Ciliary Behavior of a Negatively Phototactic
*Chlamydomonas reinhardtii*

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With an instrument that can record the motion of both cilia of the unicellular alga *Chlamydomonas reinhardtii* for many hours, the behavioral differences of its two cilia have been studied to determine their specific role in phototaxis. The organism was held on a fixed micropipette with the plane of ciliary beating rotated into the imaging plane of a quadrant photodetector. The responses to square-wave light patterns of a wide range of temporal frequencies were used to characterize the responses of each cilium. Eighty-one cells were examined showing an unexpectedly diverse range of responses. Plausible common signals for the linear and nonlinear signals from the cell body are suggested. Three independent ciliary measures—the beat frequency, stroke velocity, and phasing of the two cilia—have been identified. The cell body communicates to the cilia the direction of phototaxis the cell desires to go, the absolute light intensity, and the appropriate graded transient response for tracking the light source. The complexity revealed by each measure of the ciliary response indicates many independent variables are involved in the net phototactic response. In spite of their morphological similarity, the two cilia of *Chlamydomonas* respond uniquely. Probably the signals from the cell body fan out to independent pathways in the cilia. Each cilium modifies the input in its own way. The change in the pattern of the effective and recovery strokes of each cilium associated with negative phototaxis has been demonstrated and its involvement in phototactic turning is described. Cell Motil. Cytoskeleton 61:97–111, 2005. © 2005 Wiley-Liss, Inc.

Key words: flagella/cilia; phototaxis; motility; signal integration; nonlinear dynamic network

INTRODUCTION

In most biological cells, signals from multiple sensory and internal inputs are coupled together through a network of interactions to control multiple cell outputs that integrate together to perform some function. This is as true for unicellular organisms as it is for cells within multicellular systems. We have chosen the unicellular organism *Chlamydomonas reinhardtii* (Fig. 1) as a model for study of the nonlinear dynamic network that connects and processes multiple cellular inputs and integrates and controls outputs to provide phototaxis function. The extensive information available on this organism and the relative ease of controlling several inputs and observing several outputs and signaling intermediates on the time scale of the decisions made by its signal processing network [Josef et al., 2005 (this issue)] make this model a good choice. *Chlamydomonas* is a green alga whose main activities seem to be energy acquisition, reproduction, and phototaxis [Foster and Smyth, 1980; Foster, ...

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Input detectors include a single lateral eye, which provides an error signal for the cell’s tracking of light source direction. Rhodopsin pigments are the photoreceptors in the eye [Foster et al., 1984] and provide a signal on the time scale of milliseconds. Other photodetectors enable the cell to select among toward, away, or across (orthogonal) the direction relative to the light source [Takahashi and Watanabe, 1993]. A pigment in the chloroplast, which is responsible for photosynthesis, provides information on light quality for photosynthesis. Other potential inputs may include signals from mitochondria responsible for oxidative phosphorylation, multiple cell surface receptors in the plasma membrane, and the two ciliary receptor organelles that detect objects. The signal-processing network for phototaxis can be divided into three parts, the cell body proper and the two cilia. Principle among the outputs is the controlled motion of the pair of cilia, which steer the cell.

Communication among the parts is at least electrical [Nichols and Rikmenspoel, 1978], but probably also involves diffusion of messengers as distances of 5 μm can be reached in 100 ms. Chemical messengers could be distributed relatively quickly along the cilium by the rotating central pair acting like an Archimedes screw. Communication within each part is likely by diffusive chemical messengers such as Ca$^{2+}$ and cAMP [King and Dutcher, 1997].

Relative ease in observing the ciliary responses has been achieved with development of an instrument (Fig. 2A), which observes the cell in the near infrared and follows the motion of both cilia continuously without significantly influencing responses [Josef et al., 2005; Josef, 1994]. Cells are “butt coupled” onto micropipettes (Fig. 2B) permitting observation of the behavior of the cilia of the same cell for hours. Cilia, complete signaling systems in their own right, are a well-confined organelle with measurable inputs, electrical and diffusive messengers. They are relatively well defined structurally. They have active and observable behavior driven by a number of identified motors [Porter, 1996; Kamiya, 2002]. They are optically transparent and without obvious pigments, conditions that will facilitate observation of fluorescent labels that measure ion concentrations dynamically. They have simple flexible cylindrical geometry, which can be described mathematically. Instructions from the cell body come on a short time scale via a measurable electrical input in the form of membrane potential changes or current flows and possibly diffusive signals. Finally, they have a rich and quite sophisticated response, being temperature compensated between 4°C and 30°C, steer for phototaxis toward, away from, or across the light source direction, and show photoavoidance by switching from their normal ciliary beat to flagellar beat.

