An Electro-Optic Monitor of the Behavior of Chlamydomonas reinhardtii Cilia

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The unicellular green alga Chlamydomonas reinhardtii steers through water with a pair of cilia (eukaryotic flagella). Long-term observation of the beating of its cilia with controlled stimulation is improving our understanding of how a cell responds to sensory inputs. Here we describe how to record ciliary motion continuously for long periods. We also report experiments on the network of intracellular signaling that connects the environment inputs with response outputs. Local spatial changes in ciliary response on the time scale of the underlying biochemical dynamics are observed. Near-infrared light monitors the cells held by a micropipette. This condition is tolerated well for hours, not interfering with ciliary beating or sensory transduction. A computer integrates the light stimulation of the eye of Chlamydomonas with the ciliary motion making possible long-term correlations. Measures of ciliary responses include the beating frequency, stroke velocity, and stroke duration of each cilium, and the relative phase of the cis and trans cilia. The stationarity and dependence of the system on light intensity was investigated. About 150,000,000 total beat cycles and up to 8 h on one cell have been recorded. Each beat cycle is resolved so that each asynchronous beat is detected. Responses extend only a few hundred milliseconds, but there is a persistence of momentary changes that last much longer. Interestingly, we see a response that is linear with absolute light intensity as well as different kinds of response that are clearly nonlinear, implying two signaling pathways from the cell body to the cilia. Cell Motil. Cytoskeleton 61:83–96, 2005.

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

INTRODUCTION

Response to multiple environmental and internal inputs enables cells to seek more desirable environments. To provide an integrated response to inputs, we hypothesize that each cell has signal-processing machinery in the form of a nonlinear dynamic network that facilitates decisions and coordinates multiple responses of cell effectors. In order to characterize this network, we are using as a model the multiple photoreceptor control of the cilia1 of the unicellular alga Chlamydomonas reinhardtii. This organism uses phototaxis with rhodopsin photoreceptors [Foster and Smyth, 1980; Witman, 1993; Foster, 2001] to position itself relative to its light environment. In this report, we demonstrate the value of computer-controlled

1We use the word cilia rather than eukaryotic flagella to describe the pair of moving appendages that enable the cell to swim to emphasize that these appendages are not the more familiar rotary-motor driven flagella of bacteria. These cilia control linear motors that slide microtubules relative to each other in the axenome and are the same as the cilia of Ciliata, organisms that have provided a significant fraction of the response information on cilia. This choice of terminology follows the advice of Irene Manton [1965] and Tom Cavalier-Smith [1986] who says ‘the word flagellum should be dropped altogether from eukaryotic biology.’

Contract grant sponsor: The Research Corporation; Contract grant number: RA0289; Contract grant sponsor: National Institutes of Health; Contract grant number: GM63527.

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Received 24 November 2004; Accepted 10 February 2005

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cm.20064

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light microscope using flash photomicroscopy [Rüffer and Nultsch, 1987, 1990, 1991, 1995, 1997] and even electronic photodetectors [Smyth and Berg, 1982; Racey and Hallett, 1981, 1983a, b; Holland et al., 1997], we felt that new technologies could significantly improve hardware, software, and analysis. The setup to visually observe the cell is illustrated in Figure 1 and to collect data in Figure 2. This report describes an instrument of relatively inexpensive components that enables computerized low-resolution observation of the cilia. Our analysis has sufficient temporal resolution (about 0.25 ms) to record cell events in the same time scale as the decisions are made by the cell’s signal processing network. The components include a near infra-red (NIR) light source to which the cell does not respond, a darkfield condenser, photodetector, amplifiers and analog-digital converter, and custom analysis software. This instrument, which monitors the cilia dynamically, could reasonably be replicated in other laboratories and used to study the subtle differences of behavioral or ciliary mutants. Here we demonstrate the power of this instrument by applying it to understanding the basics of ciliary responses in the unicellular alga Chlamydomonas as revealed by step changes in light intensity.

