Photo-Induced Current Measurements in Chlamydomonas Cell Suspensions

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PART I: BACKGROUND

Introduction

All flagellated algae have the ability to track light direction and adjust their swimming accordingly. This phototactic response was first observed by Treviranus in 1817. It was later shown by Buder that such cells respond to light direction, not gradient; e.g. positive phototactic cells will swim toward light whether or not the external light intensity increases, decreases, or remains the same [1].

Such responses require the cells to have two important apparatus: one to detect the light, and another to respond to that detection and allow the cell to swim accordingly. In the case of flagellated algae, the swimming portion is accomplished by a “breast stroke” like beating of the two cilia [2]. Such ciliary action results in a swimming velocity of approximately 100µm/s for unicellular organisms, and up to 1000µm/s for large, colonial algae. Brownian motion – which randomizes the motion of a 10µm sphere (an approximate size of a unicellular algae) in roughly 6.5 minutes – is not a factor since the response time of the cells is on the order of milliseconds. Depending on what is detected, four responses are possible: positive phototaxis – the cell swims toward the light source; negative phototaxis – the cell swims away from the light source; diaphototaxis – the cell swims orthogonal to the light source; or a photophobic response, in which the cell stops and changes direction because of an abrupt change in light intensity [1,2].
The Principles of Phototaxis

In order to properly carry out phototaxis, the algae must overcome four fundamental problems:

1. They must be able to find the signal among background noise.
2. They must be able to operate over a range of light intensities present in their native environments.
3. They must be able to detect the right wavelengths.
4. They must be able to communicate accurately and quickly with the response apparatus [1].

What is the solution to these problems? A directional antenna. This antenna, which consists of a red eyespot and its associated structures, is independent of the photosynthetic apparatus and involves rhodopsin-type photoreceptors. We refer to the eyespot as a directional antenna because it is just that: due to the optical characteristics of the cell body and the photoreceptor pigment, maximum stimulation occurs when the incident light is 90° (normal) to the surface of the eyespot.

As such, it is analogous to a screen reflector antenna in which an absorbing screen is in front and a reflecting screen is in back. Furthermore, much like an antenna, the size of the eyespot is a multiple

Figure 1.1: Basic design of a Chlamydomonas cell. (a) Side view of cell. (b) End view. The incident light pattern is indicated by solid arrows. The eyespot, which lies on the plasma membrane adjacent to the chloroplast (dashed line), forms part of the antenna. Reprinted from Foster & Smyth, 1980.
of the wavelength of the light it receives. As such, the eyespot is a relatively small
patch that is located on the equator of the cell body, roughly 90° from the flagella
(see Figure 1.1) [1].

The location, size, and directivity of the eyespot allows the cell to “scan” its
environment as it swims. This scanning arises from the helical swimming path of
the cell: as the flagella beat, the cell body rotates at frequencies ranging from 1.5
to 4 Hz [5]. Therefore, as the cell swims, it receives an “error signal” that gives it
information about the direction of the light. To understand how, consider the
following two simple situations: (A) a Chlamydomonas cell that is oriented such
that the plane normal to the eyespot is perpendicular to a light source and (B)
another Chlamydomonas cell that is oriented such that this plane is parallel to a
light source. Further assume that these cells continue to swim in the same
direction regardless of the detected signal. The result, as we can see in Figure 1.2,
is that the cell’s antenna in situation (A) will receive an unchanging intensity as it
swims, while the cell in situation (B) will receive a periodic signal due to the
directivity of the antenna. This periodic signal is known as the “error signal;” its
phase gives the direction of the error and its amplitude gives the error magnitude.
The cell responds to this signal and then uses its flagella to change its orientation.
The error signal therefore changes on the next scan and the cell again makes
adjustments. The great advantage of this system is that no matter the cell’s
orientation, a correct path may be chosen [1].

Another potential advantage of this detection method is that it can be
exploited for noise suppression. As a result of the rotation, a known pattern is
Figure 1.2: A.) A Chlamydomonas cell oriented such that the direction of motion (the tracking direction) is toward a uniform light source. B.) A Chlamydomonas cell such that the tracking direction is orthogonal to a uniform light source. The photoreceptive area (the directional antenna) is indicated by the inner circle. Times (1), (2), and (3) are shown for reference. The shapes of the flagella are not meant to represent actual motion, but are only included for referencing the orientation of the cells. The graph shows the intensity of the light incident on the antenna throughout the swim cycle. Assume the cells do not change orientation throughout. As such, this is a highly simplified and idealized situation.
given to the light such that the intensity is enriched for certain frequencies. A signal processor can then select these frequencies while suppressing others, much like a band-pass filter. This is important because low frequency noise causes a shifting base that makes absolute measurements of the signal amplitude meaningless. The rate of change of the amplitude — *the derivative* — is insensitive to this, however. Furthermore, differentiation has the double advantage of making the signal independent of the average intensity of the incident light; i.e. it allows for detection over a large range of light intensities. Thus it is expected that the cell processes lower frequency noise by differentiating. High frequency noise, on the other hand, can be suppressed by averaging the input over a time period longer than the noise. In other words, it is expected that the cell would *integrate* the signal above certain frequencies. This means that the memory time of the cell must be on the same order as the rotational period. It also means that the processor as a whole must have peak response to a frequency greater than the rotational frequency [1]. If it did not, and it peaked at a frequency less than the rotational frequency, the cell would attenuate signals faster than the scan rate. This would make proper detection of the phase and error magnitude impossible.

Finally, in order for the above mechanism to work, the cell must have fast communication between the antenna and the flagella. At the very least, the communication must be faster than two seconds, which is the approximate relaxation time of many algae. (Relaxation time is the amount of time in which the swimming motion of the cell, if left uncorrected, becomes randomized. This is due simply to the mechanics of the ciliary action.) Two seconds is an extreme
upper limit; if the cell cannot react on a time scale faster than the period of the rotation (~500ms), controlling the phase error would be difficult. As a result, algae do react very quickly; the response time of *Chlamydomonas*, for instance, is less than 20ms [1].

In order for such a fast response to occur, the communication between the antenna and the flagella must be electrical. The distance between these two components is too large for a diffusion signal to be effective on that time scale. Thus, photoexcitation must trigger a cascade of rapid electrical events that begin in the plasma membrane. Since the plasma membrane is continuous with the flagellar membrane, an electrical signal can quickly pass from the antenna to the flagella in this manner [1,2]. This signal would be reflective of the detected intensity of light at the antenna. However, at some point in the cascade the signal must be processed such that the final electrical output is that which represents the raw signal plus any necessary band-pass filtering done by the cell.