While our focus is on the ciliary response system, their context as part of the whole organism’s signaling network is important. Cell orientation relative to the light is sensed by the localized lateral eye on the cell body scanning the light field with 2-Hz rotation of the cell body. The membrane potential is altered [Schmidt and Eckert, 1976] and calcium is involved in communicating that signal within the cilia. With cilia without the plasma membrane and consequently isolated from the cell body, below or at a calcium concentration of pCa 9, the cis cilium is more active than the trans 85% of the time, while above or at pCa 7, the trans cilium is 90% of the time more active than the cis [Kamiya and Witman, 1984]. At still higher calcium concentrations, one sees action potentials and photoavoidance. Signals from the chloroplast inform the cell about the quality of light and are integrated probably at the plasma membrane to signal the cilium of the cell’s decision to move toward, away from, or across the light source orientation. We anticipate that adaptation to light level and range compression of the light signal must be a function of the cell body. We anticipate on physical grounds that although both cilia respond differently, they receive the same signal.

![Fig. 1. Schematic diagram of Chlamydomonas cell showing known and hypothesized signaling pathways. As with any schematic, it is an oversimplified depiction of the cell; for example, multiple, not single, curves should model the shape of each cilium. The projection hides the fact that the two cilia are not completely planar and that the predominant beating planes are tilted. The rotation is a net rotation hiding the details of motion during each ciliary beat cycle. The net translation forward in the direction of the arrow comprises 2.3 steps forward followed by 1.0 step backward in each ciliary beat cycle [Racey and Halllett, 1981].](image-url)
from the cell body. Hence, one might anticipate a predominately common response of the ciliary measures except for specific modifications for function. Information on cell currents [reviewed by Sineshchekov and Govorunova, 2001] (Foster et al., unpublished data) suggests the cell current is likely responsible for the general form of response. As far as is known, the membrane potential change propagated around the cell is limited in the amount of information it can convey, creating a potential bottleneck in the signaling network. We don’t know how it could simultaneously signal whether the cell should respond transiently or in a sustained way to a change in light level, how much steering correction to make, which direction to go, and the light intensity level. Particularly hard to integrate with the other signals is the signal of the absolute light level that is needed for the relative phase shift of the anterior to posterior ciliary signal [Josef et al., 2005]. Hence, we wonder if there could, in addition to the electrical signal, be a diffusive signal that is proportional to the absolute light level. We anticipate that virtually all of the temperature compensation and multiple ways the cilia are regulated to give its complex response are the function of the cilia alone. Here we emphasize analysis of the complex ciliary response. Given a common input to the two cilia, we anticipate that any nonlinearities that are not present in the input signal or differences observed in either are a consequence of signal processing in the cilia themselves. We think that ciliary processing accounts for the majority of the nature of the responses described here.

In this report, we further the understanding of these intriguing organelles by analysis of the individual responses of each cilium. What will become a familiar theme are the dissimilarities in the responses of each cilium despite their identical physical appearances and receiving identical signals from the cell body. This is probably, in part, a consequence of their extraordinary...
differences in their responses to calcium [Kamiya and Witman, 1984]. Smyth and Berg [1982] first observed using square-wave stimuli that ciliary responses occur in a short time relative to the rotational scan of the cell, and hence on the time scale of the decisions made by its signal-processing network. We have examined the response of 81 cells to square-wave stimuli to survey the diversity of responses and reevaluate what has been seen by others such as Smyth and Berg [1982], Rüffer and Nultsch [1987, 1990, 1991, 1995, 1997] using flash photomicroscopy in strong orange-red light, and Holland et al. [1997] using electronic detection in the infrared. This work is intended to confirm or identify the primary mechanism of negative phototaxis and serve as a prelude to more detailed analysis of the responses and their control in wild type and mutant organisms. For this study, six parameters derived from observation of the cilia were chosen to study the diversity of response for analysis among all the cells. These were the beat frequency and stroke velocity (RMS of detectors) of the cis and trans cilia, and the relative phase or degree of synchrony of the cilia of the anterior and posterior beating pattern. For a more detailed analysis of ciliary beating, seven measures of response (see Fig. 8) were analyzed for each cilium.

