Characterization of the eyespot and hematochrome-like granules of *Euglena gracilis* by scan-free absorbance spectral imaging $A(x, y, \lambda)$ for quantification of carotenoids within the live cells

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Euglena gracilis is an edible photosynthetic single-cell alga that can synthesize carotenoids. It is highly demanded to establish the technology to select and grow individual cells capable of synthesizing more carotenoids because it contributes to safe and inexpensive production of carotenoids. In the cells of *E. gracilis*, carotenoids are mainly contained in chloroplasts and eyespots, and typical carotenoids have a characteristic absorption maximum in common. *E. gracilis* also has an organelle resembling hematochrome, which has an appearance similar to the eyespot and the absorption band spectrally overlapping that of the carotenoid although reportedly it does not contain carotenoids. To discriminate the eyespot and hematochrome-like granules and to investigate the intracellular distribution of carotenoids, scan-free, non-invasive, absorbance spectral imaging $A(x, y, \lambda)$ microscopy of single live cells was applied. It was demonstrated that this technique is a powerful tool not only for basic research on intracellular structural analysis but also for identifying difference in carotenoid content in individual cells applicable to screening of carotenoid-rich cells. By this technique, it was confirmed that carotenoids exist in chloroplasts and eyespots, and a number of characteristic absorption spectra of pigments observed specific to the eyespot or hematochrome-like granules were identified. In addition, it was found that hematochrome-like granules have a characteristic absorption peak at 620 nm as well as at 676 nm, suggesting that its origin is a component of chloroplast including Chlorophyll *a*.

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Abstract

Euglena gracilis is an edible photosynthetic single-cell alga that can synthesize carotenoids. It is highly demanded to establish the technology to select and grow individual cells capable of synthesizing more carotenoids because it contributes to safe and inexpensive production of carotenoids. In the cells of *E. gracilis*, carotenoids are mainly contained in chloroplasts and eyespots, and typical carotenoids have a characteristic absorption maximum in common. *E. gracilis* also has an organelle resembling hematochrome, which has an appearance similar to the eyespot and the absorption band spectrally overlapping that of the carotenoid although reportedly it does not contain carotenoids. To discriminate the eyespot and hematochrome-like granules and to investigate the intracellular distribution of carotenoids, scan-free, non-invasive, absorbance spectral imaging $A(x, y, \lambda)$ microscopy of single live cells was applied. It was demonstrated that this technique is a powerful tool not only for basic research on intracellular structural analysis but also for identifying difference in carotenoid content in individual cells applicable to screening of carotenoid-rich cells. By this technique, it was confirmed that carotenoids exist in chloroplasts and eyespots, and a number of characteristic absorption spectra of pigments observed specific to the eyespot or hematochrome-like granules were identified. In addition, it was found that hematochrome-like granules have a characteristic absorption peak at 620 nm as well as at 676 nm, suggesting that its origin is a component of chloroplast including Chlorophyll *a*.

Keywords: *Euglena*; carotenoid; eyespot; hematochrome; chloroplast; absorbance spectral imaging; photosynthesis; alga; microscopy;

Introduction

E. gracilis is a photosynthetic unicellular flagellate microorganism with a length of approximately 50 μm and a diameter of 8 to 12 μm inhabiting freshwater (*Jerome, 2012*). Depending on nutritional and environmental conditions, *E. gracilis* synthesizes paramylon, a β-1,3-glucan in a crystalline form, which is used as an ingredient of functional food (*Sugiyama et al.,*

2009) or produce wax ester suited for its conversion to biofuel (*Inui et al., 1982*). *E. gracilis* can grow in heterotrophic culture (Koren-Hutner medium (*Koren & Hutner, 1967*)). Also, since the cells contain about 10 chloroplasts (*Yamaji, 1980*), they can photosynthetically grow in autotrophic culture (Cramer-Myers medium (*Cramer & Myers, 1952*)). The success of mass-cultivation of *E. gracilis* has realized the commercial supply of *E. gracilis* as an ingredient of functional foods, cosmetics, and biofuel (*Suzuki, 2017*). Therefore, technology using *E. gracilis*, which enables clean, sustainable, and cost-effective production of energy and food, attracts attention in industrial and academic fields (*Yamada et al., 2016*).