Chlamydomonas is a green alga whose gametes are ellipsoids of revolution about 10 μm long and 7 μm in diameter. The organism is phototactic with a single lateral eye (light antenna of Foster and Smyth [1980], ocellus of Ehrenberg [1838]), consisting of a visually observable eyespot or stigma in the chloroplast overlaid with a rhodopsin photoreceptor [Foster et al., 1984] layer in the plasma membrane. With its pair of beating cilia, it can steer toward the light (positive phototaxis), away from the light (negative phototaxis) and in the plane that is orthogonal to the direction of the light source (diaphototaxis) [Foster and Smyth, 1980]. Forward translation of the cell during swimming is achieved with the two adjacent cilia beating in a “breast” or ciliary stroke. Being placed at the anterior end, as defined by the direction the cell normally swims, they pull the cell through the water. To better appreciate the behavior, think of a human doing a breast stroke through the water with an opaque patch covering the left eye, the right becoming a single functional eye like that of Chlamydomonas. The left arm is trans with respect to the usable eye and the right arm, adjacent to the usable eye, is cis. Now detach the left arm from the left shoulder and replace it with a copy of the right arm, noting that it now faces the wrong way. With this loss of true bilateral or mirror symmetry operating both arms in the same way, one can envisage continually rolling to the right. In Chlamydomonas (Fig. 3), following the convention of eyes being on the ventral side, viewed from the rear, the left or trans (on the side opposite with respect to the eye) cilium looks simply like the right or cis (adjacent the eye) cilium rotated about 180° around the longitudinal axis of the cell. Viewed from the rear, the cell rotates at about 2 Hz at 20°C counter-clockwise or left handed. The eye is located at the equator about 45° counter-clockwise from the plane of the cis cilium. This position anticipates or is phase advanced relative to the direction where the cis cilium beating will be when it could potentially steer the cell. Each cilium beats predominantly in a plane, but these two planes are tilted with respect to each other. Rüffer and Nultsch [1987] have noted that the trans cilium beats more out of the plane than does the cis cilium. The asymmetry of their attachment to the cell and their relatively tilted planes of action probably account for the consistent cell rotation. This cell rotation is a critical aspect of its phototaxis ability as it is used to scan the light environment. Whichever direction, cells execute either a helical or superhelical swimming path [Foster and Smyth, 1980].

Like Holland et al. [1997], we have employed NIR illumination to observe the cells, in order to avoid excit-
ing photoreceptors in the cells. Previously, Rüffer and Nultsch [1987, 1990, 1991, 1995, 1997] used very intense orange-red light (as bright as full sunlight), which was seen by the rhodopsin photoreceptor and other red light receptors. Of necessity in Rüffer and Nultsch’s studies, the cells were adapted to a high light intensity, and stimulation was necessarily of even higher light intensity. For these conditions, we have confirmed their findings [Josef et al., 2005 (this issue)]. The intense light they used also would have adapted other photoreceptors within the cell that would also have strongly affected the response.

We hold the cell on the end of a suction micropipette as did Rüffer and Nultsch [1987, 1990, 1991, 1995, 1998] and Holland et al. [1997]. This has the advantage of being able to continuously observe the behavior of a cell for hours. Achieving 8 h of continuous observation is routine, suggesting that the experimental conditions of NIR illumination and holding are quite benign.

According to Rüffer and Nultsch [1991], phototaxis is caused by an asymmetric shift in one of the cilia’s front amplitudes of its breaststroke beat (the amount of forward reach of the cilium). The ciliary beats differ between the cis and trans cilia and between a step-up or step-down in the light stimulation as experienced by the eye during the rotation of the cell. The differences in the nature of these responses result in the net differences in behavior corresponding to positive or negative phototaxis.

**MATERIALS AND METHODS**

**Cell Preparation**

Cells were grown on a high salt media (HSM) plate for 3 to 7 days under 24 h constant fluorescent illumination of 10 W/m² at 18°C. They were transferred to a solution of nitrogen-deficient minimal medium (NMM) that contained 100 μM calcium chloride [Hutner et al., 1950] with a cell concentration of approximately 2 × 10⁵ cells/ml. The cell solution was shaken at 120 rpm with fluorescent illumination of 10 W/m² for 4 h to allow cilia to grow and cells became uniformly gametes. After 4 h, the cell
solution was removed from the shaker and placed in darkness for a minimum of 30 min.