Given the above principles, we can make assumptions about the nature of the processed signal. Initially, we will assume that the system is roughly linear. Although in detail the system is probably nonlinear, this non-linearity can be considered to be static and mostly related to the conversion of light into an electrical current. In other words, any non-linearity involved probably has little to do with the processing mechanism of the cell. Therefore, all the properties of linear systems can be applied to the processed signal. These include the principles of superposition (e.g. a processed 5 Hz wave plus a processed 10 Hz wave is roughly equal to a processed (5 plus 10 Hz) wave) and scalar multiplication (i.e.
the magnitude of the impulse response is directly proportional to the intensity of
the input) [1]. Furthermore, making a linear approximation allows us to use all the
powerful tools of linear analysis – most notably Fourier analysis – when
analyzing the output signal.

Summing up the above principles of phototaxis, we can make a number of
reasonable predictions about the nature of the processed signal. These include:

1. The cell has finite memory time. Therefore, stimulation only lasts for
the duration of the impulse response. (By impulse response I mean the
response of the cell to a delta function-like pulse of light.)

2. For low frequency noise the cell acts as a differentiator.

3. For high frequency noise the cell acts as an integrator.

4. Combining (2) and (3), the cell as a whole acts as a band-pass filter.
   This filtering must result in the cell to be most sensitive to frequencies
   whose period is faster than the scan rate.

5. The more the pattern of stimulation matches the impulse response, the
greater the response.

6. The magnitude of the processed signal is proportional to the amplitude
   of the modulated input light intensity.

7. Positive phototactic cells have a positive impulse response; negative
   phototactic cells have a negative impulse response.

8. The cell can perform phase detection and phase advance. Although the
cell senses rate of change, it expected to respond approximately in
   phase with the input [1].
As we can see, in order to prove or disprove the assumptions made about how phototaxis works, we must be able to measure the output signal of the cell. In order to do this, a number of techniques have been developed.

**Measuring the Signal**

Measuring techniques can be broken up into two main categories: *intracellular* recording and *extracellular* recording. Intracellular recording involves penetrating the cell membrane with an electrode in order to measure the potential difference between the inside and outside of the cell. This method is problematic for unicellular algae, however, since such organisms do not normally get any larger than 20µm. Of those cells that were big enough, it was found that recordings could not be made when the cells were impaled, but could be made when the electrode was pressed against the cell surface. This suggested that phototaxis involves currents that are induced across the cell membrane and are highly sensitive to cell damage [2]. It also meant that extracellular methods would have to be developed in order to properly measure the processed signal.

**Extracellular recording**

To understand how extracellular recording works, assume that photoexcitation leads to the onset of local currents across a small patch of the cell membrane. The resultant circuit is closed via the rest of the cell membrane and the external solution. The current that flows through the external resistance ($R_{ext}$)
causes a potential difference (ΔV) between the interior of the membrane and the external solution which can then be picked up by a voltage amplifier [2].

Alternatively, the signal can be picked up by a current-to-voltage converter. Although the two methods are equivalent, using a current-to-voltage converter has two advantages:

1. $R_{\text{ext}}$ can be increased. This does not change the signal amplitude but does decrease Johnson noise.
2. Since the input voltage is kept at a constant level, any influences on the signal kinetics due to external capacitance is minimized [2].

Two techniques of extracellular recording are possible: recording via a suction-pipette, and recording from a suspension of cells.

*Suction-pipette*

Basically, this technique involves sucking a single cell at the end of a pipette and recording the current passing into the membrane. By stimulating the cell with pulses of light (step functions), two main components of the response have been recorded: a *primary potential difference* (PPD) and a *regenerative response* (RR). Due to localization of the current sources, the “PPD” later came to be called the *photoreceptor current* (PC) and the “RR” was renamed the *flagellar current* (FC) (See Figure 1.3) [2]. These naming conventions will be used for the rest of this paper.
The PC and FC were found to have the following characteristics:

- They are both dependent on $\text{Ca}^{2+}$ ions and as such are susceptible to calcium channel blockers.

- However, the PC is much less dependent on the calcium blockers than the FC. What this means is that the PC has a component independent of $\text{Ca}^{2+}$.

- By simultaneously observing the cell with a microscope, it was found that there is a strong correlation between appearance of the FC and undulation of the flagella. This undulation is typical of a photophobic response [3].

- The FC is all-or-nothing; i.e. it only appears if the membrane depolarization exceeds a critical level [1].

Despite these successes in using the suction-pipette, it does have problems. First, it is limited to large cells with elastic cell walls. As such, it cannot be applied to most phototactic algae. Secondly, the protoplast is deformed by the sucking action. This most likely affects the results, since the cells are
equipped with mechanoreceptors which are sensitive to such jostling[2,3]. Therefore, another technique was desired which could keep experimental conditions closer to that of the cells’ natural environment while still allowing for accurate measurements. This is what recording from cell suspensions was developed to do.

**Cell Suspension**

First reported in 1992 by Sineshchekov et al., recording via this method involves stimulating a highly concentrated suspension of algae with uniform light and recording the output of that suspension with macro-electrodes. Although the cells can be in any orientation in suspension, this method should work because single cell experiments showed that the photoelectric response is asymmetric; it leads to polarization of the entire cell. Furthermore, due to the directionality of the antennas, cells with their eyespots facing the light source will generate a maximum PC while those facing away will generate a minimum. The net result is that oppositely directed PCs are more than compensated for and so the current can be picked up by the electrodes [3]. In keeping with the suction-pipette convention, the sign of the signal of cells pointed toward the light is considered positive [2].

Two methods are possible for suspension recording: un-oriented, or *unilateral* (UL) mode, and *pre-oriented* (PO) mode. In UL mode, the setup is arranged such that the line drawn between the two electrodes is parallel to the light stimulus; the electrode furthest from the light source is considered to be the measuring electrode. In PO mode, the electrodes are rotated 90° such that the line
drawn between the two electrodes is perpendicular to the excitation source. A weaker orienting light, parallel to the electrodes’ line (i.e. in the same direction as the light stimulus in UL mode), is then applied for several seconds before the excitation flash. Once again, the measuring electrode is considered to be the one furthest from the stimulus source (See Fig. 1.4) [2, 3, 4]

If a quick excitation flash (in some of Sineshchekov’s experiments the flash could be as short as 10ns) is applied in UL mode, a strong positive spike can be recorded. This is immediately followed by a negative pulse that is much weaker as compared to the initial spike. If the same flash is applied in PO mode, the result is that the second, negative response is much larger and longer as compared to the initial positive spike. Some other features of these flash-induced responses are as follows:

- They can only be induced by blue-green light.
- There is no time delay between the onset of the flash and the appearance of the initial positive spike.
- If the intensity of the flash is decreased, the rise time of the positive spike becomes slower.
- The spikes are found to be Ca\(^{2+}\) dependent; the negative spike disappears completely if no calcium ions are present.
- If no orienting light is used in PO mode, no signal can be recorded [2, 3, 4].