We focus on carotenoid production capacity among many useful properties of *Euglena*. *E. gracilis* is one of the few microorganisms capable of simultaneously producing antioxidant vitamins such as β-carotene and vitamin C (*Takeyama et al., 1997; Kato et al., 2017*). Carotenoids have been used as a coloring and antioxidant for foods from long ago as represented by β-carotene (*Gordon & Bauernfeind, 1982; Hardeep et al., 2015; Takaichi, Mimuro & Tomita, 2006*). Certain carotenoids also have the potential to reduce the risk of cancers and eye diseases (*Johnson, 2002*). Therefore, it is also used as a health supplement and an antioxidant from demand due to recent health boom (*Joanna & Květoslava, 2014*). It is also used as a feed additive for animals (*Delia & Rodriguez-Amaya, 2015*), and also for an active ingredient of cosmetics (*Maxim et al., 2011*). Therefore, a mass production method of carotenoids which is inexpensive and guaranteed safety is a task.

Carotenoids are synthesized by photosynthetic organisms including algae and play important roles in photosynthesis (constituents of light harvesting pigment-protein complexes) and photo protection (non-photochemical quenching, xanthophyll cycle, etc.) (*Barbara, 1990; Havaux, 1998; Hashimoto, Uragami & Cogdell, 2016*). Also, most flagellate green algae exhibiting photo responses have a photoreceptive organelle, eyespot. With the eyespot, they can detect the direction of light source in the environment where they are located (*Ozasa et al., 2014*). It is known that the constituent components of this eyespot are various carotenoids (*Merete et al., 1994*). The eyespot of *E. gracilis* is an orange-red colored distinct structure with a cross section of 2×3 μm^2, located near the front end of the cell with flagella. This organelle is composed of the aggregation of a large number of granules (40 to 50) containing carotenoids with a diameter of 0.1 to 0.3 μm in the cytoplasm directly beneath the cell membrane constituting the boundary of the front end invagination called reservoir (*Mineo, 2007; Jerome, 2012; Jerome, 2013*). (This area is sometimes referred to as stigma. In this paper it is described as eyespot.) Thus, the carotenoid molecules in cells show specific intracellular localization. In order for *Euglena* to receive a modulated light signal during helical swimming, it is believed that the eyespot of *Euglena* is not itself a photoreceptor, unlike many other algae like *Chlamydomonas (Steven & Shigeoka, 2017)*, but it plays a role in casting a shadow on photoreceptor (PAB:paraxonemal body) (*Mineo, 2007; Barsanti et al., 2009; Georg, 2009*).

To improve the value of *E. gracilis* for industrial application, breeding methods for this species are established (*Yamada et al., 2016a; Yamada et al., 2016b*). Fe-ion beam irradiation efficiently induces random mutations and produces a population including various phenotypes of mutants (*Yamada et al., 2016b*). In addition, efficient selective breeding of oil-rich *E. gracilis* strain is demonstrated by isolating the desired cells from the mutant pool by using fluorescence-activated cell sorting (*Yamada et al., 2016a*). To accomplish selective breeding of carotenoid-rich *E. gracilis*, methods to measure the amount and type of carotenoids in individual cells are required. Since the aforementioned selective breeding method does not use gene recombination technology, the produced strains can be easily applicable in the industrial production process.

Conventional chemical measurement methods require a large number of cells to quantify the carotenoid content. Although there is a method of fluorescently staining substances in cells and observing them with a microscope, there is a concern that the staining themselves may affect cells (*Wojcik & Dobrucki, 2008*). Thus, non-invasive microspectrophotometry is a candidate method to solve the above problem (*Benedetti et al., 1976; James et al., 1992; Valter et al., 2007*). In recent years, a microscopic technique using stimulated Raman scattering (SRS) has been developed as a fast and unstained cell measurement technique. This made it possible to visualize lipids, paramylon, chlorophyll, and proteins contained in *E. grarcilis* in a label-free and living state (*Iwata et al., 2017*). In their approach, however, carotenoids are not imaged because the number of spectral points is reduced to four characteristic colors between 2,800 and 3,100 cm^{-1} to increase the frame rate of imaging, although Raman imaging of carotenoids is potentially feasible when the imaging rate and a plurality of types of imaged molecules are not so stringent requirement (*Wakisaka et al., 2016*). For label-free multicolor imaging of plural types of constituent molecules including carotenoids, therefore, it is desired to explore other approaches than SRS as well. One of the promising approaches is the absorption imaging. Recently, various methods of fast spatial imaging of the absorption spectra $A(x, y, \lambda)$, a three-dimensional (3-D) dataset typically called a datacube, are developed (*Matsuoka., 2002; Kester et al., 2011; Hagen et al., 2012; Lee et al., 2012; Hagen & Kudenov., 2013*).

