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Light-Induced Cell Aggregation of *Euglena gracilis* Towards Economically Feasible Biofuel Production

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Abstract

One of the most energy-consuming processes in conventional microalgal biofuel production is harvesting cells from dilute media. Cell harvesting by centrifugation or membrane filtration requires equal or more energy than is captured by photosynthesis, resulting in a negative net energy and CO_2 balance. As a cost-effective alternative to this approach, we investigated the possibility of using the inherent motility and behavioral responses of the green microalgae *Euglena gracilis* to light stimuli, to promote cell aggregation. Irradiation of cells with light stimuli of different wavelengths and intensities revealed that *E. gracilis* cells are specifically attracted to green light. The cell aggregation rate for cultures irradiated with green light for 24 h was 8.7 fold and the cell collection rate reached 70%, which is comparable to the efficiency of centrifugal separation. Utilization of green light for cell aggregation does not compete with the light absorption by chlorophylls in photosystems I and II (PSI and PSII). Therefore, the findings in the present study offer the use of green light in solar radiation, which was originally wasted energy in photosynthesis, as the energy source for one of the most energy-intensive downstream processes in microalgal biofuel production.

Introduction

Biofuel production from carbon dioxide using microalgae has been the focus of intensive research[1-3] for producing carbon neutral sources of energy. In comparison to higher plants, such as corn and sugar cane, microalgae produce markedly more fuel per gram of biomass due to their efficient photosynthetic systems.[4] However, the purification of biofuel from microalgae requires several downstream processing steps, which critically increase the total energy consumption and may negate the potential benefits of this approach.

During the typical process of algae biofuel production (Fig. 1), there are two main requirements that are associated with high energy consumption costs.[4] The first is maintaining an algal culture that is free of contamination,[5] which would have deleterious effects on the biofuel production efficiency. Although the addition of antibiotics to cell cultures reduces the risk of microbial contamination, this requirement substantially increases the total operation cost. The second costly process is the harvesting of algal biomass. When algal biofuel is produced at an industrial scale, large volumes of liquid culture are needed. As it is difficult to efficiently process large volumes of dilute media, cell aggregation is a critical requirement.[6] In a recent report by Dassey *et al*., the energy cost of harvesting algal cells using centrifugal separation is reported to range from 1 to 19 kWh/m^3, depending on the biomass harvesting rate.[7] The authors estimated that flow and energy consumption of approx. 3 L/min and 6.3 kWh/m^3, respectively, are required to achieve a cell harvesting rate of 70%. The radiation intensity of sunlight is in the order of 1 kW/m^2. Therefore, if we assume an algal reactor with a base area of 1 square meter and a height of 1 meter, the energy consumption of 6.3 kWh/m^3 corresponds to sunlight

irradiation for 6.3 h. As the photosynthetic efficiency of microalgae is 1% on average, it would take more than 1 month for photosynthesis to balance the energy consumption by centrifugal separation. Membrane filtration is considered as a leading cell dewatering method, owing to simplicity and low energy requirement. Recently, Gerardo *et al.* estimated 0.9 kWh/m^3 to achieve a cell harvesting rate of 99.9% in a pilot scale reactor by membrane filtration.[8] The value of 0.9 kWh/m^3 corresponds to the energy captured by photosynthesis for approx. 1 week with photosynthetic efficiency at 1% on average. Therefore, cell harvesting by centrifugation or membrane filtration requires equal or more energy than is captured by photosynthesis, and a distinct necessity exists for more efficient cell aggregation methods.[4, 9]

Here, we examined the potential of the microbe *Euglena gracilis* for economically feasible biofuel production because this species has several advantageous characteristics. First, the main photosynthetic product of *E. gracilis* is well suited as a precursor of jet fuel,[14] which has high commercial value. Second, because *E. gracilis* thrives under strongly acidic conditions (pH 3.5) and requires no organic metabolites in autotrophic modes of growth, it can be cultured using inexpensive medium that does not contain antimicrobial inhibitors. Third, *E. gracilis* exhibits several forms of taxis in response to stimuli such as light,[15-18] gravity,[19] and oxygen.[20] As this property may potentially be applied towards a novel cell harvesting method, in this study, we focused on the responses of *E. gracilis* to light, as this external stimulus can be easily controlled and is critical for the photosynthetic process underlying biofuel production.