Based on these and other observations of the kinetics of the responses, Sineshchekov concluded that the initial positive spike is the same as the PC that is
recorded in suction-pipette experiments, whereas the negative spike is the FC. The FC is so much weaker in UL mode because the sign and amplitude of the measured FC depends on the angular distance between the eyespot and the flagella. For most *Chlamydomonas*, the angular distance is slightly >90°. Therefore, only a small component of the FC is in the same plane as the electrodes. In PO mode, Sineshchekov reasoned that the weak light caused all the cells to orient such that their flagella (i.e. their tracking directions) were now in line with the electrodes while their eyespots were perpendicular to them. Thus a large component of the FC was in line with the electrodes and so a larger signal was recorded. This interpretation is backed by the fact that if the orienting light is not applied in PO mode, no signal is recordable (See Figure 1.4) [2, 4].

**Figure 1.4:** Left: Basic design and interpretation of: (A) UL mode, and (B) PO mode. (Reprinted from Sineshchekov & Govorunova, 2001) Top: Common measured responses. (1) UL mode, (2) PO mode, no orienting stimulus, (3) PO mode, orienting stimulus. (Reprinted from Sineshchekov et al., 1994)
Other components were also measured via this method. This included a much faster response which also had an initial positive peak followed by a negative peak. The total duration of this entire response was only about 11µs, much faster than the roughly 20ms duration of the PC/FC response. Due to the short duration and other characteristics of this fast response, it is most likely involved with photosynthesis – i.e. independent of phototaxis – and therefore can be ignored [3].

Advantages and Problems with the Cell Suspension Techniques

As stated above, using the cell suspension technique has a number of advantages. These include:

- There is no limit to the size or structure of cells that can be used; i.e. the photoelectric responses of small cells with non-elastic cell walls can be used, such as wild-type *Chlamydomonas*.

- Recording is done under more “normal” conditions.

- Any mechanical influences present in the suction-pipette technique are eliminated.

- By rotating the electrodes, electrical polarization can be measured along different axes under the same conditions [2, 3].

- A number of different stimuli, other than flash stimuli, can be applied.

- If it works, this method is much cheaper to setup and much easier to carry out than the suction-pipette technique. In other words, results can
be achieved relatively quickly and easily, opening up a plethora of different questions that can be tested and answered.

However, there are some problems with this setup. First, it is hard to know the origin of different aspects of the signal. Second, any slow components in the signal are disturbed by the motion of the cells [2]. Third, gravity can have an effect on the recordings. The cells have the capability of detecting gravitational effects, and they respond to this. Known as *gravitaxis*, this response is separate from phototaxis [1]. In suspension, the cells are susceptible to this, and so it adds another variable to be sifted out when analyzing the phototactic response. However, all of these problems can be resolved through careful analysis and by cross-correlation with other methods.

The greatest problem with this method is being able to set it up. As can be seen in Figure 1.4, the maximum signal amplitudes are only between 100-200 pA. This makes the experiment very susceptible to Johnson noise, especially since recording is done from a liquid suspension. This means that a very detailed description of how to set it up is necessary in order to properly reproduce the results. Unfortunately, Sineshchekov did not do that. As a result, in the fourteen years since it was first reported, only one other lab independent of Sineshchekov’s – that of Kenjiro Yoshimura’s – has been able to reproduce this technique. Furthermore, all of the experiments carried out by Sineshchekov only involved flash stimuli; yet many other inputs – including stimulating with sine waves – are possible. Clearly this method has been underutilized. Therefore, being able to reproduce the cell suspension method is highly desirable. This I was able to
accomplish. Having done so, it is necessary to describe the setup in detail so that others may be able to utilize this technique. That description follows in Part II.
PART II: EXPERIMENTAL SETUP

My setup was based on Sineshchekov’s UL setup. UL mode, as opposed to PO mode, was chosen because this mode is more basic; once it is accomplished, PO mode can be developed from it if so desired. Furthermore, our long term goal was to stimulate the cells using sine-wave stimuli. This would not be possible to do in PO mode since an orienting light could not be left on during such a stimulus.

Basis for the Setup

In Sineshchekov’s initial setup, the cells were placed in a cylindrical cuvette with a diameter of 1cm and a depth of 2.5cm. Two platinum wires were used as electrodes, one placed in the top part of the cuvette and the other in the bottom (i.e. the electrodes were horizontally oriented). Both electrodes were shielded from the excitation light, presumably out of worry of light-induced currents in the platinum. The signal was amplified by a voltage-amplifier and then a 10kHz or 1kHz low-pass filter was used in order to attenuate high frequency noise [3].

With this setup, it was found that the signal was inversely proportional to the conductivity of the measuring medium; if the resistance of the solution was increased, the signal amplitude increased (to a limit). Therefore, the optimum measuring medium was found to be a low-electrolyte, NMM (nitrogen minimum media) solution containing of 0.1mM K\(^+\) and 0.05mM CaCl\(_2\) and having a pH of 7. In this medium, \(\sim 5 \times 10^6\) cells/ml were suspended. This concentration was used
because it corresponded to an optical density (OD) of ~0.4 at 800nm; such an optical density was found to give maximum signal amplitudes. All experiments were carried out at room temperature (22-24°C) [3].

In later experiments, some adjustments to this setup were made. The orientation of the electrodes was changed so that they were vertical. This was because gravity had strong effects in the horizontal setup; by making the electrodes vertical, these effects were minimized. Due to this re-orientation, the cylindrical cuvette was changed to a rectangular one so that the excitation light would remain parallel when it passed through the side window. A current-to-voltage converter was also used interchangeably with the voltage amplifier, for reasons listed in Part I of this paper [4]. Most of the other parameters – such as the measuring medium and cell concentration – were kept the same. What isn’t clear is if in later setups the electrodes were still shielded from the light source and if the distance between the electrodes was still 1 cm.

Based on these parameters, I came up with the following basic setup:

**Stimulus**
- Laser
- Shutter
- Beam Expander

**Cell Box**
- Cuvette
- Cell Suspension
- Electrodes

**Current Amplifier**

**Measurement and Analysis**

**Band-pass Filter**
- High-Pass
- Non-inverting Amplifier
- Low-Pass
To prevent interference by outside electromagnetic noise, this entire setup was placed in a shielded room.