In the present research, we have adopted a non-scanning (snapshot) absorption imaging method for imaging of carotenoids which uses a fiber array (2D-1D fiber bundled array) that converts a two - dimensional (16 pixel × 16 pixel, 0.96 mm × 0.96 mm) image into a one - dimensional (256 pixels, 0.06 mm ×15.36 mm) image fitted to a spectrometer slit. This method is characterized by obtaining spatially resolved absorption spectral image $A(x, y, \lambda)$ of living cells for an extremely short time (~0.05 sec) and with high spatial resolution (about 1 μm). If we can quantify the carotenoids contained in individual cells from the absorption image of the whole one cell, we will be able to efficiently select competent cells.

As a preparatory stage, it is necessary to confirm that the distribution of various pigments present in cells and organelles can be discriminately observed. To test this, we focused on chloroplasts, eyespot and hematochrome, as these intracellular structures are reported to contain carotenoids (*Britton & Goodwin, 2013*). Hematochrome has an appearance resembling an eyespot and is known to be a plastid found in red *Euglena*, such as *Euglena sanguinea*, which changes from green to red when irradiated with intense light. It is reported that granules similar to hematochrome are also observed in *E. gracilis* (*STROTHER & WOLKEN, 1961; Dennis, 1982*), and we will refer to it in this paper as hematochrome. The hematochromes appear in *E. gracilis* cells in old culture (*Jerome, 2012*) and is suggested not to include carotenoids (*Britton & Goodwin, 2013*). Since hematochrome was also found in the mutant that cannot synthesize carotenoid and the cell cultured in a medium to which the carotenoid synthesis inhibitor was added, its origin is considered to be different from that of an eyespot, but details are unknown (*Britton & Goodwin, 2013*).

In this paper, absorption spectra inside and around these intracellular structures are measured to be compared and examined using the non-invasive and high spatial resolution absorption imaging method.

Experimental Methods

1. Sample Preparation

The wild type *E. gracilis* (Z strain) was cultured in CM medium (Cramer-Myers medium (*Cramer & Myers, 1952*), pH 3.5) for 4 weeks. Cells were kept aerobically under continuous illumination (cool white fluorescent light : 40 [μmol / m^2 / sec]) and constant temperature (26°C).

Cells were harvested by centrifugation (6000 rpm, 5 min, 4°C), then washed with purified water twice and re-suspended in purified water. This suspension of 1mL was added to 5 mL of fresh CM medium (total 6 mL). The initial cell density at this time is 1.2×10^6 [cell / mL]. This culture was kept in the glass tube with the cap sealed for 3 days under continuous illumination (cool white fluorescent light : 100 [μmol / m^2 / sec]) and constant temperature (26°C).

2. Measurement Methods

Detailed methods of scan-free absorbance spectral imaging are described in Ref. (*Isono et al., 2015*). Here, we made the major improvement as follows:

Previously (*Isono et al., 2015*), we introduced a fiber bundled array to convert a 2-dimensional image to the 1-dimensional slit image. A 50-μm core/55-μm clad/60-μm coating silica fibers are assembled to 16×16 (0.96×0.96 mm^2) array on the input side and to 1×256 (0.06 × 15.36 mm^2) array on the output (slit) side. On the side port of the microscope, the ×100 magnified image of the sample was focused on the 16×16 2D array ($x \times y$), which is rearranged in order into the 1D array to fit the entrance slit of the spectrometer. Thus, in principle, 256 spectra can be simultaneously obtained on a charge-coupled device (CCD) camera through the spectrometer for a single exposure time. Previously, however, the vertical CCD size was only 6.4 mm such that three or four times measurement is needed by shifting the 1D-array on the slit vertically.

In this paper, we have realized scan-free (snapshot) absorbance spectral imaging in the true sense of the word, by replacing both spectrometer and CCD as follows. An imaging spectrometer (MS3504i or SL100M, SOLAR TII) was replaced by that with a larger imaging area of 27 × 27 mm^2 (f=32 cm, IsoPlaneSCT-Advance, Roper Scientific). An electrically-cooled EM-CCD camera (1600 × 400 pixels with 16 μm pixel size, DU971N-UVB, Newton, Andor) was replaced by an electrically-cooled CCD camera (2048 × 2048 pixels with 13.5 μm pixel size, PIXIS2048BUV, Roper Scientific). The vertical size of both the imaging area (27mm) and the CCD (27.65mm) covers that (15.36 mm) of the 1D array of the 2D-1D converter, so that the whole 3D image $A(x, y, \lambda)$, a datacube of 16×16×2048, is obtained simultaneously without need for the vertical scan. To be precise, the vertical length in the imaging area where an aberration-corrected image is obtained is limited by 14 mm, so that the images from 22-23 fibers at both ends of the 1D array are not aberration-free. The shortest acquisition time is 0.05 second which is limited by the response time of the mechanical shutter of the spectrometer.