The negatively phototactic strain, 806, was used for all experiments reported here except the data for Figure 10 was recorded from the positively phototactic strain, 1117 [Smyth and Ebersold, 1985]. Strain 806, by 16 backcrosses, is isogenic with the wild type 1117 and is missing the agg gene (see Harris [1989], p. 477).

Microscope

Figure 1 shows the optical configuration for positioning the cell and Figure 2 shows the configuration used for data collection. The optical system consists of a Nikon Eclipse E600FN microscope (Nikon, Melville, NY) with a Nikon CFI 60X/1.00W Fluor DIC water dipping objective, working distance of 2 mm, and 1.0 numerical aperture (N.A.). The cilia were viewed visually with a 10X binocular eyepiece. A mirror inserted or removed just prior to the microscope binocular diverted the beam to the binocular for visual imaging or through the vertical port of the microscope to the detector. A custom-built optical relay shown in Figure 2 produced a secondary real intermediate image in the optical system. The relay consisted of two OptoSigma Broadband antireflection coated 25-mm glass prisms, 055-0150-PRI (OptoSigma Corp., Santa Ana, CA), two Edmund Optics NIR achromatic 100-mm focal length lenses, NT45-806 (Edmund Industrial Optics, Barrington, NJ), and four Edmund Optics protected elliptical gold-plated mirrors, NT32-088, for optimum reflection of NIR light. The prisms can be positioned to include the relay with the prisms in the optical pathway or removed to allow the beam to pass through unaffected. An adjustable pin inserted at the intermediate image plane blocks the relatively intense light scattered from the pipette and cell body. This makes it possible for the much dimmer light scatter from the cilia to be a significant fraction of the light reaching the detector (Fig. 3). A Diagnostic Instruments 0.63X image reducer (Diagnostic Instruments, Sterling Heights, MI) incorporated in the vertical port produced an overall magnification of 38X at the photodiode quadrant array detector. The optical relay also rectified the inverted image of the cell providing an image at the detector and the microscope eyepieces with the same directional sense as the actual cell. This makes it easier for the experimenter to position the cell and its stimulator.

Capturing and Holding a Single Cell

A Kopf Vertical Pipette Puller Model 720 (David Kopf Instruments, Tujunga, CA) was used to pull a 90-mm-long Kimax-51 glass capillary tube 1.8 mm OD, 1.5 mm ID (Kimble/Kontes, Vineland, NJ) to form a micropipette with a tip of approximately 5 µm OD, 3–4 µm ID. A cell suspension of 200 µl was injected into the reservoir at the center of the microscope stage and gentle suction was applied to the micropipette to capture a single cell on the tip. A specially designed manipulator with six degrees of freedom held the pipette on the microscope stage. The cell can be rotated around the axis of its holding micropipette, in the vertical plane of the micropipette and about the vertical axis of the microscope. In addition, the cell may be moved in and out with respect to the center as well as moved laterally and vertically to position the cell at the center of rotation of the stage.
Visual Observation of the Cilia

To visually inspect and position the cilia of a stationary cell, a 75 W Xenon strobe, Oriel Model 6427, and Oriel Instruments Model 68826 power supply (Spectra Physics, Mountain View, CA) illuminated the cell (Fig. 1). A fiber optic bundle of forty 1-mm diameter, 0.5-m-long strands (Edmund Optics NT02-534), with a 600-nm long-pass Kodak Wratten Filter 29 (Eastman Kodak Company, Rochester, NY) coupled the single beam source to the annular aperture of a Nikon oil immersion 1.43–1.20 N.A. dark field condenser. The strobe flash frequency, adjustable from 0.1 to 75 Hz and energy/flash set at 1,000 mJ, was adjusted to match the ciliary beating frequency providing direct observation of the 600× magnified cilia. The cell was maneuvered to obtain the proper orientation with respect to the microscope and detector photodiode array as shown in Figure 3.

