Transplant Freezing Behavior in Photophobic Responses of Euglena gracilis Investigated in a Microfluidic Device

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We found that the transient freezing behavior in photophobic responses of Euglena gracilis is a good indicator of the metabolic status of the cells. The transient blue light photophobic responses of E. gracilis cells were investigated on-chip using a new measurement, ‘trace momentum’ (TM), to evaluate their swimming activity quantitatively in real time. When blue light of intensity >30 mW cm⁻² was repeatedly switched on and off, a large negative spike in the TM was observed at the onset of the ‘blue-light-off’ phase. Single-cell trace analysis at a blue light intensity of 40 mW cm⁻² showed that 48% (on average, n = 15) of tumbling Euglena cells ceased activity (‘freezing’) for 2–30 s at the onset of blue-light-off before commencing forward motion in a straight line (termed ‘straightforward swimming’), while 45% smoothly commenced straightforward swimming without delay. The proportion of freezing Euglena cells depended on the blue light intensity (only 20% at 20 mW cm⁻²). When the cells were stimulated by four blue light pulses at the higher intensity, without pre-exposure, the transient freezing behavior was more prominent but, on repeating the stimuli after an 80 min interval in red light, the same cells did not freeze. This shows that the metabolism of the cells had changed to anti-freezing during the interval. The relationship between the interval time with/without light irradiation and the blue light adaptation was elucidated experimentally. The origin of the freezing behavior is considered to be a shortage of a metabolic substance that promotes smooth switching of flagellum movement from in situ rotation mode to a straightforward swimming mode.

Keywords: Adaptation • Blue light • Euglena gracilis • Microfluidic aquarium • Photophobic response • Transient responses.

Abbreviations: LC, liquid crystal; PAC, photoactivated adenylyl cyclase; TM, trace momentum.

Introduction

Photosynthetic microorganisms have attracted much attention of researchers due to their ability to produce oil and nutrition in an environmentally friendly manner (Chisti 2007, Schenk et al. 2008, Lardon et al. 2009, Brennan and Owende. 2010, Mahapatra et al. 2013). Some of them, such as Chlamydomonas reinhardtii (Stavis and Hirschberg, 1973, Rochaix 1995, Hegemann and Berthold 2009, Wakabayashi et al. 2011) and Euglena gracilis (Häder et al. 1981, Ozasa et al. 2013a), are motile and show kinetic responses to light, so-called photomovements (Nultsch and Häder 1979, Sgarbossa et al. 2002, Braatsch and Klug 2004), in order to stay in a moderate light suitable for photosynthesis. They have developed photokinesis (Chung et al. 2004), phototaxis (Sineshchekov et al. 2002), photophobic responses (Diehn 1969a, Bovee 1982, Matsuura et al. 1999) or photoadaptation (Greenblatt and Schiff 1959). These photoinduced kinetic responses have been investigated by counting the number of cells that change their moving speed or directions according to light irradiation (Lebert and Häder 1997). For instance, action spectra for the step-up/down photophobic responses of E. gracilis have been measured by many researchers (Diehn 1969b, Checucci et al. 1976, Matsunaga et al. 1998) and it has been shown that blue light (wavelength 350–500 nm) and UV light (250–300 nm) induce the photophobic responses effectively (Diehn 1973, Doughty 1993, Matsuura et al. 1998). One of the recent achievements for E. gracilis is the identification of the photoreceptor for their photophobic response. Iseki et al. have found that photoactivated adenylyl cyclase (PAC) is the photoreceptor (Iseki et al. 2002; Yoshikawa et al. 2005), which produces cAMP from ATP by blue light activation and mediates the photophobic response of Euglena. They also showed that the PAC molecules are useful as photocontrollable signal induction molecules for optogenetics (Boyden et al. 2005, Tsunematsu et al. 2011), demonstrating photoactivated neuron firing through genetically introduced PAC molecules (Nagahama et al. 2007, Schröder-Lang et al. 2007). Although the photoreceptor for the photophobic response of Euglena has been identified, complicated metabolic processes involved in the photophobic response of Euglena are still unclear. A micro-ethological approach will provide an insight into the metabolic processes of the photoresponses, as well as molecular level analysis of proteins/enzymes related to the photoresponses.