**MATERIALS AND METHODS**

**Ciliary Monitoring**

*Chlamydomonas* cells strain 806 (negatively phototactic, *agg*−) were grown on high salt media (HSM) [Harris, 1988] plates 3 to 7 days under 10 W/m² white light at 18°C. This strain is isogenic with 1117 (*agg*+) and a series of mutant strains. One loop was transferred to ~4 ml no-nitrogen minimal media (NMM) [Harris, 1988] shaken at 120 rpm in front of 10 W/m² white light for at least 4 h and then dark-adapted for 30 min before the experiment. Single cells held on a pipette and data recorded with the microscope and detector system (Fig. 2A,B) were described in our previous study [Josef et al., 2005]. Visual observation of the cell was accomplished with a 75-W frequency adjustable Xenon strobe approximately matched to the ciliary beat frequency. The 600-nm-long wavelength bandpass-filtered strobe output was carried through a fiber-optic bundle coupled into the annular opening of the darkfield condenser and light scattered from the cell and its cilia was observed. Proper cilia position (Fig. 2B) had the ciliary beat plane parallel to the quadrant-photodiode detector with eyespot above the beat plane and directed toward the stimulus fiber. Retractable prisms in the custom-built relay directed the image to the eyepiece or detector array. Constant 800–900 nm illumination from a 150-W Xenon arc lamp illuminated the cilia during data collection.

**Stimulation**

A 0.1-Hz green (543 nm) square-wave stimulation of 13 W/m² (35.5 photons/nm²s) was used throughout this work except as noted. This frequency of 0.1 Hz was chosen to separate the on and off responses and to distinguish sustained from transient responses. Based on preliminary work with a variety of shorter and longer squares waves, 0.1 Hz was chosen as a good compromise between limiting the interaction between the on and the off responses and the number of repetitions that could be done in a reasonable time. A Displaytech Ferro-electric Liquid Crystal (FLC) shutter, model LV050 AC with driver DR50 (Displaytech, Inc., Longmont, CO) modulated a Research Electro-Optics 1.0-mW 543-nm Helium-Neon (HeNe) laser (Research Electro-Optics, Inc., Boulder, CO) (Fig. 2A,B). A 0.98-mm core diameter, unjacketed fiber-optic strand, Edmund Optics NT534, directed the modulated output of the FLC to a point 1 mm from the cell at an angle of 25° with respect to the plane of the microscope stage and cilia. For details see Josef et al. [2005].

**Data Analysis**

From the four detector output signals, five different parameters were extracted: the ciliary beat frequency of each cilium, the overall ciliary velocity of each cilium, and the relative phase difference of cis versus trans cilia. The signals from the four quadrants (Q1, Q2, Q3, Q4) of the detector (Fig. 2B–D) are AC coupled such that they are the first derivative of the incident of light on the detector, where intensity is proportional to the light scattered by the fraction of the cilium imaged in that quadrant.

Ciliary beat frequency is the number of beating cycles the cilium performs each second. One beat cycle is comprised of an effective stroke and a returning recovery stroke. The effective stroke consists of the motion of the tip of the cilium from a maximally anterior position with respect to the cell body to a maximally posterior position (Fig. 2C–E). The recovery stroke is the returning stroke. The beat frequency for each cilium was determined from the time differences between successive signal extremes and zero crossings in the following manner. For each quadrant of the detector, the beat frequency was computed by taking the reciprocal of the time difference at four easily identifiable points of the signal: between successive positive peak values, between successive negative peak values, between successive positive-slope zero crossings, and between negative-slope zero crossings. Each calculated beat frequency was then assigned to be at the midpoint in time between the two successive decisive points. For each detector quadrant,
beat frequency values were then averaged over 24-ms time windows. To determine the trans cilium beat frequency, the beat frequency values obtained from Q1 and Q2 were averaged together. Refer to Figure 2B,E for cell image orientation. To determine the cis cilium beat frequency, values obtained from Q3 and Q4 were averaged together.

Since the signals from each detector quadrant are first derivatives of the light levels, the root-mean-square (RMS) amplitude of each signal is proportional to the rate at which a cilium enters or exits a detector quadrant. Overall ciliary velocity for each cilium was determined by computing the RMS amplitude of the detector signal. To compute the trans cilium overall velocity, the RMS amplitude values for Q1 and Q2 were averaged over 24-ms time windows. To compute the cis cilium overall velocity, Q3 and Q4 were averaged.

Relative phase between detector output signals quantified the synchrony of the cilia. For each beat cycle, time differences between Q1 and Q4 were found by comparing durations at the four easily identifiable points: positive peak values, negative peak values, positive-slope zero crossings, and negative-slope zero crossings. This calculated relative phase was then assigned in time to the midpoint between the decisive points used in the calculation. Time differences were averaged over a 24-ms time window. Relative phase was determined by multiplying the time difference (s) for each window by the corresponding beat frequency (beats/s) and converting to degrees (multiply by 360°/beat). This was repeated for Q2 and Q3.