Photodetector Cell Imaging

To illuminate a properly positioned cell for continuous monitoring of its cilia, a constant intensity Oriel Model 6253, 150W xenon arc lamp powered by a Photon Technology International MODEL LPS-200 power supply (Photon Technology International, Inc., Lawrenceville, NJ) replaced the 75-W Xenon strobe arc lamp (Fig. 2). A Melles Griot 800–900 nm reflector, 03-MHG-009 (Melles Griot, Carlsbad, CA), and a 900-mm short-wavelength mirror filtered the xenon lamp output. The beam was directed through the rear of the microscope and focused on the darkfield condenser. NIR illumination of the cell minimized the excitation of the cells’ photoreceptors.

The cell body scatters the light enormously more effectively than the thin cilia. Hence, the imaging pathway was altered with an optical relay to make an accessible secondary image of the cell. To minimize the scattered light from the cell body reaching the detector, a blocking pin was positioned at the 30× magnified intermediate image of the cell. With both the cell and blocking pin imaged in the same optical plane, it is simple to manually align them by viewing through the microscope eyepieces. This partially blocked image was then directed to the quadrant photodiode array of the detector (see Fig. 2).

Electro-Optical Detector

The 38× magnified images of the cell’s cilia were focused on a light-sensitive custom-built detector with a Hamamatsu S1557 circular quadrant photodiode array (Hamamatsu USA, Bridgewater, NJ). Each quadrant independently transformed light levels produced by NIR illumination scattered from the cilia to a voltage signal. Signals were bandpass filtered (20–80 Hz) and amplified with 250 dB overall gain so that changes in light levels in each quadrant produced nonzero signal amplitudes while constant light levels produced a zero signal amplitude [Josef, 1994]. Outputs of the detector, corresponding to changes in light levels, were digitized at 4 KHz with a Computer Boards CIO-DAS-1602/12 12 bit A/D board (Measurement Computing, Middleboro, MA) and stored in the experiment control and data collection computer, Dell GX1P 450 MHz. All cells were adapted before each recorded stimulation interval by exposing them to the green square-wave stimulation of the particular experiment for 60 sec. Data were collected under conditions of no green stimulation (dark) and green square-wave stimulation.

Light Stimulation of the Cell

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. 3). A 0.98-mm core diameter, unjacketed fiber-optic strand (Edmund Optics NT-534) directed the modulated output to a point 1 mm from the cell at an angle of 25° with respect to the plane of the microscope stage and cilia.

Data Analysis

From the four detector output signals, nine different parameters were extracted: ciliary beat frequency, overall ciliary velocity, effective and recovery stroke velocities, effective and recovery stroke durations, time cilia spend in each detector quadrant, cis-trans cilia synchrony (cis minus trans phase difference), and individual cilium shape changes (anterior minus posterior quadrant phase difference of the same cilium).

The signals from the four quadrants (Q) of the detector are AC coupled such that the signals are the first derivative of the light intensity incident on the detector, due to the light scattered by the fraction of the cilium imaged in that quadrant (Fig. 4C). This first-derivative signal is shown in Figure 4B. The effective stroke extends as shown in Figure 4C for the cis cilium from the time of maximum extension of the cilium in Q4 to the maximum in Q3. Because the signal is the first derivative, as shown in Figure 4B the effective stroke is from the negative zero crossing of Q4 to the negative zero crossing of Q3.

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. 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. The beat frequency was considered to be defined at the midpoint in time between the two successive identifiable 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

Fig. 4. A typical detector output signal. The signal for *Chlamydomonas* strain 806 cilia (A) is shown during a dark period (signal from Q1 omitted). The first 0.05 sec of A are shown in B, displaying detector output for cis-cilium anterior (Q4) and posterior (Q3) portions of the stroke. The estimated position of the cis cilium (C) is determined by integrating the output signal from each quadrant (Q4 and Q3). The situation is similar for the trans cilium (Q1 and Q2). The positions of the cilia are imaged on the photodiode array (D) at the time of each recording above (B, C). The leftmost image shows cilia at the end of the effective stroke with velocity near zero (detector signal near zero) and in Q2 and Q3. The second image shows cilia at the end of the recovery stroke with velocity near zero (detector signal near zero) and in Q1 and Q4. The two images on the right show cilia transiting the boundaries of the quadrants during the recovery stroke and effective stroke producing large positive (entering quadrant) and negative (exiting quadrant) signals corresponding to rapid changes in scattered light levels. As indicated by the arrows in A, the trans cilium occasionally executes an additional beat cycle.
obtained from Q1 and Q2 were averaged together. Refer to Figure 4D for cell image orientation. To determine the cis-cilium beat frequency, values obtained from Q3 and Q4 were averaged together. Other more sophisticated methods to compute beat frequency using power spectrum analysis were also tried; however, they were computationally more time-consuming and gave similar values.