**The Stimulus**

*Laser and Beam Expander*

A laser with a beam expander was used because the stimulus had to consist of parallel light intense enough to stimulate a large population of cells. Using parallel light is particularly important for UL mode due to the directivity of the antenna; if the light is not parallel, the cells would have trouble detecting the direction of the light source.

The laser used was an air-cooled, class 3B, National Laser Company Model 210 Argon Ion Laser with an output wavelength of 450-515 nm and a max intensity of 500mW. The wavelengths of the output were ideal since this is the range in which phototaxis is found to have peak response [1]. The laser was powered by a Laser Drive Inc. Model 9470 Argon Ion Laser Power Supply. The power supply and fan used for cooling was clamped to a separate table, away from the experimental setup, to minimize noise from vibrations.

Initially, a laboratory made beam expander, consisting of a 10x objective lens, a spatial filter, and 20cm focal length lens, was used. The resultant beam was approximately 1.8 cm in diameter. This was later replaced with an Edmund Industrial Optics 20x beam expander, which resulted in a beam that was approximately 2.0 cm in diameter.
The average maximum intensity of the beam created by the original beam expander was 4.64 mW, which corresponds to \( \sim 1.94 \times 10^{20} \) photons \( \text{m}^{-2} \text{s}^{-1} \). This was measured in clear NMM solution using a Photodyne Model 88 XLA photometer. However, since the cell suspension is optically dense, this value should only be considered an upper limit. Actual intensities as seen by the cells is dependent on their placement within the suspension: cells further away from the light source will see dimmer and more diffuse light than those closer to the source. If the concentration of the suspension is increased, this “non-homogenous” illumination of the cells becomes greater. In the 1992 paper, Sineshchekov et al. found that this results in some smoothing of the signal as compared to more diluted suspensions. However, main features of the signal were unaltered, and so the phenomena can be ignored. Unfortunately, this still means that an absolute value of the incident intensity cannot be obtained. Since these are only maximum values, the resultant intensity as put out by the newer beam expander can be assumed to be roughly the same as the original.

**Shutter**

Initially, a Stanford Research Systems Model SR540 beam chopper, which uses a spinning wheel with holes punched in it, was used to create the stimulus. The chopper was set so that pulses were \( \sim 20 \text{ms} \ (\pm 1 \text{ms}) \) in duration, with \( \sim 180 \text{ms} \ (\pm 5 \text{ms}) \) between flashes. This corresponded to a 5 Hz asymmetric square wave. Such a stimulus was chosen because it was hoped that this would result in a maximum pulse response. (This conclusion was drawn from data
reported by Yoshimura and Kamiya, 2001.) By placing tape over some of the holes on the chopper wheel, it was possible to spin the wheel fast enough so that the rise time of the pulses was ~300µs at 5 Hz. (Note that this was accomplished by placing the beam chopper between the laser and the beam expander; otherwise, illumination of the suspension would not be uniform.) To detect the signals, the beam chopper was used in conjunction with a photo-diode to trigger an oscilloscope (see Measurement and Analysis for more information).

The advantage of using the beam chopper was that the difference between flash on and flash off was a full 100%. Furthermore, the signal could be observed in real time with an oscilloscope. This was ideal for troubleshooting and optimizing the setup. However, once this was accomplished, the beam chopper became problematic since it was limited to creating asymmetric square waves. It also could not be used with a computer, which caused analysis to be dependent on the rather limited capabilities of the oscilloscope.

To expand our experimenting abilities, the beam chopper was replaced with a Displaytech Ferro-electric Liquid Crystal (FLC) shutter, Model LV050 AC, which was driven by a Displaytech DR50 Driver. (Once again, this was placed between the laser and the beam expander.) This shutter operates by using two polarizing filters (See Figure 2.1). The first filter polarizes the

![Figure 2.1: The FLC shutter.](image)
incoming laser beam. When a voltage is applied to the second filter, which is a liquid crystal, it can either become polarized in the same or opposite direction as the first filter, depending on the orientation. The great advantage of this is the shuttering can happen very quickly: < 50µs. Furthermore, it can be controlled by a computer; this allows for a wide variety of different stimuli to be tested, including sine waves of varying amplitudes and frequencies. Although the FLC can only be “on” or “off,” by switching it on and off at microsecond rates, the intensity of the light appears (to both the cells and the human eye) to modulate continuously.

The one major problem with the FLC is that the difference between off and on is not 100%. The maximum on amplitude is dimmed a bit, and at best there is a 1.4% leakage that occurs when the filters are oppositely polarized. At first I was worried that this leakage might cause the cells to pre-orient, rendering my UL setup useless. However, this was not the case, as pulse signals just as strong (and stronger) as those measured with the beam chopper were recorded. It is not clear why, but it may be the case that the leakage intensity is too low to have an effect on the cells.

**The Cell Box**

The cell suspension and electrodes were contained in an aluminum box 10.2cm wide, 7.7cm tall, and 12.7cm deep. The box consisted of two interlocking pieces – top and bottom (See Figure 2.2) – that were held at Earth ground via wires connected to the current amplifier chassis (which in turn was grounded). A hole 1.3 cm in diameter was drilled into the front of the box in order to allow light
from the stimulus to enter. If lower maximum intensities were desired, OD filters were taped over the hole (See Figure 2.3). The interior of the box was covered with masking tape and painted with flat black paint to reduce reflection of light inside. A foam pad was placed between the box mount and the optical bench to reduce any mechanical vibrations picked up through the table. This was found to have little effect, however.

The purpose of the box was to help shield the electrodes from electromagnetic radiation given off by other equipment in the room. Of course this shielding was not perfect because of the stimulus hole, but it did at least block out some noise. The box also served to shield the cells from any other light in the room. Although all the lights were turned off and the door was closed during experiments, there was still residue light from the computer monitor and reflections from the laser that could potentially throw off the cells.

**Figure 2.3:** Partially open cell box, side view.  
**Figure 2.2:** Front view of cell box. A 0.5 OD filter is shown in place.

**Cuvette**

Initially, a cuvette 5.1cm tall, 5.5cm long, 1cm wide and made of optical glass was used. This was later changed to a plastic cuvette that had two
transparent sides and two ridged sides and was 1cm long, 1cm wide, and 3 cm tall. This was held in place by a foam structure that sat on a Styrofoam base which was glued to the cell box. Exactly 1.1 ml of cell suspension – corresponding to a depth of 1.1 cm – was placed in the cuvette. All experiments were carried out with this setup.