A medium diluted to the appropriate cell concentration for observation with purified water was covered with a cover glass on the glass bottom dish (Matsunami glass D11130H). The sample was set on an inverted microscope (IX71, OLYMPUS) and observed with a ×100 objective lens of NA 0.85 from below. The light source was a 150 W Xenon lamp (Hamamatsu) to illuminate a region of 2 mm in diameter of the sample from above through a condenser. The intensity (photon flux density) on the sample was 3450 μmol / m^2 /sec for 0.5 second exposure. The transmitted light was transferred through the objective and a focusing lens to the side port of the microscope. The absorbance of the sample was calculated assuming that the transmitted light intensity in the region where only the culture solution exists is 100%. The spectrometer has automatically exchangeable three gratings, 1200, 300, and 150 grooves / 300 nm blaze. We used the 150 grooves grating with wavelength resolution of 1.1 nm at 546 nm (50 μm slit width, determined by the core size of the

fiber) and wavelength span from 390 to 790 nm. All the measurements were performed at room temperature.

Results

We observed the microscope images of more than 100 cells, among which we selected a specific cell which was slow in movement and where the eyespot and a hematochrome-like granule (we call this hematochrome hereafter) were accidentally located close to each other and not overlapped with a chloroplast. Figure 1 shows a bright-field microscopic image of that cell and Figure 2 shows the absorbance spectral images A(x,y,488 nm) of the eyespot and hematochrome in the enlarged view. Figures 3 and 4 show the local absorbance spectra of the eyespot and hematochrome at the positions labeled in Figs. 2(b) and 2(c), respectively.

1. Absorption Image at the Wavelength of the Maximum Absorbance of Carotenoids (Figure 2)

The data show that absorption is found in hematochrome, chloroplast and eyespot regions in the absorption image at the carotenoid absorbance maximum wavelength 488 nm. Although hematochrome of *E. gracilis* does not contain carotenoids, absorption at this wavelength is large. Therefore, it was suggested that it may be an error factor for imaging the distribution of carotenoids in the absorption image of the whole cell.

2 Absorption Spectra of Eyespot (Figure 3)

The bright-field microscopic image of the eyespot shows that various warm colors of yellow, orange, and pale red are distributed variously in the eyespot. Depending on slight changes in the depth of focus, the colors of eyespot varied diversely. It is observed that the area near the boundary of the eyespot is pale red and the interior of it shows yellow or orange (Figure 1). The absorption spectra near the center of the eyespot has a broad absorption maximum in the region of 420 to 510 nm, which are consistent with the literature (*James et al., 1992; Jerome, 2012*).

(a) E is an absorption spectrum of the chloroplast adjacent to the eyespot which has an absorption peaks derived from Chlorophyll *a* (Chl-*a*) at 436 and 676 nm and a peak derived from carotenoids at 488 nm. G is a chloroplast spectra existing at a location distant from the eyespot and has a spectrum resembling the spectrum of E and each absorption peak is at about the same wavelength. G has a higher absorbance at the absorption peak of Chl-*a*, which suggests that G has constituents of chloroplasts present at higher density than E. In addition, comparing the increase ratio of absorbance at the absorption peak of carotenoids with the Chl-*a* of G against E, the ratio of increase was larger for the carotenoids. The ratio of increase in absorbance in the wavelength other than the periphery of the absorption peak was also larger than that of Chl-*a*. Particularly, from 600 nm to 520 nm, the absorbance of the spectrum of E decreases toward shorter wavelengths, but the spectrum is nearly flat in the region corresponding to it. This suggests that the presence of accessory pigment (carotenoids) is greater in places where chloroplasts are dense, and that the light harvesting system is functioning more efficiently. For carotenoids, it is suggested that G has a higher density of carotenoids by comparing the

absorption maximum at 488 nm. F shows the absorption spectrum near the border of the chloroplast dense area, which has a single absorption peak characteristic at 405 nm.

(b) The spectrum of the outline of the eyespot is shown. Unlike the general spectrum of the eyespot, the spectrum in the region A1 to A7 has a single absorption maximum at 516 nm. Corresponding to the bright-field microscopic image, it shows that this region is pale red. On the other hand, since the spectrum in the region from A8 to A9 shows a typical spectrum of the eyespot, it is suggested that the composition of eyespot's pigments is changed greatly with the boundary between A7 and A8 as the border. Among the carotenoids contained in the eyespot of E. gracilis, canthaxanthin (467) and echinenone (458) are known to have a single absorption maximum (Numbers in parentheses indicate wavelengths in diethyl ether (*Heelis et al., 1979*)). Although the absorption spectrum of the pigments extracted from purple cabbage at pH 3.2 most closely agrees with the spectrum and the color, a report that the eyespot contains anthocyanin has not been found. (cf. The pH of the culture solution in this experiment is 3.5.)