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.

The effective-stroke velocity for each cilium was computed by separating the positive and negative detector output signals. For a given quadrant, positive signal values indicate part of the cilium is moving into that quadrant while negative signal values indicate part of the cilium is moving out of the quadrant. For the cis cilium, positive signal RMS amplitude values of Q3 were averaged with negative signal RMS amplitude values of Q4. Refer to Figures 3 and 4 for cell image orientation and corresponding detector signals. RMS amplitude values were then averaged over a 24-ms time windows. The effective stroke velocity of the trans cilium was similarly calculated from the signals from Q2 and Q1.

Recovery-stroke velocity for each cilium was computed in a similar manner as the effective-stroke velocity. For the cis cilium, positive signal RMS amplitude values of Q4 were averaged with negative signal RMS amplitude values of Q3. The values for the trans cilium were calculated similarly except using signals from Q1 and Q2, respectively.

The duration of the effective stroke was found from analysis of the successive zero crossings of the detector output signal (Fig. 4B). For the trans cilium, the time between the positive-slope zero crossing and the next negative-slope zero crossing of Q2 and between the negative-slope zero crossing and the next positive-slope zero crossing of Q1 were used.

These times were averaged over 24-ms time windows. The time of the effective-stroke duration was defined to be at the midpoint between the successive zero crossings. The values for the cis cilium were calculated similarly except using the zero crossings of Q3 and Q4. The duration of the recovery stroke was computed in a similar manner to the effective-stroke duration as it simply involves the other half of the stroke.

A cilium was estimated to be entering a quadrant when the signal output of that quadrant was at a maximum positive value and exiting the quadrant when the signal output was at a minimum value. Time cilia spent in each quadrant was determined by finding time differences between each positive-signal peak value and the next negative-signal peak value.

Relative phase between detector output signals quantified the synchrony of the cilia. For each beat cycle, time differences between Q1 and Q4 (the cis phase minus the trans phase) 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. If the cis-ciliary stroke reaches maximally forward a little earlier than the trans stroke, then the relative phase will be positive. The relative phase so defined was taken as occurring at the midpoint between each identifiable point. Time differences were averaged over a 24-ms time windows. Relative phase was determined by multiplying the time difference for each window by the corresponding beat frequency and converting to degrees. This was repeated for Q2 and Q3.

Change in the ciliary shape or orientation of the cilium was assayed by the anterior phase minus the posterior phase for each cilium. This relative phase of the Q1 phase minus the Q2 phase for the trans cilium, and the relative phases Q4 minus Q3 for the cis cilium were determined in a similar manner to the phase differences obtained for the synchrony of the cis and trans cilia.

RESULTS

Typical Synchronized and Asynchronized Ciliary Beating

Figure 4A illustrates typical detector outputs with signal-to-noise ratio in excess of 20 dB. As a cilium alternated between two adjacent quadrants (Fig. 4D), signal amplitude alternated between positive and negative values that were proportional to the rate the cilium entered (positive-signal amplitude) or exited (negative-signal amplitude) each quadrant. Relative phase of approximately 180° between the two waveforms (Fig. 4B) monitoring the cis cilium (Q3 and Q4) arises from the cilium’s entering one quadrant while exiting the other. Changes in light levels in each quadrant should be extremely large, producing signal spikes during the effective stroke as cilia cross the boundary between the two anterior and posterior quadrants. However, bandpass filtering (20–80 Hz) of the detector limited the frequency range and smoothed the signals. A small phase difference between Q2 and Q3 signals (Fig. 4A) and Q1 and Q4 (not shown), indicates effective and recovery strokes are
synchronized. Spontaneous, short-duration asynchronous beating does occur as depicted in Figure 4A and has been observed by others [Rüffer and Nultsch, 1987]. Note the cilia lose synchronization at midpoints of effective stroke (left arrow in Fig. 4A) and regains synchronization at midpoints of recovery stroke (right arrow in Fig. 4A). Figure 5F shows the two cilia generally remain synchronized over long periods without green stimulation. However, there were intervals of spontaneous short-duration swimming-mode changes and/or stops (arrow in Fig. 5F) by the cilia reminiscent of bacterial turns [Berg, 1975]. Parameters extracted from detector output signals (Fig. 5) remained stable over long periods in the dark, i.e., in the absence of red or green stimulation.