The detailed ethological study on the photoresponses of photosynthetic microorganisms is also important to achieve the well-controlled growth/culture of the microorganisms and to understand the mechanisms of metabolic adaptation.
to photointensity changes. For this purpose, single-cell level studies on the photoresponses of the microorganisms with millisecond resolution are required. Recently, we have developed an optical feedback system, in which optical monitoring/stimulation can be performed dynamically with arbitrary intensity of light and a high spatial resolution (Ozasa et al. 2011, Ozasa et al. 2013b, Ozasa et al. 2014). We demonstrated that the spatial distribution of *Euglena* cells confined in a microfluidic device can be controlled via optical feedback through the photophobic response of *Euglena*. The system also enables us to measure quantitatively the photophobic response of *Euglena* cells in situ under various light intensities and to trace the photophobic movements of single cells.

Here we report an investigation of the photophobic responses of *E. gracilis*, including transient freezing behaviors, variations of photoresponses and blue light adaptation. By confining a batch of *Euglena* cells in a microfluidic device, their swimming activities were measured quantitatively in real time under blue light repeatedly switched on and off while increasing its intensity. The detailed movements of single cells in the same batch were subsequently traced through video analysis. Blue light adaptation was also investigated by measuring the transient freezing behaviors after the interval of blue light pulse exposures while changing the interval time and light irradiation condition.

**Results**

**Dependence on blue light intensity**

Fig. 1b and c shows temporal changes of ‘trace momentum’ (TM; see the Materials and Methods) observed in response to blue light pulses of constant duration and progressively increasing intensity. The blue light, whose spectrum is given in Fig. 1a, was switched on and off at 75 s intervals. There were approximately 100 swimming cells in the micro-aquarium. The upper limit of the TM envelope obtained during the blue-light-off period slowly increased between 10 and 30 min and then gradually decayed between 30 and 60 min. This increase was attributed to an increase in swimming speed evoked by the blue light (Melkonian et al. 1986), whereas the rate of the decay varied from experiment to experiment, for unidentified reasons. As the blue light intensity increased above approximately 2 mW cm\(^{-2}\), the photophobic response to blue light was observed as a decrease in TM during the blue-light-on period. The difference in the TM value between the blue-light-on and blue-light-off periods increased as the blue light intensity was increased, up to approximately 30 mW cm\(^{-2}\). Above 30 mW cm\(^{-2}\), the recovery of TM during the blue-light-off period (step-down response) became slower and a downward spike was observed at the onset of each blue-light-off period. The slower recovery and negative spike are clearly observed in a comparison between expanded sections of the record obtained at 20 and 40 mW cm\(^{-2}\) (Fig. 1c). The negative spike revealed that the change in the locomotion of *Euglena* cells from rotational tumbling to forward motion in a straight line (termed ‘straightforward swimming’) in response to blue-light-off was not instantaneous or smooth for blue light intensities >30 mW cm\(^{-2}\). The TM recovery in Fig. 1c was fitted to an exponential curve with a time constant of 6 s at 20 mW cm\(^{-2}\) and 17 s at 40 mW cm\(^{-2}\). A small delay of 1.5 s was inferred in the exponential recovery at 40 mW cm\(^{-2}\), corresponding to the downward spike of TM.

The downward spike observed at the onset of each blue-light-off period was to be categorized as a step-down photophobic response, according to the terminology recommended...
by Diehn et al. (1977). However, the downward spike is a part of the recovery process from the step-up photophobic response for relatively higher light intensities, and differs from the conventional step-down photophobic response in which *Euglena* cells in a moderately illuminated area turn their swimming direction at the boundary between moderate light and a dark area to avoid going into the unfavorably dark area. In order to avoid confusion, we described the response we observed in this study just as the photophobic response. The illumination intensity of 40 mW cm$^{-2}$ with a blue light spectrum centered at 465 nm (Fig. 1a) corresponds to a photon fluence rate of 1,760 μmol (m$^2$·s$^{-1}$), relatively higher than the conventional fluence rate [typically, 0.1–200 μmol (m$^2$·s$^{-1}$)] investigated for the step-up photophobic response of *Euglena* cells (Matsunaga et al. 1999). The onset of the step-up photophobic response observed in Fig. 1b was approximately 2 mW cm$^{-2}$ [90 μmol (m$^2$·s$^{-1}$)].