(c) From the boundary of the eyespot toward the center, the absorption maximum wavelength of the spectra similar to (b, A1 to A7) gradually shift to a shorter wavelength, and the shapes of the maxima becomes broader. By correspondence with the bright-field microscopic image, it is indicated that the vicinity of the boundary of the eyespot is pale red and the vicinity of the center is yellow. Therefore, it is suggested that the spectrum having this single absorption maximum is a pigment with pale red. In addition, since each slope on the longer wavelength side of the maximum of the spectrum (B1 to B8) is smoothly connected to each other, this pigment was suggested to be related to the main carotenoids constituting the eyespot.

(d) The data shows the spectra of a typical eyespot. This shows that the absorption decreases from the center of the eyespot towards the boundary while the shapes of the spectra remains almost the same.

(e), (f) In the vicinity of the center of the eyespot, a typical absorption spectra of the eyespot are shown, but in the region of 410 to 540 nm, the absorbance of the spectrum of (e) increases toward longer wavelengths and that of (f) decreases toward longer wavelengths.

(g), (h) The data shows that Chl-*a* is present in these regions since all the spectra have peaks at about 676 nm. Since the characteristic absorption of carotenoids is shown in the spectra of D3 to D8, it is suggested that a greater amount of carotenoids is contained than that contained in chloroplasts. In the region of D9 to D13, it is considered that the carotenoids are contained in a slightly larger amount than that contained in the chloroplast. This is because in these spectra the absorption maximum of carotenoids at 490 nm is large relative to that of the carotenoid free spectrum (D22). These graphs show that as a result of the absorption of carotenoids, the absorbance of the spectra increase toward shorter wavelengths. However, since it does not show the shapes of the spectra overlapping with the spectra of the typical eyespot, there may be carotenoids of a different type from eyespot, or in a different form.

3 Absorption Spectra of Hematochrome-like Granule (Figure 4)

The absorption spectrum obtained when hematochrome is observed with the focus on the coloring of warm color like eyespot has a basic structure like the Chl-*a* spectrum with an additional characteristic peak at 620 nm. Every time the same hematochrome is measured,

different spectra are obtained. They generally show spectra with (C 10, C 11, D 9, D 10, etc) in Figures (e)~(l).

Since the hematochrome which is observed is located in the region without chloroplasts, it is considered that there is no contribution by absorption of Chl-*a*. In addition, although hematochrome does not necessarily appear in a specific area, the hematochrome of present observation is located near the eyespot, so that it was adopted for easy comparison with the eyespot (Figure 2. (a), (c)).

(a) The data shows typical absorption spectrum of carotenoids contained in the eyespot. Therefore, the absorption region in the vicinity is the eyespot. (Figure 2. (B)).

(b) The data shows the absorption spectrum in the region slightly away from the hematochrome. This shows the same spectrum as the region exhibiting vivid green color in the vicinity of the eyespot (Figure 3. (A). E).

(c) The data shows the absorption spectra of the boundary of hematochrome. They are spectra similar to Chl-*a* and have an absorbance offset over the entire wavelength range, which is consistent with showing dark green color in bright-field microscopic image.

(d) The data shows the spectra showing the dark green regions at the hematochrome boundary. They have clearer minor peaks at 490, 580, 625, and 715 nm in addition to the major peaks at 676 nm and 440 nm characteristic of the absorption spectrum of Chl-*a* compared to (e). Therefore, they are clearly different spectra from the absorption spectrum of the chloroplast.

(f) The data show that the absorbance above 730 nm for the absorption peak at 676 nm increases from the center of hematochrome toward the boundary. The largest spectral change was observed between the C12 and C13. This is considered to correspond to the boundary between the dark red (C12) and dark green (C13) regions of the bright field observation image (Figure 2. (A), (c)).

(k) The spectra show that the absorbance of hematochrome at 620 nm increases from the boundary to the center (F1 to F3). However, the absorbance at 620 nm stops increasing at a certain value (Absorbance = 0.17), whereas the absorbance increases at the range of 400 to 600 nm and 650 to 730 nm (F4 to F12). As a result, since the absorbance in the region of 580 to 650 nm and 730 nm or more becomes relatively small, it is expected to exhibit warm color. In fact, the bright-field microscopic image of hematochrome in this region shows that it is warm color. Spectra (Figures i, j, l) in the peripheral region of F1 to F10 also exhibit absorption spectra similar to them. The fact that the absorbance in the entire spectrum shifts largely upward towards the center of hematochrome and that the difference between the absorbance at 620 nm and 676 nm decreases is consistent with the fact that this region is dark red in bright-field microscopic image. On the other hand, the spectrum in Fig. (k) has a small upward shift and the absorbance at 600 to 650 nm is small, suggesting that it shows a bright warm color.

