I designed a simple method to preliminarily test the effect of sound waves on the Chlamydomonas cell population, using phototaxis behavior as an indicator. The experiment quickly showed that the mechanosensor response competed with the photoresponse. I also showed that sound waves increased the ciliary BF of the negatively phototactic *Chlamydomonas*. 
Introduction

*Chlamydomonas reinhardtii* is a green, biflagellate single-celled organism 10 µ long. It is a primitive life-form of evolutionary significance. *C. reinhardtii* has been studied intensively over many decades and offers a complete toolset for genetic manipulation. Figure 1.1 shows transmission electron microscopy (TEM) images of *C. reinhardtii*. The *Chlamydomonas* oval cell has a glycoprotein cell wall, a stigma (eyespot), and usually a cup-shaped, pigment-containing chloroplast. The stigma is to track the light for phototaxis (swimming toward or away from light), and the chloroplast is the machinery for photosynthesis. This major organelle within the *C. reinhardtii* cell has two main light-absorbing centers: photosystems I and II. The greatest absorption wavelength is 678nm. To stimulate the cell, I used a 670-nm red-laser light, which is close to its maximum absorption wavelength. Figure 1.2 shows two cilia attached to the cell body. Cilia are tiny hair-like cellular appendages, 12 µm long and 200 nm in diameter. They are important cellular sensors that are sensitive to changes in the environment, such as fluid flow, light, temperature, pH, etc. Ciliary beating propels and steers sperm, larvae and microorganisms. Cilia with the same structure are found in various human organs, such as in the lung to repel dust and bacteria, in the kidneys to measure fluid flow, and in sperm so that they can propel themselves. Therefore, defective cilia can lead to diseases or malfunctions in these organs.

A cross section of a cilium is shown in figure 1.4. The main structure is the axoneme (about 180 nm diameter), which is composed of nine doublet microtubules arranged around a central pair of microtubules. The dynein motors lie between adjacent doublet microtubules. Doublet microtubules slide when the dynein proteins on one side of the axoneme are active and the dynein proteins on the other side are less active. Their sliding cause the axoneme to bend as though the sliding were restrained by springs. Radial spokes which link doublet microtubules
with the central core of the axoneme also constrain axoneme bending by adding elasticity and friction.

For the red-light effect experiments, my model cell was the mutant cpc1-2, which is missing the part of the central-pair complex containing the enolase enzyme, one of the ciliary glycolytic enzymes that produce ATP in the cilia. This means that in the dark (no red light stimulation), cpc1-2 cilia become depleted of ATP supplied from the cell body by photosynthesis. According to Adulrattananuwat (2011), 670-nm red laser light increased the BF of cpc1-2 from a steady state at 30Hz to a new steady state at 50 Hz. However, it has been argued that the red-light effect turns on certain enzyme on the cilia, which results in an increment of the beating frequency (BF). Therefore, the goal of this experiment was to examine whether the BF response to red light is caused by ATP produced from photosynthesis or by the activation of an enzyme on the cilia.

Strathmann (2007) found that sea urchin larvae concentrated particles without filtration, by using mechanosensitive cilia to temporarily and locally redirect current. Strathmann’s results imply that the cilia of many organisms are responsive to mechanical stimuli. Sangadkit (2013) measured sound wave effects on _C. reinhardtii_ motility and phototaxis, using the automated cell tracker in our lab. Inspired by their results, I proposed to study the effects of sound waves on the ciliary motion of a held cell by using an EMCCD camera and quadrant photodiode detector (ciliary monitor). Before doing the experiment using the ciliary monitor, I designed a simple experiment to manually observe the effects of sound waves on population phototaxis in a small Petri dish.

For the computational projects, my goal was to understand the benefit of the design of the _C. reinhardtii_ cilia. Figure 1.3 is a diagrammatic drawing of the ciliary beating mechanism. By
changing only one physical parameter, the static curvature, the beating pattern is transformed from a flagella beating to a ciliary beating. My animation model predicts the effect of the static curvature. In addition, the stiffness of ciliary beating is frequency-dependent. Thus, I proposed a simple model consisting of: a dashpot, which represents the effect of the frequency-dependent property; and a spring, which represents the effect of the longitudinal stretching of the nexins and the bending of the axoneme (see figure 1.3). The proposed model can be fitted to the actual cilia model, with an agreement of a 0.98 regression coefficient.