The reason the larger cuvette was replaced with the 1cm x 1cm one is that in this cuvette almost the entire suspension was illuminated by the stimulus beam. This is especially important when using strong negative phototaxis algae – such as 806 strain *Chlamydomonas* – because such strains will swim out of the way of the light source. Even with *ptx* – a strain of *Chlamydomonas* that cannot phototact at all – the recorded response amplitude from a 20ms pulse was nearly twice as large and less noisy in the smaller cuvette than in the large. In short, for best results, it is essential that the entire suspension be illuminated.

This smaller cuvette did have some drawbacks, however. The plastic carried surface currents which added to the noise. In fact, no signal was recordable if the measuring electrode touched the cuvette wall (it did not matter if the ground electrode did). Since there was only 1cm from the front to the back of the cuvette, this limited the distance between the electrodes to 0.6 – 0.8 cm. The
shorter the distance, the less \( R_{\text{ext}} \) is and thus more noise is present. As such, an improvement on this setup might be to have a cuvette made of optical glass that is wider. Glass is a better insulator than plastic and being wider, the distance between the electrodes could be increased. However, increasing the distance still might not be optimal, since as the suspension gets larger, the stimulus light is attenuated more. Either way, it would be advantageous to at least test the results of increasing this distance.

**Cell Suspension**

In keeping with Sineshchekov’s reported optimum conditions, cells were suspended in low electrolyte NMM solution consisting of 0.05mM CaCl\(_2\) and 0.1mM potassium phosphate buffer. We did try other solutions in which we attempted to increase \( R_{\text{ext}} \) by decreasing Ca\(^{2+}\) concentration; we even tried using distilled water. None of these solutions worked as well as the NMM solution, although the reduced Ca\(^{2+}\) solution did have some interesting results (see Part III for more information). The resistance of the NMM solution was measured to be 4.5-5 M\(\Omega\).

The cells were prepared by growing them in a liquid high salt medium (HSM) in continuous fluorescent light at a constant 18 °C for three to five days prior to being transferred to the NMM solution. It was found that optimal signals were produced when the cells were shaken in the NMM medium for one day (~24 hours) before being used for experiments. Although signals could still be recorded two and even three days after being transferred, the signal amplitude would
decrease and become more unstable with time. Most importantly, the cells should not be tested on the same day that they are transferred to NMM. This can result in the signal amplitude to be very weak or non-existent, and the kinetics to be altered.

Differing methods of preparation were also tested. This included taking cells directly from HSM agar plates and growing them in HSM liquid for three to four days before transferring them to NMM. Alternatively, cells were transferred from HSM to HSM solutions every three days for up to three generations before being put in the NMM medium. These two methods were carried out on 806 and 1117 (a positive phototaxis strain) *Chlamydomonas* and tested using 20ms pulses. The results were somewhat inconclusive, but it did appear that cells transferred from agar plates were more sensitive to the same light intensities than cells transferred from liquid solution (data not shown). This is another variable that should be tested more to further optimize the technique.

For most of the experiments, the density of the suspension was kept at ~1.5 x10^7 cells/ml, which was found to be approximately optimum. This is about 3 times the concentration that Sineshchekov used. However, since the signal is also dependent on the distance between the electrodes and intensity of the stimulus, raw density numbers are not as important, especially since the distance between my electrodes and the max intensity of my stimuli may have been different from that of Sineshchekov’s experiments (it is not entirely clear what the specs of these last two parameters were in his setup). In fact, we found that for densities between 0.5 and 2.0 x10^7 cells/ml the signal amplitude could vary more
for the same densities than between densities. However, above $2.0 \times 10^7$ cells/ml, noise would become a problem and the overall signal quality would degrade (data not shown).

**Electrodes**

In my initial setup, 2 platinum wires, 0.2 mm in diameter and roughly 4.7 cm long were used as electrodes. The electrode farthest from the light source was used to measure currents while the near one was set as the reference; as such, the reference electrode was held at Earth ground via a wire attached to the cell box. The wire used for measuring was later replaced by a platinum strip 1.2 mm wide, < 1 mm thick and 3.8 cm long (see Figure 2.4). The result was that the signal was much stronger as compared to the two wires: the amplitude of a response to a 20 ms pulse was as much as 2.5 times as great. This, despite the fact that the strip, being nearly a centimeter shorter than the wire, was only submerged 4 mm into the suspension. The noise was also greater with the strip than with the wire. However, after performing signal averaging (a process necessary for all experiments – see

**Figure 2.5:** Electrodes. Wire (ground) / Strip (measuring) configuration shown.
Measurement and Analysis), the noise was the same and so this was not a problem.

Given the small amount of the strip submerged in the suspension, I thought it might be better to try soldering a strip onto a wire, thereby increasing the amount of strip that could be submerged. (I did not have longer strips at my disposal.) Since this would increase the surface area of the measuring portion of the strip, I thought that the recorded signal amplitude and resolution would be better. However, I found that the differences between the two were negligible. This means that the observed improvement of the strip over the wire is probably more due to the inherent resistances of each than to the amount of the electrode submerged. The wire, having a smaller surface area than the strip, has a higher resistance than the strip. Therefore, while noise is suppressed, the signal is also suppressed; this explains the observed increase in signal and noise for the strip. Since there was little difference between the wire/strip configuration and the wire/strip-soldered-to-wire configuration, the two were used interchangeably. The wire/strip configuration did have the advantage of being less susceptible to be bent out of place. As a result, the wire/strip configuration eventually came to be used exclusively.

Regardless of the configuration used, the electrodes were always soldered to a female BNC connector which was screwed into the top of the cell box. Also, the measuring electrode was always kept farthest from the light source. This was particularly important for the strip configurations because if it was flipped the other way around, the strip would block light to the cells.
Initially, I was worried about the effects the stimulus light might have on the electrodes: it could induce a current in them. As stated above, Sineshchekov mentioned this in his first paper, but did not say anything about it in subsequent papers, despite changing the setup. Thus it is unclear whether or not he continued to shade the electrodes. Either way, I found that this effect did not matter. There were no measurable signals when the electrodes were submerged in cell-free NMM solutions and exposed to the stimulus light.

On the point of electrodes, my setup differs a bit from Sineshchekov’s. In his experiments, two platinum wires 1mm in diameter were used. It may be the case that using a strip is an improvement on the setup. Clearly the strip was an advantage over the 0.2mm wire, but since I did not have wires 1mm thick, it is hard to know how a strip compares to such a wire. Also, it isn’t entirely clear whether or not Sineshchekov kept the reference electrode at Earth ground, or if he measured the difference between the electrodes using a difference amplifier.