RESULTS

Cis and trans cilia for 81 cells were examined individually by stimulating each cell with green square-waves of 13 W/m² (35.5 photons/µm²·s, 543 nm). Figures 3A,B through 5A,B are, respectively, typical beat frequency, stroke velocity (labeled as RMS of raw signal), and relative phase responses; Figures 3C,D through 5C,D are summary block plots of the diversity of responses seen. A thick dashed line outlines the time course of the square wave used as stimulus. If a certain number of cells responded positively and transiently to the increase of the light level, then a positive block with a height proportional to that number was placed just after the increase of the light level. Similarly, a block downward indicates the number of cells responding negatively and transiently to an increase of light. If there was a transient response at the off of the stimulus, then blocks were also indicated. Cells responding to both the on and off transitions of the stimulus would have two blocks and these cases are shown by their identical patterns. Cells that responded in a sustained way are indicated by the bars covering the full width of the stimulus between the on and the off of the square wave.

Each cell’s cis and trans cilium exhibited 8 different types out of the 10 possible combinations of beat-frequency response. Responses included transient and sustained beat frequency increase and/or decrease following the positive and/or negative transition of the stimulus (Fig. 3C,D). Common beat-frequency responses observed for either cilium (Fig. 3A,B) were consistent with earlier works [Smyth and Berg, 1982; Rüffer and Nultsch, 1990]. However, a wider variety of responses was observed. The cis cilium of 64 cells responded with a transient beat frequency response, 13 displayed a sustained response, and 4 did not respond (Fig. 3E). The trans cilium of 53 cells responded with a transient beat frequency response, 21 displayed a sustained and 7 did not respond. Cis- and trans-cilia beat frequencies responded in the same sense for 57 of the cells examined and oppositely for 16 (Fig. 3F). While the patterns of responses are quite similar, there are significantly more sustained responses of the trans versus the cis cilium.

Common stroke-velocity responses observed for either cilium are shown in Figure 4A,B. Of the 10 possibilities, there were 8 different characteristic velocity responses observed for the cell’s cis cilium (Fig. 4C). A somewhat different set of 8 responses were observed for the trans cilium (Fig. 4D). Of the 81 cells, 56 cis and 61 trans cilia responded with a transient velocity response; 9 of cis and 10 of trans cilia displayed a sustained increase in velocity during the stimulus pulse (Fig. 4C,D). With respect to the cis- and trans-cilia velocities, 43 cells responded in the same sense while 38 responded differently. The pattern of response is similar to that of beat frequencies. There are, however, no negative sustained responses and the frequency of same responses for both cilia is reduced.

Common cis-trans cilia relative phase responses observed for either cilium are shown in Figure 5A,B. Eight characteristic relative phase responses were recorded, with each cell exhibiting one type of response (Fig. 5). Cis-trans cilia relative phase was determined for both anterior and posterior sections of the cell (see Fig. 2B for orientation). Anterior sections of 39 cells and posterior sections of 47 cells exhibited transient phase responses (phase change from dark baseline); 30 anterior sections while 19 posterior sections responded with a sustained phase increase or decrease from the dark baseline during stimulus “on” intervals (Fig. 5C,D). Compared to the beat frequency and stroke velocity responses, the relative phase responses are much more likely to be sustained although transient responses are still dominant.

Green square-wave stimulation at various frequencies (Fig. 6) provided an estimate of how the cis- and
trans-cilia response magnitude depends on stimulus frequency, known as system gain. The ciliary beat frequency (Fig. 6A–E) and the magnitude of the velocity (Fig. 6F–J) change varied with stimulus frequency. Also seen in Figure 6 is that the response peaks shifted with respect to positive and negative stimulus transitions. Beat frequency and velocity responses peaked near 1-Hz green stimulus frequency and fell off at higher frequencies (Fig. 6E,J). Note that at the peak of response, the cis cilium has a 50% higher beat-frequency response than the trans cilium. On the other hand, the magnitude of the stroke-velocity response of the trans cilium is about 170% higher than that of the cis cilium at the peak of response. The 0.1-Hz periodic square-wave stimuli (Fig. 7D–F) provided a similar estimate of the spectral response with stimulus frequency as that estimated from the method of Figure 6. Cis and trans cilia exhibited a similar gain.