The movements of *Euglena* cells at four selected times (A–D in Fig. 1c) at 40 mW cm$^{-2}$ are compared in the trace images shown in Fig. 2. At the end of the blue-light-on period (Fig. 2A), 91 traces of *Euglena* cells were counted. Most were tumbling rotationally, but about 10 were still swimming. At the onset of blue-light-off (Fig. 2B), 64 traces were counted, and 18 cells were straightforward swimming. Because the blue light was turned off, the tumbling cells were recovering to straightforward swimming as indicated by the increased number of swimming cells. In contrast, the decrease in the total number of traces revealed that at least 27 cells that were moving at time A had stopped moving at time B. The negative spike observed in Fig. 1c was caused by this decrease in the number of moving cells. As TM recovery proceeded, the number of traces and the number of straightforward swimming cells were increased to 68 and 48, respectively at time C, and to 89 and 76, respectively at time D. From these observations, we infer that the recovery process at 40 mW cm$^{-2}$ (Figs. 1c, 2) was comprised of (i) transition from rotational tumbling to straightforward swimming and (ii) freezing of locomotor activity and awakening from the freezing. The
freezing observed here may correspond to ‘delayed agitation’ mentioned but not fully described in previous reports (Diehn 1969a, Diehn 1973).

**Single-cell analysis**

To elucidate the photoresponses of the cells, nine representative traces of individual cells were tracked in a series of trace images and the TMs of these cells were plotted (Fig. 3a). All nine cells showed irregular TM changes during the blue-light-on periods, corresponding to continuous or intermittent rotational tumbling movements. At the onset of the blue-light-off period, the cells could be separated into three distinct types based on their photophobic (step-down) responses. Type-A cells continuously and smoothly recovered their original TMs, type-B cells froze activity for 2–30 s before recovering their original TMs, and type-C cells ceased moving during the blue-light-off period. The transient freezing behavior of type-B cells constituted a complete arrest of motion, which resulted in the disappearance of their traces and hence the decrease in cell number observed in Fig. 2 at time B. The typical swimming behavior of three types of cells are plotted in Fig. 3b in blue light of intensity 40 mW cm$^{-2}$. Swimming speeds of type-A cells were relatively high compared with those of type-B and type-C cells, as shown in Fig. 3a and b. Apart from their different swimming speeds, the traces of type-A and type-B cells were similar, i.e. straightforward swimming during the blue-light-off phase and rotational tumbling and intermittent swimming during the on phase. Of course, the transient freezing of type-B is unobservable in the traces in Fig. 3b. Type-C cells drifted with rotational tumbling during the blue-light-on phase and became motionless during the off phase. We suggest that the type-C cells may be in diapause, or have a deficiency that prevents straightforward swimming.

We attributed the gradual recovery of TM shown in Fig. 1c at 40 mW cm$^{-2}$ to the frequency distribution of the freezing durations of type-B cells. The TM recovery in Fig. 1c was a good fit to a single exponential curve, from which we deduced that the frequency of freezing decayed exponentially with a time constant of 17 s and a delay of 1.5 s. As seen in Fig. 3a, the freezing duration was not constant for individual cells. This implies that release from transient freezing occurs stochastically with an equal probability for unit time.

The numbers of cells of types A, B and C can be counted by checking the behavior of each cell before and after the onset of the blue-light-off period. We have counted the number of cells of each type for 15 individual experiments, as shown in Fig. 4. For blue light of intensity 40 mW cm$^{-2}$, there were 45% type-A cells on average, 48% type-B and 4% type-C, with 3% indeterminate. Fewer type-B cells were observed for blue light at 20 mW cm$^{-2}$, i.e. 76% type-A cells, 20% type-B and 2% type-C, with 2% indeterminate. The decrease (increase) in the number of type-A (type-B) cells indicates that the photophobic (step-down) response of *E. gracilis* changes from type-A to type-B when the blue light intensity increases. The gradual increase of negative spikes in TM with blue light intensity suggests that the threshold level of the transition from type-A to type-B varies from cell to cell.