(m), (n) Spectra with the shape similar to the absorption spectrum of the chloroplast was obtained. This suggests that there are chloroplasts nearby, so influenced by them.

Discussion

In the eyespot and hematochrome, various absorption spectra were obtained depending on the location, while almost the same spectra are obtained in the green region of *E. gracilis* where the chloroplast exists. These results indicate that the eyespot and the hematochrome are characteristic intracellular structures recognizable by microspectrophotometry. In particular, since hematochrome resembles an eyespot in appearance and its absorption spectrum resembles a chloroplast, so that attention must be paid when measuring the absorption by microspectrophotometry of the eyespot where chloroplasts are present in the surroundings. Actually, there is a report that seems to mistake a hematochrome for the eyespot, and the spectrum of the eyespot indicated in that report was very similar to the spectrum of hematochrome in the study (*Werner et al, 1990*).

There is also a report that various spectra were obtained for each measurement at the same eyespot by microspectrophotometry (*Jerome, 2012*). This was considered to be caused by the change in the focus at every measurement and the change in the position and distribution of the carotenoid granules contained in the eyespot everytime the cell shape deforms by the euglena movement, since the granules are not composed of a rigid structure (*Benedetti et al., 1976*) and are irregular sequences and the granules are cytoplasmic side organelles bound to the cell membrane (*Kivic & Vesk, 1972*).

It is considered to be difficult to identify each carotenoid constituting the eyespot from the spectra of the eyespot obtained by microspectrophotometry because the absorption bands of each carotenoid are almost the same (*Jerome, 2012*). There are two reports that carotenoids contained in the eyespot or in the whole cells of *E. gracilis* were chemically analyzed. Each showed different types of carotenoids and their content ratios (*Norman & Timothy, 1960; Heelis et al., 1979*). Although we could not identify carotenoids contained in each intracellular structure in this study, we could confirm the presence or absence of pigments with characteristic spectra.

Although the origin of hematochrome has not yet been elucidated, there is a hypothesis that its origin is an eyespot (*Jerome, 2012*). But the absorption spectra in the regions where the absorption of the hematochrome is large have an absorption peak at 676 nm characteristic to Chl-*a*, suggesting that its origin may be chloroplast. In addition, many spectra of hematochrome obtained had large absorption in the absorption band of carotenoids although there is a report that hematochrome does not contain carotenoids (*Britton & Goodwin, 2013*). If this is true, it is necessary to exclude the region of hematochrome when analyzing the absorption image of carotenoids contained in the whole cells, because the absorption of hematochrome is large at 488 nm which is the absorption maximum of the carotenoids (Figure 2. (C)). However, since the chloroplast contains carotenoids, this study alone cannot prove the hypothesis that the origin is chloroplast. Therefore, it is suggested that another approach such as chemical analysis targeting only hematochrome is required as a future task.

Conclusion and Prospects

The present measurement method enabled us to simultaneously and comprehensively obtain absorbance information of various microscopic regions of an organelle in a single measurement. By contrast, the conventional methods allow the cell to move or the pigments to bleach owing to a long measurement time.

The absorption spectrum of hematochrome of *E. gracilis* showed a distinctly different shape from the spectrum of the eyespot, characterized by a peak at 620 nm and that at 676 nm coinciding with the absorption peak of Chl-*a*. This indicates that hematochrome is an organelle composed of a pigment different from the eyespot. This distinction made it clear that this technique can be used for fundamental research on intracellular structural analysis and applied research for screening of carotenoids-rich competent cells.

As a next step, we would like to pursue the conditions to grow the cells which contain more carotenoids by selecting such cells to have the higher maximum absorbance values of carotenoids than the average: The average is determined from the distribution of the maximum carotenoids' absorbance of individual cells, which is extracted from the absorbance spectral image of each whole cell excluding the regions occupied by hematochromes.

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Figure 1

(a) A bright-field microscopic image of *E. gracilis*. (b) Enlarged view near the eyespot. (c) The colors of the eyespot and hematochrome-like granule are changed as the depth of focus is slightly changed.