**The Current Amplifier**

A Keithley Model 428-PROG current amplifier was used to pick up the signal from the electrodes. A current-to-voltage converter was used for the reasons stated in the *Extracellular Recording* section of this paper. The current amplifier was attached directly to the cell box via a female/male BNC converter; i.e. there was no cable in between. This eliminated the possibility of triboelectric effects altering the signal, as well as reduce the chances of picking up electromagnetic waves in the cable, which can act like an antenna.
The maximum possible gain of the 428 was found to be $10^9$. Any higher would result in an “overload,” meaning the 428 was trying to put out a voltage $>10V$. This means that the unfiltered input current was between 1 and 10 nA. Yet this is 10-100 times the magnitude of what the peak amplitude of the signal is expected to be. It was quickly realized that this current was due to noise and not the signal; in order for the signal to be seen this noise would have to filtered out and the output would have to be amplified even more.

**The Band-Pass Filter**

The signal, now converted to a voltage and amplified by $10^9$ by the 428, was next passed through a laboratory made band-pass filter with a non-inverting amplifier. The amplifier and the low-pass filter portions were powered by a GW instek GPS-4251 4 Channel Laboratory DC power supply. All circuitry was placed on a breadboard.

**High-Pass Filter**

The passive high-pass filter (HPF) was built using a 5µF ceramic capacitor and a 32kΩ resistor, giving a cutoff frequency of 1 Hz. The HPF was included because a relatively strong, “slow” response was present in experiments. Measured with the oscilloscope, the voltage (i.e. current) of this response was found to drop and then rise steadily when exposed to the light stimulus (See Chart 2.1 in the Appendix). Although this response was “slow” as compared to the pulse response, it could change by as much as 0.55 pA/ms when first exposed to the
light stimuli. The effect of this was that the signal was flattened out when the data was averaged. Since averaging is a key factor in detection (see Measurement and Analysis), this slow response made recording a signal impossible.

Another problem with the slow response is that it could create an offset voltage of 1V or more at a gain of $10^8$. Ideally, the total gain should be $10^{10}$ to put the ~100 pA signals on the order of a volt. Yet with such an offset, this is implausible, since it would result in a dc offset output of 100V. In other words, we are presented with a shifting electrical base, much like the algae are presented with a shifting light-intensity base when trying to detect the light source. Ironically, in order to overcome this, we must employ the same technique that the cells use: we must differentiate at lower frequencies. Hence, we use a high pass filter.

The cause of this slow response is unclear. It doesn’t appear to be related to phototaxis, nor does it appear to be caused from the electrodes reacting with the light. It could be the result of the photosynthetic apparatus in the cells, but this has to be investigated more. Oddly, Sineshchekov never mentioned it, or how he dealt with it, despite the fact that it appears to be the single biggest barrier to properly recording signals. Whatever the cause may be, because of the slow response, the HPF is probably the single most important element in this setup; nothing was recordable before it was inserted.

However, the HPF does have some draw backs. It is also not possible to measure any long-lasting, slow changing currents that might be present during longer square wave pulses. Furthermore, when using sine wave stimuli, the HPF
makes it difficult to measure the response for frequencies under 1Hz. For these reasons, the cause of the slow response should be investigated more in order to figure out alternative ways to knock it out.

**Non-inverting Amplifier**

Since the shifting base caused by the slow response is now set to ground, it is possible to further amplify the signal. This is accomplished by the non-inverting amplifier. Originally, a switch was included so that the gain could be set to 11 or 101, depending on whether the 428 was set to $10^9$ or $10^8$. However, it was found that much better signals are recorded when the 428 is set to $10^9$ and the amplifier is set to 11. Therefore, the switch was taken out and all subsequent experiments have been carried out with this setup.

**Low-Pass Filter**

A Texas Instruments TLCO4 Butterworth Fourth-Order Switched Capacitor integrated circuit acted as the low-pass filter. The switched capacitor was operated by a Hewlett Packard 33120A 15 MHz Function/Arbitrary Waveform Generator. The cutoff frequency was set to 5 kHz. As such, it did not have much effect on the observed amplitudes of the signals I recorded, since there were no components that changed this quickly. It did have a strong effect on phase shifting though, and this has to be considered whenever the phase of the response is analyzed.
Measurement and Analysis

As stated above, an oscilloscope was initially used to measure and analyze the signals. This was done by a Hewlett-Packard 54600B Oscilloscope. The photo-diode that was used to trigger the scope was activated by reflecting a small amount of light from the bottom of the stimulus beam and into the photo-diode via a mirror positioned in front of the cell box. The first detectable signals were measured in this manner.

Using the Oscilloscope, it was found that the signal could only be seen if the recordings were averaged together, despite all the filtering. At least 32 records had to be averaged together in order to make out the response to a 20ms pulse. Averaging 64 records was found to be optimal, however, since averaging less resulted in much more noise while averaging more did not decrease the noise much, but did decrease the signal amplitude.

Despite its usefulness, the oscilloscope was eventually replaced by a computer for reasons stated in the Shutter section of this paper. That computer was a Dell Dimension XPS D300 with a Pentium II running at 300 MHz and 128MB of RAM. The computer was fitted with a Computer Boards, Inc. CIO-DAS 1600 A/D board. With this board and computer, the maximum sample rate of a pulse was found to be 50μs. For sine waves, the sample rate was decreased to 200μs due to limitations on the shutter. If the sample rate was set too high on a sine wave stimulus, the computer would try to open and close the shutter faster than it could move, resulting in a random signal.
All software used to run the stimulus and record signals were written by Keith Josef, a graduate student in Foster and Saranak’s lab. Since individual signals had to be averaged together, the software would run a stimulus program – four sine waves, for example – record to the RAM, then send that data to the hard drive when the stimulus was over. It would immediately repeat this, and would continue to do so up to specified limit, usually 64 times. A short delay was introduced in between each of these 64 records due to the time it took to send the data to the hard drive. Once the stimulus file was over, the 64 records were then averaged together by another program and so this would result in one file. Files were then plotted and evaluated using Poly Software International, Inc. Psi-Plot for Windows. For many of the plots, additional smoothing was required to filter out further noise (usually in the form of 60 or 120 Hz sine waves coming from the power supply). Some results of this setup are shown and discussed in Part III.
PART III: RESULTS

Once the cell suspension method was established and perfected, many different kinds of experiments could be and were performed. In fact the possibilities were so numerous that at times I found myself collecting new data before former data were fully analyzed. However, in the interest of time and consistency, only three experiments are presented here. Those three are as follows:

1. 806 and 1117 Chlamydomonas cells were flashed with 20 ms pulses at different intensities. These different intensities were accomplished by either placing a 2.0, 1.5, 1.0, or 0.5 OD filter in front of the cell box hole. Maximum intensity (no OD filter in place) was also tested. Starting at 2.0 OD, the intensity was increased to no OD, and then decreased again to 1.0 OD and 2.0 OD, for comparison. Results are shown in Charts 3.1 and 3.2 in the Appendix.