Although the photoresponses of *E. gracilis* were first described more than 100 years ago,[21] the present study is the first to apply this phototactic ability towards the directed migration of cells. Although several studies have performed cell tracking experiments to examine the movement of individual cells over several seconds to minutes,[19,22-27] here, experiments were conducted from a macroscopic perspective by evaluating the movement of the entire cell population in 9-cm petri dishes over the course of several hours. Based on optical density, the amount of cells used in each experiment was estimated to be in the order of ~10^6 cells. Although *E. gracilis* exhibits several responses to light, including phototaxis, photophobic response, and photokinesis,[22] these different types of responses can only be discerned microscopically. For this reason, the term "photoresponse" is used in this report to collectively refer to these responses.

Results

As *E. gracilis* shows behavioral responses to gravity and oxygen levels,[19,20] we first examined the movement of cells irradiated with horizontal light in a petri dish. Using this approach, only the photoresponse of cells, rather than the response to a mixture of stimuli, could be evaluated.

The cell distribution patterns within the petri dish before and after light illumination with different wavelengths are shown in Fig. 2. Prior to irradiation of the cell suspensions, the cells were uniformly distributed (Fig. 2A). Irradiation with blue light (400 nm, 1.5m W/cm^2) for 24 h resulted in a cell distribution characterized by the formation of an intense band of cells with a "C" shape around the light source and a wide zone of relatively few

cells (Fig. 2B), whereas in response to irradiation with green light (530 nm, 1.5 mW/cm^2), the cells were selectively attracted to the light source (Fig. 2C). These results clearly show that the cells exhibited a wavelength dependent photoresponse to visible light, a finding that was further confirmed by the cell distribution returning to the initial state within several hours of turning off the light source.

To determine the contribution of cell growth to the observed cell distribution patterns, the amount of cells present at the beginning and end of the experiment was estimated spectrophotometrically (Fig. S1). Because the total amount of cells in the petri dish remained nearly constant, it was confirmed that the cell distribution observed after light irradiation was due to the movement of cells in response to light.

It was also noted that the cells displayed different responses to the same wavelength of light over time. Figs. 2D, E, and F show the cell distribution patterns after 2, 6, and 9 h, respectively, of 530 nm light irradiation. On comparison of the initial and final cell distributions, it appears that the cells first moved away from, and then were attracted to, the light source. This finding suggests that not only do different wavelength give different cell distribution, but the time elapsed also affects the cell distribution as well.

During oxytaxis in *E. gracilis*, cells form a clear band perpendicular to the oxygen gradient, indicating that the cells are attracted to a specific oxygen concentration.[20] Presumably, this concentration corresponds to their oxygen consumption rate, as excess oxygen can damage cells. Based on this oxytactic response, we hypothesized that photoresponses of *E. gracilis* are also influenced by an optimum light intensity (OLI), at which the optimum cell activity can be maintained. At the OLI, cellular photosynthetic

activity would be sufficiently high to maintain required energy levels, and the cells would not be adversely affected by photoinhibition. Therefore, it is expected that cells will migrate to areas with this OLI, where they will ultimately form a visible band of cells. Thus, the OLI represents the border between positive and negative photoresponses. According to this hypothesis, if a cell senses that the light intensity is too weak, it will migrate towards an area with higher light intensity. Although a stronger light will affect a larger area, cells will retreat from the light source if the intensity is too high. Therefore, the OLI is not only the optimum light intensity for cellular metabolic activity, but it also represents the optimum intensity for promoting cell aggregation. Based on this assumption, the OLI should show a wavelength dependency, as not all wavelengths of light are photosynthetically active. To investigate this hypothesis, we measured the light intensity at the place of highest cell concentration with light of various wavelengths and intensity.