**Anti-freezing adaptation**

When blue light pulses of high intensity were applied without pre-exposure to increasing intensity, much larger negative spikes were observed in TM. Fig. 5a shows the changes in TM produced by four pulses of blue light of 40 mW cm$^{-2}$, applied without pre-pulses. The TM at the onset of blue-light-off decreased to 50% of the original TM level and the number of moving cells in the micro-aquarium was reduced.
from approximately 55 to 30. Combining the observations shown in Figs. 1c and 5a, we conclude that the threshold for transient freezing was low at the beginning of the Fig. 1b experiment but gradually increased as blue light pulses of increasing intensity were applied, resulting in the relatively small negative spikes at around $40 \text{ mW cm}^{-2}$.

An important observation in Fig. 5a is that the TM was increased by blue light pulses. The TM initially decreased from 4,000 to 2,700 at the first pulse of blue light but it recovered during the light pulse and increased further to 5,000 after the blue light was turned off. It is well known that the swimming speed of *E. gracilis* is increased by blue light, a behavior termed photokinesis (Wolken and Shin 1958, Melkonian et al. 1986, Lebert 2001). The TM increase could be partly attributed to photokinesis but also partly reflected an increase in the number of moving cells (Fig. 5a) (Melkonian et al. 1986). The number of moving cells increased from 55 to approximately 60 during blue light pulses, revealing that some cells had been inactive before the first blue pulse. After the last blue pulse, the TM gradually decreased, reaching the original level after about 7 min. This decrease paralleled a decrease in the number of moving cells to approximately 50. We speculate that some resting cells were awakened by blue light pulses to escape from the light and returned to the resting state when the blue light was terminated.

Fig. 5b shows the changes of TM obtained during a second test with four blue light pulses performed on the same *Euglena* cells, commencing 80 min after the last pulse of the first test (i.e. an 80 min interval between the blue light pulses). Although the up-down trend of TM in Fig. 5b is almost identical to that in Fig. 5a, no large negative spikes are apparent in Fig. 5b, indicating that transient freezing behavior was suppressed after the 80 min interval. This difference suggests that the *Euglena* cells had acquired a capability to recover straightforward swimming without freezing (anti-freezing capability) after the interval. Type-B cells comprised 49% of all cells in the first experiment of Fig. 5a, but only 8% in the second experiment of Fig. 5b.

### Adaptation vs. interval time

The adaptation to strong blue light was examined by changing the interval time between the first and second test. Fig. 6 shows the dependence of the spike ratio on the interval time in red light, measured in similar experiments to that in Fig. 5 with various interval times. The spike ratio was defined as the depth of the negative spike to the TM level before the spike.
for the fourth pulse in the second test. During the interval time, the micro-aquarium was continuously irradiated with red light of 16.8 mW cm\(^{-2}\). The dependence shows that the adaptation, i.e., the acquisition of the anti-freezing capability proceeds faster at the beginning of the interval, and gradually slows down with time. When the red light was not irradiated during the interval time, large negative spikes were observed even after an 80 min interval, indicating that the cells do not acquire the anti-freezing capability in the dark. This observation reveals that the blue light adaptation sensitively reflects the metabolic status of *Euglena* cells, which is changed by the photosynthesis in red light.

**Discussion**

**Transition between type-A and type-B**

We consider that the metabolism of type-B cells was changed to that of type-A during photosynthesis under the red light (Fig. 6). The rather scattered data of the spike ratio in Fig. 6 suggest that the adaptation may also be influenced by the circadian rhythm of *E. gracilis* (Hagiwara et al. 2002, Bolige et al. 2005), since the examination in this study was carried out without culture synchronization (Osafune et al. 1975). The adaptation to strong blue light is presumably intimately related to the survival strategies of the micro organism. When the same *Euglena* cells were kept in the dark for >24 h, large negative spikes were again observed, indicating that some of the type-A cells were transformed back to type-B. Interestingly, the freezing of the flagellum of *E. gracilis* was also found when the cells encounter sudden gravity change from hyper- to micro-gravity (Strauch et al. 2010), which may also be related to the survival strategies of the micro organism.