Adaptation

Figure 6 shows the importance of conditioning the cell to green stimulation for measurement of steady-state response as the cell shows habituation to the stimulus. A cell suddenly introduced to green stimulation after being in the dark for a long time shows a transient response. To make measurements at a steady state, therefore, requires an adaptation period. Figure 6 shows that changes in beat frequency following the sudden shut-off of the light stimulus tended to decrease relative to the dark baseline over the stimulation period as the cell adapted to the stimulus. The time course of the beat-frequency response changed as well.

Effect of Green Stimulus Intensity

The cell examined in Figure 7 responded with a small transient increase in beat frequency of the cis cillum at the positive transition of the step stimulus. It also responded with a decrease in beat frequency at the negative transition of the step stimulus. Similar responses were reported by Rüffer and Nultsch [1990]. Note the nonlinearity here. The response to a positive step is much smaller and less light dependent and, hence, is not the opposite of the response to a negative step. Beat-frequency percent change after the positive transition of the green-step stimulus increased linearly with stimulus intensity with slope of 0.017%/W/m² and linearly with slope of −0.09%/W/m² after the negative transition. The trans cillum has also increases and decreases in frequency to step-ups and step-downs, but is in detail different and does not maintain synchrony with the cis. RMS amplitude (overall velocity) decreased after both transitions of the step stimulus. Again, note the nonlinearity of this response. If the system were linear, the responses would have been equal and opposite. The percent change from the dark RMS baseline after both positive and negative transitions of the green-step stimulus was virtually independent of step stimulus amplitude. The RMS amplitude decreased by 10% for all positive transitions of the step stimulus. After all negative transitions of the step stimulus, RMS amplitude decreased by 30%. Similar responses were reported by Rüffer and Nultsch [1991].

Light Intensity Dependence of the Anterior/Posterior Relative Phase

The electro-optical detector monitored each cillum during the anterior portion of the beat cycle (Q1, Q4) and posterior portion of the beat cycle (Q2, Q3). It was configured such that if the stroke were planar and in a constant orientation, then there would be a constant phase difference between anterior and posterior portions of the stroke for each cillum. In fact, without green stimulation, each cillum maintains constant phase difference (Fig. 5E) over a long time. However, in Figure 8, the anterior-posterior trans-cillum relative phase (Q1 versus Q2) shifted from the constant dark baseline to new levels during the stimulus “on” intervals. This light-intensity dependent relative phase shift was maintained during the “on” interval, and returned to the dark baseline during stimulus “off” intervals (Fig. 8A). The anterior-posterior relative phase from the dark baseline increased linearly with stimulus intensity with slope of 0.31°/W/m² (Fig. 8B).

Momentary Beat Pattern Changes

Nearly all cells in the absence of light showed occasional random spontaneous changes such as stops or low amplitude strokes as seen in Figure 5. Such responses are enhanced by a green square-wave stimulus as shown in Figure 9A and B. Under the conditions of cells of Figure 9, about one quarter of the 81 cells examined exhibited frequent momentary (one to four beat cycles) low amplitude (short stroke) ciliary pattern changes in response to square-wave stimulation. Others have made similar observations [Rüffer and Nultsch, 1985, 1987]. The frequency of momentary pattern changes increased transiently following a positive transition of the green stimulus. After a negative transition of the stimulus, the cell stopped beating for a short period (shaded area in Fig. 9B) followed by a transient increase in the frequency of pattern changes, again for a number of seconds.