Fig. 3A shows the plots of OLI against the wavelength between 400 and 700 nm. In the obtained spectrum, blue and red light, which are photosynthetically active, had a lower OLI compared to light with wavelengths between 500 and 600 nm (blue: 0.1; green: ~2.5; and red: ~0.1 mW/cm^2). One possible explanation for this result is that *E. gracilis* cells avoid high-intensity photosynthetic light because it is more effectively absorbed by chlorophyll, leading to cell damage. Instead, cells concentrate in regions that avoid cell damage, but still permit photosynthesis to proceed, thereby forming a pattern similar to that observed in Fig. 2B. In contrast to blue and red light, the cells were attracted to all but the strongest intensities of green light (Fig. 2C). This result correlates well with those from an experiment in which the light intensity of blue light directed at the cells was reduced from

1.5 to 0.15 mW/cm^2 (Fig. 3B-1 to 3B-4). From these images, it is clear that the cells were increasingly attracted to blue light as the light intensity was reduced. Therefore, it appears that *E. gracilis* cells have an OLI for supporting cell growth.

According to the obtained OLI curve, we determined that 530-nm light has the greatest potential for promoting the aggregation of *E. gracilis* cells. Although light of stronger intensity forms a gradient over a wide area, cells are not attracted to areas higher in light intensity than the OLI. Using 530-nm light, the light intensity can be increased to 2.5 mW/cm^2 without inducing photoinhibition of *E. gracilis*, allowing for the operation of larger bioreactors. Notably, this value would support an economically feasible biofuel production process, because green light of this intensity can be easily obtained by the spectral diffraction of sunlight. Due to the inherent motility of *E. gracilis* cells, cells can even be attracted from areas which are not directly illuminated (Fig. S2, S3).

We next examined the effect of light on the aggregation of *E. gracilis* cells in a three-dimensional (3D) bioreactor system. As opposed to the previous experiments conducted using a thin layer of suspended cells in a petri dish representing a two-dimensional system, industrial-scale bioreactors are three-dimensional, and the effects of gravity and oxygen concentrations may therefore be larger than that of light. To test the feasibility of light-induced cell aggregation in 3-D systems, we examined the concentration of cells in 20-ml size serum bottles after 24 h under light-irradiated and dark conditions (Fig. 4). For the analysis, light was irradiated from the bottom of the serum bottle, and the supernatant was progressively sampled from the top to the bottom of the bottle and examined spectroscopically in 96-well plates.

Under dark conditions, the color of the culture medium did not change between samples, indicating that cell concentration did not exhibit a gradient in the vertical direction (Fig. 4B). However, in the case of green light, the color of the sampled liquid was almost transparent, with the exception of the last four samples. As the final four samples were collected from the bottom of the bottle, they contained cells that had accumulated at the bottom of the reactor during light irradiation. The cell concentration in the collected samples was examined indirectly by performing absorption measurements at 680 nm, which corresponds to the absorption of chlorophyll. The graphs obtained from the measurements of the green-light-irradiated and dark-cultured cells are shown in Fig. 4C, along with the results of samples generated using white light (non-monochromatic light) of the same intensity.

We had anticipated that cells would accumulate at the bottom of the serum bottles due to the effects of gravity for both light conditions; however, as can be seen in Fig. 4A and 4B, a cell concentration gradient was not observed in the absence of light. Furthermore, although white light promoted cell accumulation, a higher concentration of cells at the bottom of the reactor was achieved with green light. A possible explanation for this difference may be because white light consists of light with different wavelengths, each of which has a different OLI and therefore attracts cells to different regions of the reactor. This would result in only the broad attraction of cells to the light penetrating from the bottom of the bottle. This photoresponse is in contrast to the acute cell distribution at the bottom of the bottle observed in the case of green light. Based on the absorbance of the last four samples collected from the serum bottles after 24 h of green-light irradiation (Fig. 4C),

the cell accumulation rate for the cultures irradiated with green light was 8.7 fold and the cell collection rate was 70%, which is comparable to the efficiency of centrifugal separation.[6] The intense cell accumulation was also seen from optical microscopic images of the sampled liquid from the bottle with light (Fig. 4D).

Discussion

The primary aim of the present research was to develop an energy-efficient approach for harvesting algal cells during biofuel production. We demonstrated that the accumulation of *E. gracilis* cells was induced with 530-nm light at an intensity of 2.5 mW/cm^2, which can be obtained from the monochromatized solar spectrum. In 2012, Mochiji *et al*.[28] reported the phototactic migration of *Chlamydomonas* cells using a combination of reactive oxygen quenching reagents and light. An approx. 10-fold increase in the culture density was demonstrated by light illumination in the presence of dimethylthiourea. In the present work, no reagents were added to enhance the phototactic activity of *E. gracilis* cells. In this respect, this report is the first to demonstrate cell accumulation by microalgae utilizing only an inherent photoresponse, which avoids the potentially negative effects of physiologically active reagents both on medium recycling and the quality of the generated products.