Lastly, data are presented for seven measures of response of each cilium for the most common response type that we believe is associated with negative phototaxis (Fig. 8). Note that the responses depicted here are...
different measures of the same single response of a single cell. There were two motivations behind Figure 8. First was to revisit the question of how does the cell turn in negative phototaxis and second was to obtain an initial estimate on how much of the signal processing and optimization is taking place in the cilia themselves. Our interpretation of Figure 8 for how the cell turns is in the Discussion. The question of where is the signal processing taking place can be analyzed by consideration of the unique nonlinearity present in the signal. Sequential linear processes cannot be ordered, but if the signal passes through a nonlinearity it is forever changed.

First, given the frequent responses to step-ups and -downs (Fig. 8), the cell body must provide a signal that the light level has increased and decreased. Also immediately evident is that the responses of the cis and trans cilia are different in the majority of cases although the stimulus from the cell body is the same. In certain cases, the responses to the step-down are not in general the opposite of the responses to the step-up, indicating that

Fig. 4. Distribution of cilium-velocity change in response to green square-wave stimulation. Responses for 81 stationary cells independently subjected to 100 cycles of 0.1 Hz 13 W/m² (35.5 photons/nm²s) green (543 nm) square-wave stimulation were classified and totaled. Examples of cis or trans cillum response with stimulus form overlaid: (A) typical transient response, (B) typical sustained response. C, D: The distribution in number of cells of velocity change from dark baseline. Square-wave stimulus is overlaid in C and D as dashed lines denoting positive and negative transitions. E: Summarizes velocity change. Transient change (increase or decrease) to positive or negative stimulus transition included cells that responded transiently at one or both transitions. F: Compares cis- to trans-cillum velocity response for the 81-cell set. In comparing cis to trans cilia, velocity response in the same sense indicates that both cilia velocities increased, decreased, or remained unchanged. Some velocity changes could not be compared due to different responses at the positive and negative transition of the square-wave stimulus. For example, if cis-cillum velocity decreased after both square-wave transitions while trans-cillum velocity increased after the positive transition and decreased after the negative transition, the comparison was considered as mixed.
the responses are nonlinear. Some of this nonlinearity is probably present in the signal from the cell. However, the \textit{cis}-cilium’s effective-stroke velocity response (Fig. 8A) appears to be quite linear, response to step-up is positive and to step-down is negative, which might be two nonlinearities making a response look linear, or the cell stimulus is fairly linear. The corresponding \textit{trans}-cilium responds in the same way to step-up and -down and is apparently nonlinear. The recovery stroke velocities (Fig. 8B) follow the same pattern. Another example of same responses to step-up and -down is the \textit{cis}-cilium recovery stroke duration (Fig. 8D).

Reviewing Figures 3 through 5, one may also see many examples where a portion of the cells respond in the same direction for step-ups and step-downs. Another kind of nonlinearity is the unique delay of the \textit{cis}-cilium’s time spent in the anterior quadrant following a step-down (Fig. 8E). Many times there is a suppression of response, for example, on step-up, the \textit{trans} effective-stroke duration (Fig. 8C) and time spent in posterior quadrant (Fig. 8F) and the \textit{cis} beat frequency (Fig. 8G). Also on step-down, the \textit{trans} effective stroke duration (Fig. 8C) and time spent in the posterior quadrant (Fig. 8F) and the \textit{cis} effective stroke duration (Fig. 8C) is suppressed. One final nonlinearity is that the magnitudes and time courses of responses to step-up and -down are not the same.

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**Fig. 5.** Distribution of \textit{cis-trans} cilia relative phase changes in response to green square-wave stimulation. Responses for 81 stationary cells independently subjected to 100 cycles of 0.1 Hz 13 W/m$^2$ (35.5 photons/nm$^2$s) green (543 nm) square-wave stimulation were classified and totaled. Examples of \textit{cis-} or \textit{trans}-cilium response with stimulus form overlaid: (A) typical transient response, (B) typical sustained response. C, D: The distribution of \textit{cis-trans} cilia relative phase change from dark baseline phase (interval with no stimulation) in number of cells. Square-wave stimulus is overlaid in C, D as dashed lines denoting positive and negative transitions. E: Summarizes relative phase change. Transient change (increase or decrease) to positive or negative stimulus transition included cells that responded transiently at one or both transitions.
DISCUSSION

We have examined the detailed responses of 81 cells of a single strain raised as well as we could under identical conditions. We studied in most detail the beat frequency (Fig. 3), stroke velocity (Fig. 4), and relative phase (Fig. 5) responses. In hindsight, we found several factors that altered responses. Principle among these seems to be the history of exposure to red light. This situation came about because during setup the cells were exposed for variable amounts of time to red light so that we could catch the cells to micropipettes. In all subsequent work, we have set up in the near infrared and exposure to red light is deliberate and known rather than