Spontaneous Trans-Cillum Beat Frequency Shift

Trans cillum of wild type (agg⁺), 1117, has been observed to spontaneously insert an additional beat without external stimulation (Fig. 10). Here, the trans cillum beats 1.43 times as fast as the cis cillum for 2.79 beat cycles of the cis cillum (between point A and point B in the figure) resulting in four trans beats to three cis beats before they become synchronized again. This was studied in detail by Rüffer and Nultsch [1998]. Each cillum would also appear to have its own natural oscillation frequency in the absence of entrainment by the other,
with the trans cilium always being faster than the cis. During normal synchronous beating, this entrainment mechanism forces the trans cilium to have the same beat frequency as the cis. This has previously been reported by Rüffer and Nultsch [1987] and by Takada and Kamiya [1997]. Note that synchronization is lost about midway during the recovery stroke (Fig. 10 at A) and regained about midway in the effective stroke (Fig. 10 at B). While
we believe there are strain-specific differences, it is premature to come to a generalization on this point.

DISCUSSION

The electro-optical method developed to monitor the cilia of a single *Chlamydomonas* cell has several advantages over previous approaches. A stationary cell body eliminates effects due to rotation of a free-swimming cell, and permits accurately quantified light stimuli to be delivered to the photoreceptor. The two cilia are monitored separately, providing a means of comparing cis-trans cillum beat frequency, stroke velocity, stroke duration, relative phase, and time spent in each detector quadrant (Fig. 5). The system is capable of recording in excess of 100,000 consecutive beat cycles of each cell with resolution to measure transient changes in beat pattern or amplitude occurring in a single cycle (Figs. 4 and 9). Recordings of single cells have spanned 8 h, providing a means of investigating long-term effects of the environment, stimulation, circadian rhythm, and metabolic changes on the cell.

In addition to square-wave stimuli, stimulus amplitude may be modulated as sine waves, ramps, pseudorandom noise, pulses, or any pattern that might resemble actual modulation that a free-swimming cell might encounter at any orientation with a light source. It is possible to simultaneously stimulate the photoreceptor with multiple stimuli of different wavelengths with independent amplitude modulation. The green stimulation intensity required to obtain measurable ciliary responses in this work (1–13 W/m²) was significantly lower than in earlier work (500–1,000 W/m²: the equivalent of full sunlight) [Rüffer and Nultsch, 1985].

Green stimulation
at 543 nm stimulated Rhodopsin photoreceptor in the normal operating range of the phototaxis system, although variations in magnitude of the responses suggest sensitivity to green stimulation had appreciable variability among the cells examined. There are many factors contributing to the ciliary responses to green stimulation including age of the cell culture, illumination quality, and exposure time of the cell culture, duration as gametes, and stimulation history of cell.

Previous work [Rüffer and Nultsch, 1985] using high-speed photography to observe the cilia required intense xenon lamp flashes filtered to pass through 560 to 750 nm light (orange-red, 100 W/m²). As a consequence, unavoidably stimulation of the chlorophyll and phytochrome receptors occurred that presumably affected the ciliary responses to the green stimulus. In this work, the problem was minimized by illumination of the cell in NIR (800–900 nm). We also tried to be more specific with our stimuli than prior work [Smyth and Berg, 1982; Rüffer and Nultsch, 1985,1987,1990,1991,1998]. The use of broadband stimuli may have complicated the analysis by stimulating the blue-absorbing rhodopsins, the cryptochrome blue receptors, and the green-absorbing rhodopsins. We used 543-nm light, which is quite selective for the green-absorbing rhodopsins.

The automated experimental system is capable of observing ciliary responses in almost the same detail as one could analyze from cinematographic [Rüffer and Nultsch, 1985, 1987, 1990, 1991, 1998] or other electronic [Smyth and Berg, 1982] image analysis. We could further take advantage of rapid statistical analysis of large quantities of data. Results obtained are consistent with these earlier works.

The nonlinearity of the responding system is quite apparent from the data of Figure 7. Responses to positive stimuli are not opposite those following negative stimuli. Different measures of response as comparing beat or stroke frequency with the velocity of the stroke show significant and consistent differences with increasing and decreasing light. Further, they show different dependencies on intensity and other parameters. Over the range of intensities used, the velocity of the power stroke did not vary much with intensity, while the beat frequency response changed significantly.