Despite the potential of this approach, the underlying mechanisms and properties of *E. gracilis* motility are not well established, particularly the specific attraction of *E. gracilis* to 530-nm light and the unique time dependence of the photoresponses to the same wavelength of light. Although fishermen have been using green light to attract plankton and in turn, to attract fish, the reason why many photosynthetic microbes are attracted to green

light is still unclear. In the case of *E. gracilis*, Iseki *et al*.[29] identified a flavin-bound adenylyl cyclase that initiates a signal for photophobic responses after absorbing blue light. Although this finding was a major breakthrough towards understanding photoresponses by *E. gracilis*, the chromophore responsible for our present results remains elusive, as no chromophore or pigment with an absorption peak at 530 nm has been found to date in *E. gracilis*.[30-32] Although the carotenoids and rhodopsins located at the eyespot are major candidates, further experiments are necessary to confirm this speculation.

Furthermore, in the case of green light, cells initially retreated from the light before exhibiting positive photoresponses (Fig. 2D, E, F). However, the retreating behavior could not be observed when cells were pre-irradiated with green light for 24 hours (Fig. S4). This behavior cannot be simply attributed to photosensitive pigments or a one-way signal transduction system, because differential responses cannot occur in a pure photochemical reaction. Therefore, these results suggest that at least one other factor, such as proteins involved in redox homeostasis and/or photosynthesis, or a transcriptional factor controlling gene expression, is responsible for controlling the photoresponses of *E. gracilis*.[24,26, 27, 33-36] Over a 24-h period, the activities of these potential factors may fluctuate; therefore, it is possible that other factors strongly interact with the signal transduction system associated with photoresponses.[37,38]

Utilization of green light for cell aggregation is attractive, as it does not compete with the light absorption by chlorophylls in photosystems I and II (PSI and PSII, Fig. 5), allowing green light to penetrate into the cell suspension twice as efficiently as blue or red light (See Fig. S5). To raise the theoretical limit of photosynthetic efficiency, reengineering

the absorption energy of chlorophylls is the subject of intensive research,[2] as the two photosystems compete for the same wavelengths of light, reducing overall photochemical efficiency. In other words, the absorption spectra of photosystems will be optimised in a way to utilize green light for harvesting a broader spectrum of solar radiation, making biological tandem cells. However, as described in this work, when the total process of microalgal biofuel production is considered, green light does not have to be used for photosynthetic processes. Instead, it can be used in the most energy-intensive downstream processing steps, which potentially turns a negative net energy and CO_2 balance into a positive one.

Conclusion

In conclusion, we have demonstrated for the first time that the photoresponse of *E. gracilis* can be utilized to promote cell aggregation in liquid cultures. Upon irradiation with green light, the cell density of the medium was increased 8.7 fold and the cell collection rate reached 70%. As the light intensity necessary for this photoresponse was 2.5 mW/cm^2, which can be achieved by splitting the solar spectrum, this method has the potential to contribute to the economic feasibility of algae biofuel production using *E. gracilis*. We anticipate that this method can also be applied to other microorganisms that show a similar photoresponse, as long as the external stimulus, such as wavelength and light intensity, is properly applied. Our present experiments also highlighted unique time dependence of the photoresponses, in which cells initially retreated from the light before exhibiting positive photoresponses.

Thus, it will be of great interest to examine the mechanisms of this phenomenon to shorten the irradiation time required for cell aggregation. The investigation of gene expression and signal transduction associated with the eyespot will be of importance to this end.

Experimental

Cell cultivation: *Euglena gracilis* strain Z was provided by Euglena Co., Ltd. and was routinely cultivated until the stationary phase in Cramer-Myers medium in 60-ml test tubes at 29°C under constant illumination by a 20-W fluorescent lamp. The medium was bubbled constantly with 5% CO_2 in air during cultivation.