Fig. 6. Ciliary response to various green square-wave stimuli. For each stimulus frequency, the cell was subjected to 100 repetitions of a square-wave stimulus of zero to 1 W/m² (2.7 photons/nm²s, 543 nm) with form overlaid, beat frequency (A–D), and ciliary velocity (E–J). They were computed and averaged for over a time of two stimulus periods. E: The magnitude of the beat-frequency response (gain, on log scale) was computed for each stimulus frequency shown by a circle (trans) or triangle (cis) by determining the power in the beat-frequency change from the dark (non-stimulated interval) baseline beat frequency. J: The magnitude of the velocity response (gain, on log scale) was computed at each stimulus frequency by determining the power in RMS amplitude change from the dark baseline.
Another significant reason for variability is that while the dominant response is negative phototaxis in this range of light intensities, positive phototaxis and diaphototaxis (orthogonal to light) are present to some extent among the individual cells. This variety of responses was also seen previously by Rüffer and Nultsch [1985] probably for these same reasons, except that they kept the red light high in all cases. In fact, a population of cells under these conditions viewed in the microscope doesn’t appear to be stampeding in one direction as one would expect for uniform behavior, but some individual cells can be moving in any direction. Other reasons for variability might include the age of the cells, the time of dark adaptation, time in NMM, and the positioning of the cell is not the same as thought (rarely, the position of the eye might be mistaken) leading to misinterpretation of which cilium is cis or trans. However, what might seem a problem has turned to our advantage in this study because we have been able to identify the types of ciliary responses that are available to these cells. For each of the 3 types of response studied, there were eight different response patterns observed, making a total of 512 possible types of response. While we didn’t attempt to enumerate the incidence of all these patterns, it is clear that only a few dominate what happens, but that “few” is more than the three phototaxis categories we have listed so far. We anticipate a variety of subcategories will be characterized in the near future.

A freely swimming cell rotates counter-clockwise (CCW) (in the left-handed sense) at approximately 2 Hz viewed from the rear of the cell (Fig. 1) [Foster and Smyth, 1980]. Light incident on the photoreceptor is modulated at this rotation frequency in the presence of a source that is not directed along the axis of the swimming direction. Therefore, green stimulation modulated near the cell-rotation frequency should be relevant for phototaxis and produce appreciable ciliary responses. This assumption is supported by the beat frequency and overall velocity gain (Figs. 6, 7) peaking at about one-half of the rotation rate. The peak frequencies and rate at which the response falls off at higher frequencies gives insight into the rate constants and number of reactions in the signaling pathway. However, square-wave stimuli do not adequately represent modulated light detected by a freely swimming cell [Foster and Smyth, 1980]. The modulation amplitude and duty cycle (time “ON” to stimulus period) depend on swimming angle relative to light source direction, inherent noise due to rotational diffusion of the cell body, rocking motions caused by out-of-plane motions during the effective and recovery strokes, and environmental factors. Square-wave stimuli also include higher-order frequency stimulus components not generally found in the cells’ natural environment. The best approximation to what the cell sees occurs when rotating oriented at 90° with respect to the light direction. The responses for a 35.5 photons/nm²s (543 nm) are maximum at about 1 Hz, which is comparable to 3.1 Hz for the current response to half-sines at 500 nm 24 photons/nm²s [Yoshimura and Kamiya, 2001]. In spite of their deficiencies, square-wave stimuli are a useful analytical tool revealing most of what we can learn from stimulation.
Long-period square-wave stimulation can be used to estimate the response to a theoretical, short pulse of light known as the impulse response [Milsum, 1966]. Figure 7 displays the derivative of the square-wave response or the impulse response and corresponding gain (in the frequency domain) for beat frequency, overall velocity, and \textit{cis-trans} cilia relative phase difference. The lack of an extensive latency period indicates initialization of a response is on the order of one beat cycle after the absorption of a photon and signifies a short signal processing time. Each impulse response for the different response measures has a different shape, peak time, and duration suggesting the cell controls each parameter independently and in the cilium. Impulse response duration is shorter than a single cell rotation, which it does by differentiating the incoming light signals on a short time scale and integrating on a longer time scale. The calculation of the impulse response presented assumes that the phototactic signaling pathway is a linear system [Milsum, 1966]. For a linear system, changes at each transition of the long-period square-wave stimulus have the same shape, magnitude, duration, and direction with respect to the stimulus change. Transient responses (Figs. 3A, 4A, 5A) show changes in beat frequency, stroke velocity and phase to only the negative transition of the square wave, indicating the response is strongly nonlinear. Sustained responses (Figs. 3B, 4B, 5B) are, however, approximately linear suggesting that a relatively linear signal from the cell body also can exist.