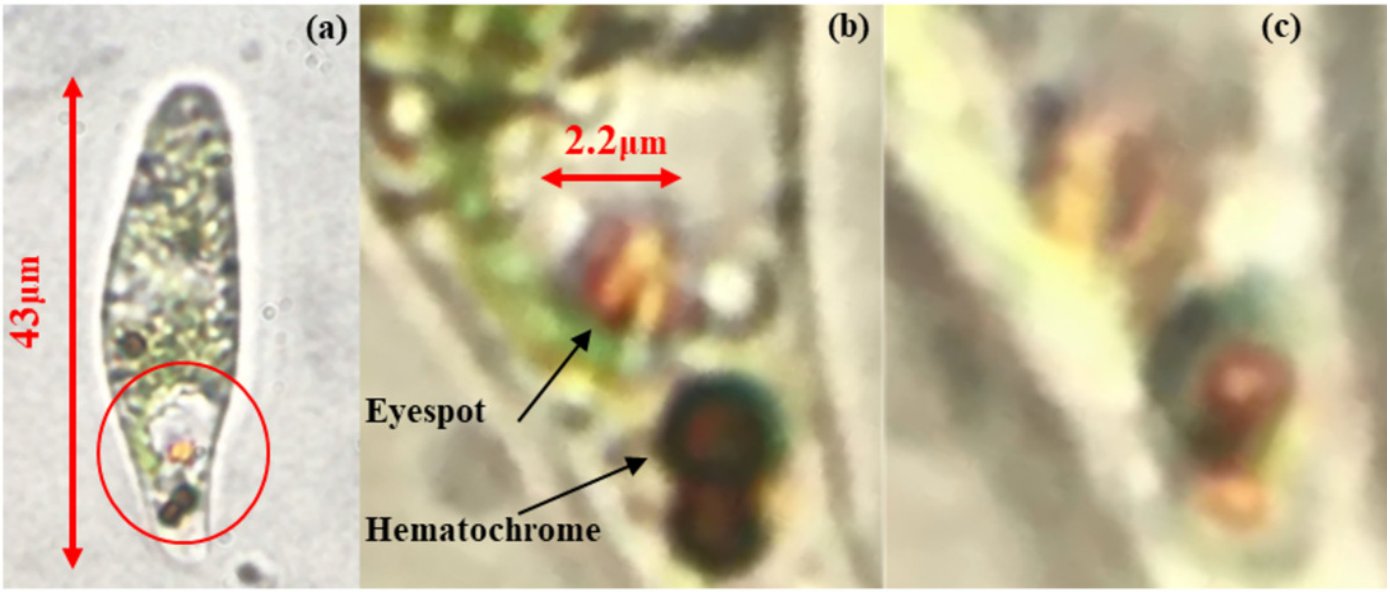

Figure 2

(a) A bright-field microscopic image of *E. gracilis*. (b) Absorption image (at 488 nm) inside the eyespot and in the surrounding area. (c) Absorption image (at 488 nm) inside the hematochrome-like granule and in the surrounding area. Du

Note: The absorption maximum wavelength of carotenoid is assumed to be 488 nm.

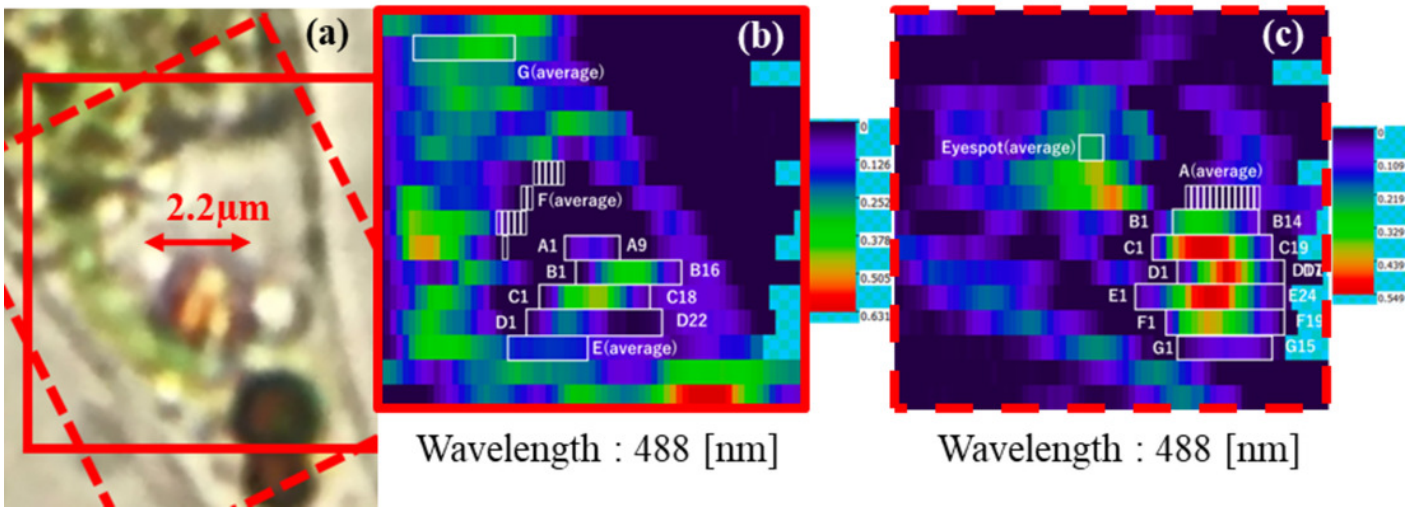

Figure 3

.Absorption spectra of the eyespot of *E. gracilis*.