Methods
Preparation of cells

*Chlamydomonas* cultures, cpc1-2 (for red-light experiments) and 806 (for sound wave experiments), were grown on a high-salt medium (HSM) agar plate (MgSO$_4$•7H$_2$O 81 μM, NH$_4$Cl 9.3 mM, CaCl$_2$•2H$_2$O 0.1 mM, K$_2$HPO$_4$•3H$_2$O 8.27 mM, KH$_2$PO$_4$ 5.3 mM, NaAcetate 15mM, and agar 1.5%), under constant white light (10W/m$^2$) at 16° C for 3-7 days. Before the experiment, the cells were harvested by using a sterilized inoculation loop and suspended in a liquid nitrogen-deficient minimal medium, or NMM (MgSO$_4$•7H$_2$O 81 μM, CaCl$_2$•7H$_2$O 0.1 mM, K$_2$HPO$_4$•3H$_2$O 4.13 mM, KH$_2$PO$_4$ 2.65 mM, and Trace 0.1%), in order to have the cells regenerate cilia and differentiate into gametes. The cell suspension was placed in a shaker (125 rpm) under constant white light for 3-5 hours.

Experiment setup

The setup is shown in figure 2.0. It was composed of a Nikon Optiphot microscope with a 60x water-immersed objective. A micropipette holder on the stage was controlled by a micromanipulator with 6 degrees of freedom to adjust the position of the cell held at the micropipette tip. The cell chamber, with a clear glass bottom, contained 1 ml of cell suspension. The red laser (670 nm) was brought close to the cell body by a small optic fiber. The microscope was connected with an EMCCD camera controlled by a computer. A *Chlamydomonas* cell was captured by air suction through the micropipette tip and then positioned until the two cilia were in good focus on the same plane. It was held in darkness for 30 min. The ciliary beating behavior with and without the red light was then recorded at 500 frames/sec, for a duration of 1 to 10 seconds. A modified program VirtualDub was used to play the recorded video clip at a slow speed, so that the BF could be perceived and counted by the human eye.
Figure 2.0. The microscope setup (left) and a close-up (right), showing the cell chamber on the stage, with a micropipette, the optic fiber and the 60X water-dip objective lens.

**Sound stimulation**

A waterproof earphone (see Figs. 3.2.1 and 3.2.2) was used to generate sound waves. At the beginning of the experiment, the earphone was first sterilized using 70% alcohol, then positioned on the cell chamber at a distance of 1.1 cm away from the glass pipet tip. The other end of the earphone was attached to a function generator that gives sinusoidal signals. Effective
sound wave stimulation for *C. reinhardtii* was in the range of 1, 3, 5, 10, 20 Hz, and at amplitudes of 1, 2, 3, 4 V (Sangadkit, 2013).

**Results and Discussion**

**The Red-Light Effects**

If the red-light effect is mainly due to photosynthesis, in the absence of DCMU, one should expect to see an increase in BF; and in the presence of DCMU, one should expect see a lesser or no increase in BF.

For the first protocol, 5 cpc1-2 cells were recorded. DCMU was not given; only continuous red light at an intensity of 30 W/m$^2$ intensity was used to stimulate the cells. For each cell, the BF without red light was first recorded after a 90-second dark adaption. Next, the BF with red light was recorded after a 90-second exposure to the red light. Then the sample was kept in darkness for 15 minutes before the next BF recording. Such on-off operations were repeated for 4 times for each cell. Some cells lost activity after the red-light stimulation. The results of this protocol (table 2.1) showed no increase in cpc1-2 BF by red light. Since there was no red-light effect detected in this set of experiments, the DCMU effect was not investigated.

My next question was whether the DCMU would change the cpc1-2 BF with or without red light stimulation. Therefore, I conducted another set of experiments to study the DCMU effects. DCMU was given cumulatively in 0, 1, 4, 16, and 64 μM to each held cell for BF recording. Two conditions: “red laser on” and “red laser off,” with the same protocol, were tried with a given DCMU concentration. One typical data set is shown in figure 2.2. For each DCMU concentration, the BF was similar under both conditions: when the red light was on and off. These data are consistent with previous set of data, which showed that the red light did not increase the cpc1-2 BF recorded by the rapid EMCCD camera.
In contrast to the previous results obtained by Adulrattananuwat (2011), under my experimental conditions, the BF of the cpc1-2 did not increase significantly with the stimulation of red light. The stimulation by impulses of red light give a blast of sudden intensity that might cause some harm to the cpc1-2 cells. There was a sign that some cpc1-2 cells might have been damaged, since the cilia stopped beating at the middle or end of the experiments. Usually, held cells can stay active for hours. An optional modification is to use a laser module to convert the red light impulse into a sinusoidal signal, which would provide a slow increase in the light intensity for the cpc1-2 strain.

As a by-product of this project, I was able to observe and record a newly discovered ciliary behavior of cpc1-2 that was not known before. A process depicting how cilia behave in recovering their beating was unexpectedly observed in some of the video records by the EMCCD camera. In the attached video, the time was slowed 32 times compared to real time. At the beginning, the cilia were not beating and stayed in a fixed position. When the cell was stimulated by red light, the cilia started vibrating and then beating. The mechanism of how cilia start beating has not been observed or studied. My video recordings of 3 cpc1-2 cells will initiate further exploration of this phenomenon.