The anterior-posterior relative phase of the trans cilium, as shown in Figure 8, showed a linear positive dependence on the amplitude of the stimulus intensity. This response does not show adaptation; it lasts as long as the stimulus input of 5 sec, disappears immediately when the stimulus is removed, and does not carry over an adapted level into the future as shown by the strict linearity of response with stimulus size. Based on simulation of what the detector would show as a result of simple conceivable changes in the beat pattern, the most probable change is a repositioning of the orientation of the base of each cilium as a function of the green light intensity. This might also change the beat plane. Since far-red light does not elicit this response, we think it probably is due to a rhodopsin. Despite uncertainty, it is an intriguing behavioral adjustment without a proven function. One possible function is that it compensates for the decreased reaction time observed for responses with increasing average light intensity. This reaction time also does not adapt. A decreased reaction time alters the plane in which the cell turns relative to the eye. Perhaps the reorientation of the base of the cilium corrects for this in either the helical or superhelical phototaxis mode. The critical nature of this reaction time or delay is shown by the fact that superhelical tracks are
left-handed for positive phototaxis cells but right-handed for negative phototactic cells [Foster and Smyth, 1980]. This implies that the plane in which the cell turns is rotated clockwise as compared with the counter-clockwise cell rotation as viewed from the rear and too early for precise correction of the track that would lead to a helical path. Note that the modes of phototaxis have not yet been systematically studied with respect to the critical parameters of light intensity and angular distribution of light.

The momentary changes illustrated in Figure 9 show that other relatively long-term changes over seconds also result from changes in the light level. This means that although the memory time for phototaxis adjustments is a few hundreds of milliseconds there are underlying changes that must be modeled on a much longer time scale of seconds. The role, if any, of these momentary beat changes in phototaxis is unknown. What causes them is also unclear at present.

In Figure 10 with strain 1117, an extra beat is inserted on the trans side with the trans beat frequency more than 40% faster than that of the cis cilium. This was also observed in Figure 4 with strain 806 suggesting
that it is true for both negatively and positively phototactic cells. This behavior was previously noted by Rüffer and Nultsch [1987] and extensively studied by Takada and Kamiya [1997]. Interestingly, after the two cilia have been beating at constant different rates when the two cilia become in phase, they instantly become synchronized with the same rate and phase. In the future, it should be possible to determine if there is a preferred point in the beating cycle for the resynchronization to occur. The advantage for phototaxis of having the trans cilium oscillator consistently faster than the cis remains a mystery. In terms of phototaxis response, it means that the trans frequency response leads the cis by a 20 to 35° phase shift (data not shown).

A number of studies are currently in progress using this stationary single-cell monitoring system. Responses to green square-wave stimulation are being used to gain insight into the ciliary changes involved during phototactic turning. Green sine-wave and pseudorandom noise stimulation are used to characterize the ciliary response and provide information about the green-light temporal frequencies to which the phototactic system is sensitive. Analysis of ciliary motion in the absence of external stimulation, including spontaneous pattern and mode changes is underway. Responses to red light stimulation are being analyzed to investigate the red receptor influencing different parameters of ciliary movement. Differences in \( \text{agg}^- \) and \( \text{agg}^+ \) and other mutants’ responses and simultaneous green and red light stimulation will provide insight into the phototactic pathway.

CONCLUSIONS

An instrument was developed to monitor the ciliary beating of the trans and cis cilia of *Chlamydomonas reinhardtii*. Every beat of each cilium could be individually observed for hours on a single individual cell. Stimulation light may be conveniently programmed from the computer so that the behavior and stimulation may be easily correlated over a long time. Nine different measures of response can be quantified and evaluated. To test the value of the cell system for future extensive studies, the appropriate conditions for system stationarity and the effect of the absolute light intensity was determined. To indicate the power of the recording system, several brief studies confirming already observed phenomena as well as a several new discoveries have been presented.

ACKNOWLEDGMENTS

Professors Edward Lipson (Syracuse University) and David Mitchell (SUNY Upstate Medical University) provided discussions and comments on the manuscript. We acknowledge the work of machinists John Barden, Charles Brown, and Lester Schmutzler of the Physics Department Shop.

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