Photoresponse measurements: Cells were removed from the incubator immediately prior to the photoresponse measurements. Monochromatic light was irradiated horizontally onto a transparent plate containing approx. 15 ml of cell suspension at an OD_{680} of 0.4 (dry cell weight per liter = 0.2 g) unless otherwise stated. The light source was generated with a 300-W Xe lamp (Asahi Spectra, MAX-302) equipped with a UV cut-off filter (L39, transparent at wavelengths larger than 390 nm; Irie Seisakusho) and interference filter to obtain monochromatic light. The light had a half-width of 10-15 nm and projected in a cone-like shape, rather than a straight gradient. A heat filter was not used because the light strength in the IR region was negligible. After the cell suspension was irradiated for 24 h, the light source was turned off and a photograph of the plate was taken.

Optimum light intensity calculation: The light intensity in the region of cell suspensions with the highest cell concentration was measured using a USR 45 Spectro-radiometer

(Ushio). In cases where the light intensity was too high to be measured directly, ND filters (Asahi Spectra) were used. The light diffraction or dispersion at the edge of the plate holding the cell suspension was not accounted for. The light intensity at this point was termed the optimum light intensity (OLI), as it was considered to be the optimal light condition for cell growth.

Cell aggregation measurements: Twenty-milliliter cell suspensions at an OD_{680} of 0.4 (dry cell weight per liter = 0.2 g) were added to 20-ml serum bottles, which were then either irradiated from below at an intensity of 2.5 mW/cm^2, or wrapped in aluminum foil as a control experiment for cells kept in the dark. After 24 h, 0.4-ml samples of the suspensions were taken from the surface layer to a 96-well plate until the entire cell suspension had been collected. The absorption at 680 nm of each well was measured using a plate reader (Tecan Infinite M200 PRO) to estimate the cell concentration in each sample.

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Electronic Supplementary Information (ESI) available:

Fig. S1 Cell amount before/ after light irradiation

Fig. S2 Light intensity at different regions of the petri dish

Fig. S3 Photo-induced cell aggregation at low light intensities

Fig. S4 Effects of pre-irradiation on photo-induced cell aggregation

Fig. S5 Diffused-transmission UV-vis absorption spectrum of *E.gracilis* cells

See DOI:

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Figure Caption

Figure 1. Typical procedure for algae biofuel production. Cell aggregation is needed for the medium exchange and cell harvesting steps.

Figure 2. Cell distribution before and after light irradiation. A: Before light irradiation. B: 24 h after 400 nm irradiation from the left. C: 24 h after 530 nm irradiation from the left. D,E,F: 2,6,and 9 h after 530 nm irradiation, all from the top right. Experiments A-C (OD_{680} = 0.4) and D-F (OD_{680} = 1.0) were conducted at different times, hence the difference in the color of the photograph. However, the general trend remains the same. The light strength was 1.5 mW/cm^2 in all cases. The dark spots observed in the petri dishes of Fig. 2 D-F is due to the local aggregation of the cells.

Figure 3. Determination of the optimum light intensity (OLI). A: Wavelength dependency of the OLI in the visible region. B: Difference in cell distribution according to the intensity of 400 nm light. B-1 to B-4 correspond to light intensities of 1.5, 0.75, 0.33, and 0.15 mW/cm^2, respectively.

Figure 4. Results of 3-D experiments using serum bottles. A: Schematic illustration of the photo-aggregation experiments. B: Photograph of the 96-well plate containing the sampled

liquid. The top 3 rows show samples collected under dark conditions, and the bottom 3 rows are samples of cell suspensions irradiated with green light. Samples were added to plates starting from the top left to the bottom right. C: Cell amount in relation to the sampled amount of liquid from the bottom of the bottle. D: Optical microscopic images of the sampled liquid from the bottle with light. Bottom layer (D-1), top layer (D-2).

Figure 5. Schematic illustration of (A) an absorption spectrum of chlorophyll and (B) optimum light intensity for photo-induced cell aggregation (OLI).