<table>
<thead>
<tr>
<th>Ciliary beating frequency of 5 cpc1-2 cells (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red laser on</td>
</tr>
<tr>
<td>26.1 Hz ± 1.71</td>
</tr>
</tbody>
</table>

Table 2.1
The Sound Effect Experiment

A suspension of microspheres 1 µ diameter in distilled water was put in the cell chamber to test the sound wave effect on the mechanical vibration of small particles. It was confirmed that these particles vibrated at the same frequency as the sound wave. The negative phototactic strain 806, which has a very stable BF of 50 Hz, was used for this project. Using the same apparatus as in the red-light study, the cell was captured and adjusted to a focused, planar position, and the BF was recorded in the dark, without and with sound waves. The preliminary data (Table 3.1) suggested that the BF of 806 was increased by the sound wave.

I conducted another set of experiments to test the sound wave effect on the negative phototaxis of 806. I separated the cell suspension (in NMM) into two samples of 1.5 ml in two small Petri dishes. One waterproof earphone was immersed near the edge of one of the dishes.
(see figure 3.2.1 and figure 3.2.2). A function generator generated a 3-volt, 3-Hz signal for the earphone. A white fluorescent lamp with low-light intensity was placed on the side opposite to the earphone. After 3 minutes, the phototaxis behavior was observed and recorded, as shown in figure 3.2.1. The cells in the dish without sound stimulation moved away from the light (negative phototaxis) more than the cells with sound stimulation. In another, separate trial, the observation was done 20 minutes after the light exposure (figure 3.2.2). In the dish with the sound wave stimulation, the cells were spread out in the solution evenly (no negative phototaxis). In the dish without the sound wave, the cells migrated in the direction opposite to the light source. The results showed that the sound wave decreased the phototaxis of 806 cells.

A separate set of experiments conducted by my lab-mate, Wipavadee Sangadkit, using the automated cell tracker showed similar results: that sound waves reduce the cell phototaxis (figure 3.3).

<table>
<thead>
<tr>
<th>Ciliary BF(Hz)</th>
<th>Sound wave stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without sound</td>
<td>4v,3Hz</td>
</tr>
<tr>
<td>63</td>
<td>69</td>
</tr>
</tbody>
</table>
Table 3.1. Effects of sound waves on ciliary BF of a 806 cell

Figure 3.2.1
Effects of sound waves on the negative phototaxis of an 806 cell population. Cool white light was opposite to the microphone.

Figure 3.2.2

Figure 3.3. Sangadkit’s (2013) results showing the effects of sound waves on the phototaxis of 806, recorded by automated cell tracker.

\[
\text{% Reduction} = \frac{(\text{Net displacement at control} - \text{Net displacement light after off sound})}{\text{Net displacement at control}} \times 100
\]
Both Sangadkit’s and my results consistently show that sound waves at amplitudes of 1 to 4 volts and a frequency range of 3 to 10 Hz affect the net displacement (decrease the negative phototaxis) of the 806 strain. My results in table 3.1 show that the 806 cells responded to sound by increasing their BF. However, in order to align these two parameters, the ciliary beating pattern needs to be studied. Non-rhythmic ciliary beating pushes the cell at a much lower speed than rhythmic ciliary beating. A further investigation would be to use a photodiode quadrant detector to record the beating phase of the two cilia simultaneously.

The Computational Projects

**Animation.** I have simulated a ciliary beat model via MATLAB & SIMULINK (see the attached video). This animation describes how a flagellum beat starts from a straight line, then becomes ciliary beating. I obtained a bending cillum shape via SIMULINK in MATLAB by the following steps: I integrated a periodical square-wave function $f(s)$ along $s$ to obtain the periodical triangle $dc/ds$ wave (where $f(s)$ is the shear force, and $s$ is the arc length); after making it symmetrical about zero, I integrated it to obtain the curvature wave; after making it symmetrical about zero, I integrated it, to obtain the shear angle wave $\psi(s)$; then I plotted the cillum’s shape in $X$-$Y$ Cartesian coordinates, where the $Y$ axis is $\int s \sin (\psi(s)) \, ds$, and the $X$ axis is $\int \cos (\psi(s)) \, ds$. Finally, I wrote a program to propagate the $\psi(s)$ versus the $s$ wave, and at the same time, transfer the wave from the arc length domain to the $x$-$y$ axis domain. In addition, my program enabled a change from flagellar beating to ciliary beating by adding a ramp function to the shear angle wave $\psi(s)$.