![Fig. 6](image-url) Dependence of the spike ratio on the interval time in red light, measured in similar experiments to that in Fig. 5 with various interval times. The spike ratio was defined as the depth of the negative spike to the TM level before the spike, for the fourth pulse. Circles indicate the average for each interval time, whereas squares represent the result of each independent experiment. The dependence shows that the acquisition of the anti-freezing capability proceeds faster at the initial stage of the interval and slows down with time. When the red light was not irradiated during the interval time, large negative spikes were observed even after the 80 min interval, indicating that the cells do not acquire the anti-freezing capability in the dark.

**Origin of type difference and adaptation**

The observation in this study revealed that the mechanism of freezing behavior differs from that of the conventional photophobic response of *Euglena*, since the photophobic response continues even after the freezing behavior disappeared after the red light illumination of 80 min. We consider that the metabolic status of *Euglena* cells evolves by red light illumination for a short period of some 10 min, and then the cells are able to avoid freezing after the light (type-A). The difference between type-A and type-B is distinct, and no intermediate response was observed. This indicates that freezing behavior is an independent mode of flagellum motion, differentiated from straightforward swimming and in situ rotation (tumbling).

A reasonable mechanism for the freezing behavior is that a certain metabolic substance produced by light is required to switch the mode of flagellum movement from in situ rotation to forward swimming immediately. The shortage of the substance (type-B) results in delayed ignition of the swimming; the cells stop in situ tumbling when the blue light is terminated but cannot start swimming, i.e., they freeze for 2–30 s. The photo-produced substance will be decomposed gradually during the night-time (dark situation), resulting in the transformation from type-A back to type-B.

The correlation of the freezing behavior to a metabolic substance is still speculative and not conclusive at the moment. Specific experiments are required to determine the effect of blue light on the metabolic activity of *E. gracilis*, such as co-immunoprecipitation studies to look at what binds to PAC following activation in the presence of blue light and in its absence, gene expression profiling of common metabolic proteins and mitochondrial proteins, or quantification studies on glucose utilization induced by blue light. Unfortunately, these molecular-level studies are hard for non-sustainable phenomena including transient freezing behaviors investigated in this study. Instead, we are trying to determine the signal path related to the transient freezing by employing an enzyme knock-down technique, expecting that the enzymes responsible for photophobic response and for the mode of flagellum movements will be made clear.

The effect of photosynthesis on freezing behavior will be examined indirectly by employing chloroplast-free *Euglena* cells, or by using DCMU to suppress photosynthesis. Further, the oxygen concentration in the culture medium or in the cells, or other photoreceptors such as phytochrome for red light (Bolige and Goto 2007), may play an important role to induce/suppress the freezing behavior. We will perform the further experiments on the freezing behavior (i) with chloroplast-free *Euglena* cells; (ii) with DCMU; (iii) under reduced red light to test the contribution of phytochrome; and (iv) with excess oxygen in the culture medium by adding hydrogen peroxide. The results of these experiments will be reported as a separate paper in the near future.

In conclusion, transient behaviors in the blue light photophobic response of *E. gracilis* were investigated with the introduction of the TM measurement to evaluate the
swimming activity of Euglena cells. Three types of transient behaviors at the time the blue light was turned off were observed by single-cell trace analysis: smooth recovery from tumbling to straight line swimming (type-A); transient freezing for 2–30 s before starting swimming (type-B); and slow tumbling only under blue light (type-C). The same Euglena cells that experienced transient freezing for blue light pulses did not show the transient freezing behavior after an 80 min interval in red light, indicating that they acquired anti-freezing metabolism during the interval. From the analysis of the dependence of blue light adaptation on interval time and red light, we concluded that the adaptation is determined by metabolic change evoked by photosynthesis. The adaptation suggests that a metabolic substance, as yet unidentified, is produced by light illumination and promotes the smooth switching of the mode of flagellum movement from in situ rotation to straightforward swimming. The analysis of transient freezing is needed to determine the signal path responsible for the photoinduced responses of green algae, and provides wide application for photosynthetic production of biomass.