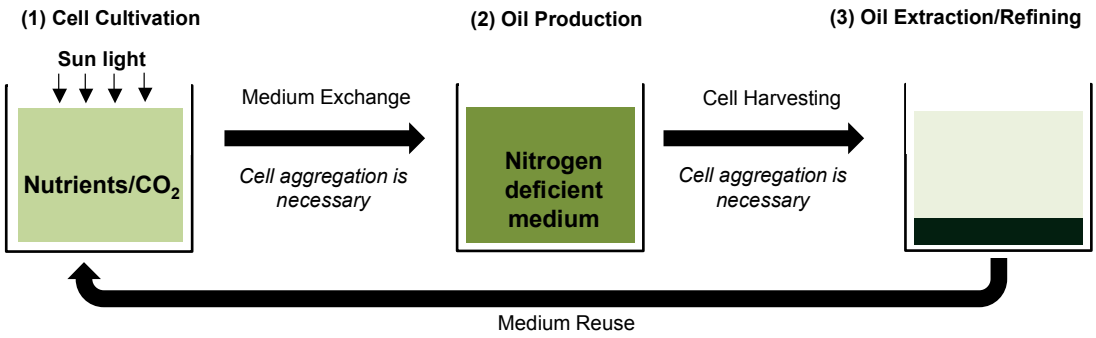


Fig. 1.

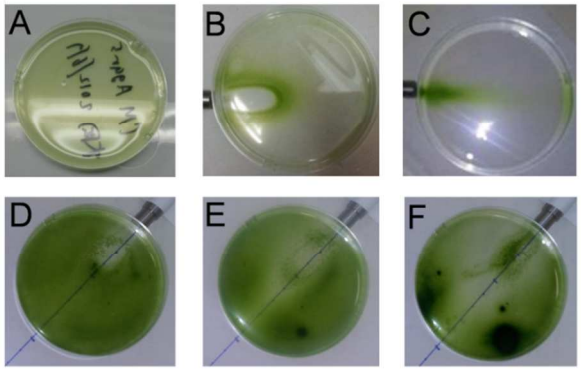
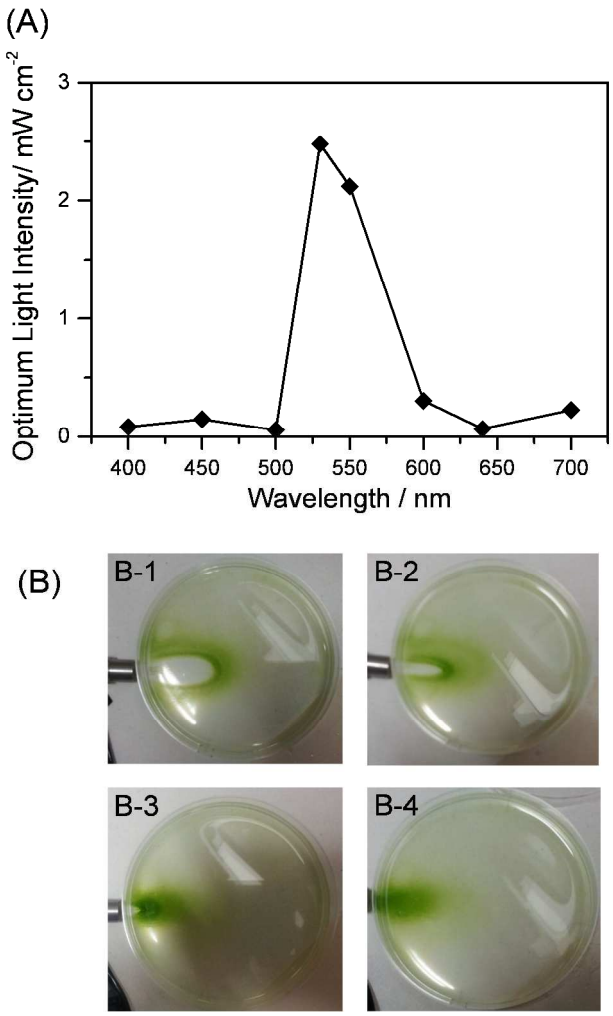


Fig. 2.