The many different “responses” as seen by the different measures of response in Figure 8 suggests that the cell may be communicating a common signal, which is then being modified according to what is needed. Such a signal should be easily modifiable into all the other patterns observed. On this basis, we suggest that the \textit{cis} cilium response in Figure 8A,B,E (except for delay on the light off) and F is proportional to the common signal coming from the cell body. Our work with cell current measurements suggests that the common signal is very similar to the integral of the cell current (Foster et al., unpublished data). While the \textit{trans} cilium partially mimics the \textit{cis} cilium, it is significantly modified from the \textit{cis} response, presumably for a functional reason.

Responses of \textit{cis} and \textit{trans} cilia also provide insight into the mechanisms that produce changes in swimming direction during phototaxis. The cell is immersed in a fluid with a very large viscous force compared to inertial force supplied by the cilia. The Reynolds's number is the ratio of inertial force to viscous force [Vogel, 1996]. For \textit{Chlamydomonas}, the maximum Reynolds’s number the cell experiences occurs during its forward stroke and is about $4 \times 10^{-5}$. This implies that the cell has small inertia and the motion is relatively independent of the velocity and relative phase of the cilia [Purcell, 1977]. A very slow cilium stroke will accomplish the identical motion as the same stroke performed very quickly. A stroke with identical shape in the opposite direction will produce the same motion in the opposite direction. A cilium oriented perpendicular to the direction of motion, as during the most of the effective stroke, has 1.5 times the drag of a cilium moving parallel to the direction of motion as during the recovery stroke [Vogel, 1996]. The ratio of forward to backward motion of the cell for one beat cycle has been measured to be about 2.3 and the forward speed is twice the backward speed [Racey and Hallett, 1981]. Orientation to a light source that is initially perpendicular to the swimming direction will take several hundred beat cycles corresponding to 5–7 cell rotations to align the cell path with respect to the light sources.

Different beat frequencies for each cilium would produce turning. However, beat frequency changes to green square-wave stimulation (Figs. 3C,D, 6A–E) reveal the two cilia respond in the same pattern and magnitude for 75% of the cells. This has been interpreted to suggest that phototactic turning is not a direct consequence of beat frequency changes for these cells [Rüffer and Nultsch, 1990]. However, the \textit{trans} cilium responds significantly faster to changes in light than does the \textit{cis} so that probably the beating frequency does play a role in phototaxis (Josef et al., unpublished data).

If the cilia are assumed to beat predominantly in their own plane, the net transient torque producing a turn should depend on the shape and length of the effective and recovery stroke. A detailed study of how one cell performed yielded insight into how changes in the pattern of ciliary beating contribute to how a cell turns. Figure 8 shows percent changes from dark (no external green stimulation) baseline values in response to stimulating with 0.2-Hz green square waves of 13 W/m². For a positive stimulus transition, the \textit{trans} cilium experienced mainly transient changes rather than a sustained change. The velocity decreased (Fig. 8A,B) while beat frequency increased (Fig. 8G). This implies effective and/or recovery stroke duration must have decreased and Figure 8C,D indicates effective stroke duration had minimal change while recovery stroke duration decreased after a positive stimulus transition. In addition, time spent in the anterior quadrant (Q1) (Fig. 8E) decreased, suggesting \textit{trans}-cilium anterior amplitude decreased. The combination of decreased \textit{trans}-cilium velocity and no change in time spent in the posterior quadrant (Q2) (Fig. 8F) suggests a more posterior effective stroke. Examination of 0.4 s of the posterior \textit{trans}-cilium detector output (Q2) (Fig. 8H) following the step-up shows broad, constant, lower amplitude negative response peaks during the stimulus “on” interval as compared
Figure 8.
with the preceding stimulus “off” interval. Since detector signals are a measure of the rate of change of light levels, the constancy of the wide negative peaks of Q2 suggests the trans cilium is moving during this negative peak out of Q2 at constant speed. This suggests that in a recovery stroke, the trans cilium moves forward parallel to the anterior-posterior axis of the cell body. The extra duration and lower amplitude of the constant signal suggest a longer recovery stroke beginning with a more posterior position closer to the body at the end of the effective stroke. The cis-cilium response at the positive transition (ON) of the stimulus is mostly opposite (see Table in Fig. 8).