Next, I modified this model by adding a part that displayed how the wave was generated. In the old model, $\frac{d\psi(s)}{ds}$ versus $s$ is a propagating polynomial wave with a constant amplitude.
and wave length. Now, I made this wave amplitude and period increase linearly with time from an initial condition with the wave as a straight line. Then I conducted similar computation on $\frac{d\phi(s)}{ds}$ to obtain a plot of the cilium’s shape in X-Y Cartesian coordinates. Once the wave’s amplitude and period reached the maximum values, it became a stable, oscillating wave. Finally, the ciliary beat-initiating model was continued by the old model.

**Computation.** A numerical calculation showing the relationship between moment and curvature was conducted, and the results are shown in figure 4.0. The dots in the figure correspond to evenly spaced intervals along the arc length. The solid dot in the middle of graph is the starting point at the basal end, then the dot goes counter-clockwise and back to its original point.

![Figure 4.0](image)

**Figure 4.0**

Relationship between the stiffness of the cilium and its beating frequency
It can be shown by the plots of the beating frequency versus the propagation velocity squared that $E$ (the total energy available) in a wave is constant for cilia. Since $E$ is constant, it can be assumed that $\frac{\lambda}{\theta^2}$ is proportional to $\kappa_x + \kappa_s\lambda$, where $\lambda$ is the wavelength and $\theta$ is the peak-to-peak tangent angle, $\kappa_x$ is the flexure rigidity of the cilium, and $\kappa_s$ is a spring constant. A plot of the raw data from the literature is shown in figure 4.1 (Brokaw, 1998, table 5).

![Figure 4.1. Raw data of $\lambda/\theta^2$ versus $\nu$](image)

For the maximum $K_s$ value possible versus the minimum $K_s$ value possible, refer to figure 4.2. The standard for choosing the range of $K_s$ values follows two rules: first, there must be a linear fit $R^2$ greater than 0.9, and second, the residual should be greater than zero, which guarantees that $\frac{\lambda}{\theta^2}$ is always positive.
After substract a constant Ks*wavelength

Figure 4.2. The plot of $\frac{\lambda}{\Theta^2}$ versus $\nu$ after subtraction of a constant $Ks^*$wavelength

$$y = 0.0429x + 0.0343$$
$$R^2 = 0.9916$$

$$y = 0.0245x + 1.5536$$
$$R^2 = 0.9088$$

Figure 4.3. The model I used was composed by a dashpot parallel with a spring.

The equation that describes this system is $(t) = \eta \dot{\theta} + \theta + k$, where $(t)$ is the function of force, $\eta$ is the dashpot constant, and $\theta$ is displacement. When the force is oscillating at the same
frequency as the cilia, this equation can be written as \( f_0 \cos(\nu t) = \eta \dot{\theta} + \theta \kappa \), where \( f_0 \) is maximum force possible and \( t \) is the time. I created a MATLAB program to calculate the variables \( f_0 \), \( \eta \) and \( \kappa \). The idea of my program was as follows:

For every valid value of \( Ks \) (\( Ks \) can be 0.057, 0.058, 0.059, 0.060...0.079), a random constant is chosen from (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1) to replace \( \kappa \); a random constant is chosen from (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) to replace \( \lambda \); and a random constant is chosen from (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) to replace \( f_0 \). After the substitution, my program continues to apply the equation \( f_0 \cos(\nu t) = \eta \dot{\theta} + \theta \kappa \), in order to compute and plot \( \lambda/\nu^2 \) versus \( \nu \). The regression coefficient \( R^2 \) is then computed by fitting the computed data plot to the literature data plot shown in figure 4.2. For each \( Ks \) value, my program returns the maximum \( R^2 \) possible and a corresponding combination of \( f_0 \), \( \eta \) and \( \kappa \). To sum up, for a fixed \( Ks \) value, there are 1,000 possible combinations of \( f_0 \), \( \eta \) and \( \kappa \) values; and, since there are 22 possible values of \( Ks \), there are 22,000 fittings tried.

The calculation results are shown in figure 4. The optimum fitting seems to be when \( Ks \) is 0.079 with an \( R^2 \) of 0.99. This model implies that the stiffness of cilia is frequency-dependent. This model is similar to the model studied by J.C. Snowdon (1968). He proposed a model illustrating that the dynamic shear modulus of neoprene is proportional to frequency for a given frequency range. His model also shows that the neoprene becomes stiffer as the frequency increases.

<table>
<thead>
<tr>
<th>( R^2 )</th>
<th>( Ks )</th>
<th>Spring</th>
<th>Dashpot</th>
<th>Force</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.816636</td>
<td>0.057</td>
<td>8</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>0.758306</td>
<td>0.058</td>
<td>8</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>0.706096</td>
<td>0.059</td>
<td>5</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>0.671919</td>
<td>0.06</td>
<td>9</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td>0.816299</td>
<td>0.061</td>
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<td>4</td>
</tr>
<tr>
<td>0.894278</td>
<td>0.062</td>
<td>9</td>
<td>0.4</td>
<td>4</td>
</tr>
</tbody>
</table>
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