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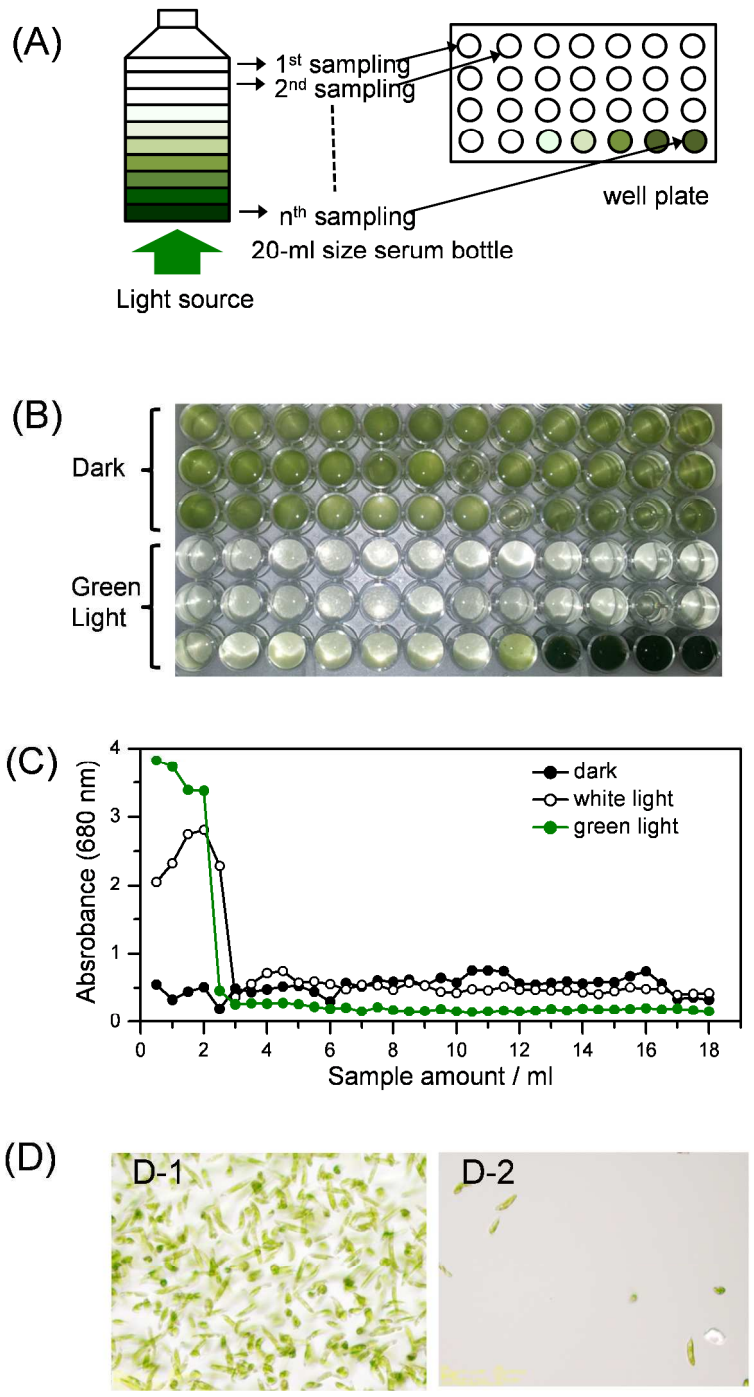

Fig. 4.

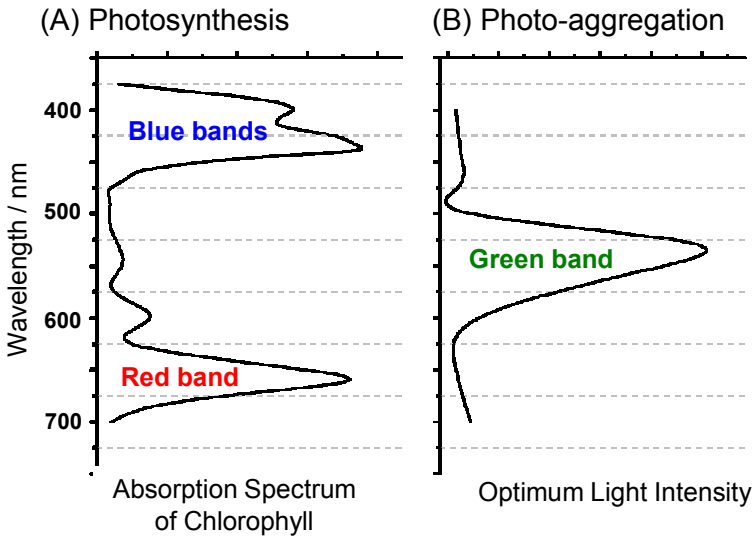

Fig. 5.

Graphical Abstract

Using the photoresponse of the green algae *Euglena gracilis*, we demonstrate a novel and economically feasible method of cell aggregation.