Materials and Methods

Euglena gracilis Z strain were supplied by Euglena Co., Ltd. as a suspension cultured in Cramer–Myers’ (CM) medium (without sodium citrate) (Cramer and Myers 1952). The suspension was stored in a conical microtube (1.0 ml) before experiments; the tube was kept at room temperature without agitation under white light illumination of approximately 0.15 mW cm⁻² (daytime) and 60 nW cm⁻² (night-time). The cells were in the stationary phase with possibly less oxygen due to complete confinement in a plastic microtube. For each experiment, an approximately 10 μl droplet of CM medium containing Euglena cells was placed on a poly dimethyl siloxane (PDMS) circular micro-aquarium 2.49 mm in diameter and 140 μm deep (0.7 μl). The micro-aquarium was then sealed by a cover glass, and the excess volume of 9.3 μl overflowed away from the microchip. The micro-aquarium was then sandwiched between two cover glasses and the space between the two glasses was filled with pure water to prevent evaporation (Fig. 7a). Approximately 50–200 cells were confined in the micro-aquarium. Oxygen supply was recovered when the cells were...
transferred to the microchip, since the microchip permeates oxygen well. The micro-aquarium was then placed in a 30 mm diameter dish with a glass top and bottom and sealed with plastic tape to maintain internal humidity. The cells are able to survive for >2 weeks in these evaporation-resistant surroundings. The photophobic response in this study was also observed even 2 weeks after transferring the cells from the microtube into the microchip. The increase in the cell number can be ignored for the time periods used in this study.

Transient behavioral responses of *E. gracilis* to blue light stimulation were examined with the 2D optical feedback system that we used previously for the demonstration of the spatial and dynamic control of the density of *Euglena* cells in a micro-aquarium (Ozasa et al. 2011). The system was the combination of an upright optical microscope (BX51; Olympus), a video camera (JVC-HC200C; Trinity), a data processing computer (PC, MG/D70N; Fujitsu), a liquid crystal (LC) projector (LP-XU86; Sanyo) and optics, as shown in Fig. 7c. The micro-aquarium was placed on the microscope stage and illuminated from below by the LC projector through reduction lenses. The illuminated area was 5.1 mm × 3.8 mm. During experiments, the whole area of the micro-aquarium was illuminated constantly by red light (570–700, 16.8 mW cm⁻²) to observe the movements of the cells. The stimulating blue light (430–520 nm) was also provided from the LC projector whose intensity and duration were controlled by the data processing computer. The spectra of the red and blue light provided by the LC projector are given in Fig. 1a. Because the micro-aquarium was only 140 μm deep, the swimming directions of all *Euglena* cells were perpendicular to the direction of the stimulating light.

Real-time raw images of *Euglena* cells swimming in the micro-aquarium were taken by the video camera through a × 5 objective lens (MPFLENSX; Olympus) at intervals of approximately 0.1 s at a resolution of 200 pixels mm⁻¹ and an image size of 800 × 600 pixels. To quantify the swimming activity of *Euglena* cells, the raw images were processed according to the flow chart shown in Fig. 7d. We produced a differential image from two consecutive raw images and converted the differential image into a binary image by thresholding. We superimposed *n* (n = 3–10) binary images to generate a ‘trace image’, which showed the swimming traces of *Euglena* cells as footprints during *n* time intervals. Finally, a measurement, termed the TM, was calculated by counting the number of pixels covered by *Euglena* traces in each trace image. Each TM value approximates the scalar summation of the swimming speeds of *Euglena* cells in the micro-aquarium. Typically, one swimming *Euglena* cell produced a TM value of 60–70, whereas one rotating and tumbling cell produced a TM of 40–50. In contrast to particle tracking techniques (Wu et al. 2006), which require extensive calculation and are weak in trace identification, the TM calculation can be performed in real time and reliably allows for crossing and disappearance of swimming cells in the trace image. The forward motion of *E. gracilis* in a straight line (termed ‘straightforward swimming’) under red light and the ‘rotational tumbling’ motion under blue light are presented in Fig. 7b.

The results described herein have been reproduced for three independent cell samples of *Euglena* gracilis Z strain supplied from three different organizations.

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**Disclosures**

The authors have no conflicts of interest to declare.

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