2. 806 and 1117 Chlamydomonas cells were placed in a medium with a Ca\(^{2+}\) concentration reduced to 10\(^{-9}\) M (5x10\(^{-5}\) M in the low electrolyte NMM solution). These were compared to cells placed in a normal NMM solution. The cells were flashed with a 2s pulse, again at light intensities of 2.0, 1.5, 1.0, 0.5 or no ODs. Results for no OD for 806 and 0.5 OD for 1117 are shown in Charts 3.3 and 3.4 in the Appendix.
3. 806 and 1117 *Chlamydomonas* cells in NMM were exposed to a sine wave stimulus at different frequencies. This experiment is described in detail below.

For experiments (1) and (2), the strip soldered to the wire electrode configuration was used. In (3), the strip/wire (as shown in Figure 2.2) configuration was used. Air temperature was kept between 24-26 °C for all experiments.

**Experiments (1) and (2): Intensity and Calcium Dependency**

First and foremost, in order to verify the validity of my setup, it was necessary to show that the signals that I was measuring were similar to those measured by Sineshchekov. Although this was done prior to experiments (1) and (2), these two experiments provided data that can be used for this justification.

As can be seen in Charts 3.1 and 3.2, the response of both 806 and 1117 increased as the intensity of the stimulus increased. Since the response again decreased when the intensity was decreased, the observed effects are clearly only related to the intensity changes, and not to any conditioning that may occur with successive trials. Other important observations include:

- All signals begin with a positive spike. This spike has no time delay, but its rise time does decrease with decreasing intensity.

- For both 1117 and 806, the positive spike is followed by a negative spike that is weaker in amplitude and longer in duration than the positive one.
In charts 3.3 and 3.4, we can see that the positive spike for both the 806 and 1117 decreases with a decrease in calcium ion concentration. This was observed across all ODs (since the result was the same, only 1 OD is shown for each).

All of these observations are in agreement with the results Sineshchekov reported for UL mode (see *Extracellular recording: Cell Suspension*, in Part II). Therefore, we can assume that the measured signal is related to the photoresponse of the cells. The initial positive spike seen is probably the photoreceptor current, and the negative portion is probably the flagellar current. Although the pulses that were given are much longer than the ones Sineshchekov used (20ms and 2s as opposed to 10ns), we can still be confident in this conclusion because of the strong correlations between intensity and calcium response.

In the above experiments, both 1117 and 806 were used because we wanted to see if there would be a difference in the electrical signal between the positive and negative phototaxis. As stated in Part I of this paper, it was thought that negative and positive phototaxis strains would have opposite impulse responses. Thus we thought that the measured signals between 1117 and 806 might be opposite. However, it can be clearly seen in Charts 3.1-3.4 that this is not the case. This further backs the claim that the positive and negative spikes are due strictly to the photoreceptor and flagellar currents. Since the two strains do react differently to light, there should be some differences in the processed signal. This difference probably occurs somewhere in the flagella. Since no difference is
seen, we can be confident that the measured pulse responses are probably from the photoreceptor.

There does appear to be one major difference between the two strains: their sensitivity to light. Comparing charts 3.1 and 3.2 we can see that the 1117 started to react to the light at lower intensities than the 806. At high intensities, we can also see a second positive spike appear after the negative one for 1117. What part of the cell is responsible for the generation of this spike is not entirely clear, as no one has reported it previously. It also isn’t clear if the 806 would generate the same spike; it could be the case that they would if the intensity was increased higher. This is not possible with my current setup, however, for a stronger laser would have to be used. Still, it is a point that deserves future investigation.

**Experiment (3): Sine-Wave Stimulus**

Although my initial goal for this thesis was simply to recreate Sineshchekov’s setup and confirm his results, this success has made it possible to attack many scientific questions. One such question related to the frequency response of the cells. The transient responses to the step stimulus observed in these two experiments resemble that of the derivative of a step function: taking the derivative of a step up results in a positive delta function-like spike while taking the derivative of a step down results in a negative delta function-like spike. This is essentially what we see in the response curves. Such a phenomena was expected: as stated in Part I, the cell is expected to act as a differentiator. To further confirm this theory, it became desirable to stimulate the cells with sine
waves of different frequencies. If the cells do indeed act as differentiators at low
frequencies and integrators at high frequencies, the resultant response should
resemble a sine wave whose amplitude peaks at some frequency greater than that
of the rotation. Furthermore, the data from such an experiment would lend itself
nicely to evaluation by Fourier analysis. This analysis could easily confirm or
reject the cellular band-pass theory by plotting the logarithm of the resultant
amplitude versus the logarithm of the frequency.

Curiously, despite the benefits of such an experiment, none like this had
been carried out on cells in suspension before. Therefore, the following
experiment was recently performed:

Setup

The setup for this experiment was the most recent configuration described in Part
II of this paper. Two stimuli were used:

1. A 20ms pulse that was the same as the stimulus used in Experiment (1).
   This was used as a control to compare to past pulse experiments.

2. Sine wave: This stimulus consisted of the following frequencies: 3.125
   Hz, 4.032 Hz, 5.000 Hz, 6.098 Hz, 8.064 Hz, 10.000 Hz, 12.500 Hz,
   15.625 Hz, 20.833 Hz, 25.000 Hz, 31.250 Hz, 41.667 Hz, 50.000 Hz,
   55.556 Hz, 62.500 Hz, and 71.429 Hz. The frequencies were run back to
   back, 5 cycles a piece, in increasing and then decreasing order, giving 10
   total cycles of each frequency per record. 64 records were taken in
   sequence and then averaged together, as is always done. Since each record
was ~15s long, one file lasted ~17 minutes. The sample rate was set to 200µs for all frequencies.

Procedure

1. Two batches of 806 and 1117 were prepared in the usual fashion and placed on a shaker after being transferred to the NMM medium one day before the experiment.