For a negative stimulus transition (OFF), the trans cilium experienced mostly transient rather than a sustained change in response. Time spent in Q2 (Fig. 8F) remained the same while increasing for Q1 (Fig. 8E). Recovery stroke duration increased, indicating the effective stroke began more anteriorly as shown in Figure 9B. Beat frequency (Fig. 8G) decreased in accordance with a velocity decrease (Fig. 8A) for both cilia. Time spent in the anterior quadrant of the cis cilium (Q4) (Fig. 8E) decreased while increasing for the posterior quadrant (Q3) (Fig. 8F) that presumably resulted from a less anterior stroke shown in Figure 9B.

Figure 9 depicts the deduced cilia stroke length changes for a negatively phototactic cell in response to step-up and step-down light intensity. A free swimming-cell rotating at approximately 2 Hz, exposed to a step-up in light intensity occurring when the eyespot rotates toward the light source will execute a longer cis-cilium and shorter trans-cilium stroke producing a turn toward the trans cilium and away from the light due to the longer cis-cilium stroke. A free swimming cell exposed to a step-down in light intensity occurring when the eyespot rotates away from the light source will execute a longer trans-cilium and shorter cis-cilium stroke producing a turn toward the cis-cilium side and away from the light. The results indicate that the posterior to anterior length of the stroke is the primary factor in phototactic turning along with the shape of the cilia during effective and recovery stroke. Our interpretation of the effect of shape change on the cilia-mediated turning is similar to that of Rüffer and Nultsch [1991].

There are other factors that contribute to phototactic turning. The cilia must have three-dimensional motion to produce 2-Hz cell rotation. Three-dimensional motion can be inferred by slight adjustments in focus necessary during visual observation to bring different parts of each cilium into focus, also seen in previous
work [Rüffer and Nultsch, 1991]. The relative phase changes to square-wave stimulation occurring between the anterior and posterior quadrants of a single cilium [Josef et al., 2005] suggests either a nonplanar motion or a change in the orientation at the base of the cilium that is proportional to absolute light intensity. The detector signal amplitude and, therefore, velocity recorded will reflect changes in the ciliary beat plane. This non-planar motion or changes in cilia plane during a beat cycle presumed to occur in response to changes in light levels are likely to be involved in phototactic turning. Subtle changes are anticipated to compensate for the decreased latency at higher light intensities. The helical motion of a free-swimming cell may also participate in the phototactic response [Foster and Smyth, 1980].

The beat frequency graded response to different stimulus intensities [Josef et al., 2005] may, in part, be a result of graded stroke length changes required to achieve optimal orientation for different relative angles between light source and swimming direction. As stroke length increases, time required to execute the stroke may increase resulting in a beat frequency decrease.

So far, the correlation among the beat frequency, velocity, and cis-trans relative phase response is not clear. Future studies with this monitoring system will use more sophisticated stimulation and analysis techniques to more precisely quantify this correlation. The nonlinear aspects of each response will also be investigated. The responses of mutants will be compared to elucidate the phototactic signaling network.

CONCLUSIONS

It seems likely that both linear and nonlinear signals come from the cell body to control ciliary motion. The linear signal, which is proportional to the light intensity, is hypothesized to give rise to the sustained responses as well as the phase shift between the posterior and anterior quadrants. The nonlinear, which is probably the electrical signal, gives rise to the transient responses on the “on” and “off” of the light. Then each measure of response of each cilium modifies the common input coming from the cell body in its own way. The beat frequency, stroke velocity, and relative phase responses appear to be relatively independent of each other in their responses, suggesting such a common input followed by a fanning out to multiple effectors. Tentatively, we suggest that this nonlinear signal from the cell body is transmitted by a temporal pattern of membrane potential change to the electric field sensitive ion (probably calcium) channels of the cilium. This ion signal fans out to several independent pathways affected by calcium and/or cAMP or magnesium, which in different ways influences the response of the cilia. One may anticipate that the conserved signaling proteins located in the central pair and radial spoke structures mediate these complex patterns of changes [Smith and Yang, 2004]. The stroke shape change as confirmed here and as suggested in the past [Rüffer and Nultsch, 1998] is likely an important aspect of the phototactic response. Nonplanar motions, helical swimming, the relative phase of the cilia and their beating frequencies probably also contribute to the net phototaxis. How these different subtle responses make phototaxis a robust and optimized behavior still remains to be clarified.

REFERENCES