(Each spectrum corresponds to the labeled position in Figure 2. (b).)

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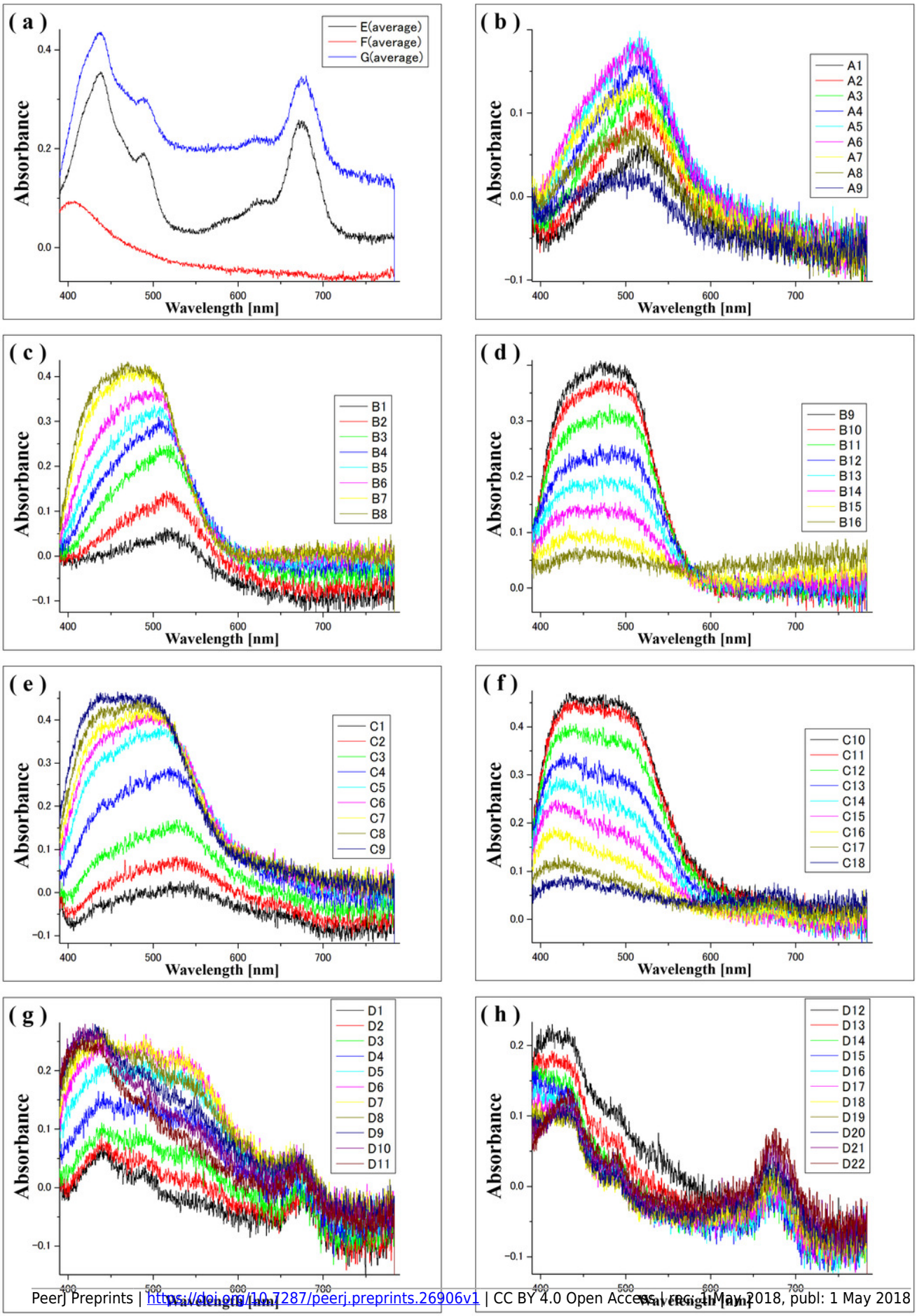

Figure 4

Absorption spectra of the hematochrome-like granule of *E. gracilis*.

(Each spectrum corresponds to the labeled position in Figure 2. (c).)

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