2. Two 1.1 ml samples were taken from the 806 suspension and placed in a cuvette. One sample (C) was only flashed with 20ms pulses, while the other (S) was stimulated with sine waves and pulses. The following was done with no OD filters covering the cell box (i.e. stimulus was at full intensity):

3. The C-sample was flashed with the pulse file while the S-sample left in dark.

4. The S-sample was then placed in the cell box and recorded with three consecutive sine wave files. In between each file, the cells were taken out of the box and mixed. Although the C-sample had been left in the dark during this time it was also re-mixed each time S-sample was mixed. A red light was used to see during mixing; the rest of the time the room was kept dark.

5. After the third sine wave file, the S-sample was mixed and flashed with the pulse stimulus.

6. The C-sample was mixed and flashed with the pulse stimulus.

7. Steps 3-7 were repeated for 806 with 0.5 OD filter in place. This was again repeated with 1117 with 0.0 OD and 0.5 OD filters in place. Once steps 3-7
were carried out on a sample of cells, that sample was disposed of and a fresh sample was taken from the suspensions left on the shaker.

Results/Analysis

The results of the pulse files were similar to that of the ones shown in Experiment (1) and so are not shown here. As such, the cells used in this experiment could be considered to be representative of the ones used in past experiments.

The results from the sine-wave stimulus differed between 1117 and 806. For 806, no clear response was observable for either 0.5 OD or no OD (see Chart 3.5 in the Appendix for a plot of one entire record at no OD). The 1117, on the other hand, gave a very nice response at both ODs and for all trials. The only difference between trials was the response amplitude decreased slightly with successive trials. Little difference was seen between the front half of a stimulus file and the second half. Therefore, only the first half of the first trials of 1117 at no OD and 0.5 OD are shown (see Chart 3.6, Appendix).

As can be seen in Chart 3.6, the responses at both intensities do appear to increase and then decrease as the frequency increases, as expected. However, the strong pulse-like responses that are witnessed at lower frequencies for no OD were somewhat unexpected. These pulses added a non-linearity to the response that made Fourier analysis of the lower frequencies of this file unreliable. Since these pulses were much weaker for 0.5 OD, Fourier analysis of this file did result in the expected log(amplitude) vs. log(frequency) plot; this plot is shown in Chart 3.7. In the plot, we see that the response amplitude peaks at around 8 Hz. The
slope on the first half of this peak is roughly +1, while the slope on the second half of this peak is between -1 and -2.

These results agree nicely with the predicted response in Part I. First of all, the amplitude does peak at a frequency greater than the rotation frequency, which is 4 Hz, max. Secondly, a slope of +1 is characteristic of a differentiator, while a slope between -1 and -2 is characteristic of an integrator [6]. In agreement with theory, the differentiator is seen at low frequencies while the integrator is observed for high frequencies.

Conclusions

While the amplitude response of the 1117 agrees with theory, many more questions have to be answered. First, the phase response should be analyzed in order to verify the predictions about phase advance. (This is currently being done.) Second, it is unclear why no response could be recorded by the 806, especially since identical pulse responses were recorded by the same batch of 1117 and 806 both before and after the sine-wave stimulus.

Finally, questions abound as to what point in the signal processing network of the cell these sine wave responses represent. Since the origin of the pulse response is well known and since the sine wave response is consistent with the pulse response, they probably have the same origin. Therefore, the sine-wave response most likely reflects a cell body current that controls the plasma membrane electric field. If this is true, then by combining this with information about the total signal processing, the processing that takes place in the individual
cilia can be inferred. Interestingly, the signal that appears to be in common for both flagella and both the stroke velocity and beat frequency response is very similar to my observed current response (Josef, Saranak and Foster, unpublished).

In short, this work with cell suspension measurements will aid other researchers looking for biochemical and genetic evidence for the signal processing components (Capano, Saranak and Foster, in preparation).
Chart 2.1: Slow response measured in a suspension of 806 Chlamydomonas. Stimuli were created using the beam chopper; results were recorded from the oscilloscope. The left vertical line represents when the cells were exposed to the stimulus (accomplished by opening a cover on the cell box) and the right vertical line represents when they were shielded from the stimulus.
806 Intensity Results

A: 2.0 OD  B: 1.5 OD  C: 1.0 OD
D: 0.5 OD  E: No OD
F: 1.0 OD  G: 2.0 OD
Stimulus: 20ms pulse (20 ms high, 280 ms low)

Chart 3. 1: Shown responses were smoothed using Savitzky-Golay method (available in Psi-Plot). The stimulus is indicated by the square wave. All the graphs are on the same scale. The plot is inverted in order to maintain convention and make up for the inversion of the signal by the 428; as such, the stimulus pulse is also inverted. Recorded 12/14/04
1117 Intensity Results

A: 2.0 OD  B: 1.5 OD  C: 1.0 OD
D: 0.5 OD  E: No OD
F: 1.0 OD  G: 2.0 OD
Stimulus: 20ms pulse (20 ms high, 280 ms low)

Chart 3.2: Shown responses were smoothed using Savitzky-Golay method (available in Psi-Plot). The stimulus is indicated by the square wave. All the graphs are on the same scale. The plot is inverted. Recorded 12/14/04
Chart 3.3: Black: Cells in normal NMM medium; Grey: Cells in decreased calcium ion medium; No OD filter was in place; Stimulus: 2s pulse (2s high, 2.5s low). The response curve was smoothed via Savitzky-Golay method using Psi-Plot. The plot is inverted. Recorded 2/17/04
Chart 3. 4: Black: Cells in normal NMM medium; Grey: Cells in decreased calcium ion medium; 0.5 OD filter was in place; Stimulus: 2s pulse (2s high, 2.5s low). The response curve was smoothed via Savitzky-Golay method using Psi-Plot. The plot is inverted. Recorded 2/17/04
Chart 3.5: 806 Sine wave response, no OD. The smooth dark line represents the stimulus. The response curve was smoothed with Psi-Plot. Recorded 4/15/05.
Chart 3. 6 (1): The smooth sine wave represents the stimulus. All plots are plotted on the same amplitude scale, but the time scales differ for the different frequencies. All times are in milliseconds. Responses were smoothed with Psi-Plot. Signals are not inverted to make up for inversion by the 428 and are not corrected for phase shifting by the band-pass filter. Therefore, phasing is not exact.

*Recorded 4/15/05*
Chart 3. 6 (2)
Chart 3. 6 (3)
**1117 Frequency Response**

*Chart 3.7:* Plot obtained from Fourier Analysis of 1117 cells exposed to sine wave stimuli with a 0.5 OD filter in place. Frequency of the stimulus ranged from 3.125 Hz to 71.429 Hz; therefore, only 3 to 72 Hz is shown. Black lines represent an approximate linear fit. Amplitude was smoothed with Psi-Plot before being graphed.
REFERENCES


