

TWO-DIMENSIONAL AND THREE-DIMENSIONAL RAMAN SPECTROSCOPIC IMAGING OF *Spirulina platensis* CELLS**

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Two-dimensional (2D) and 3D Raman spectroscopic imaging of *Spirulina platensis* was carried out to investigate its applicability in studying the chemical distribution and morphology of algal cells. The *Spirulina* trichome was easily damaged by laser illumination unless the laser parameters were properly adjusted. Raman spectra exhibited an evident sign of fluorescence, which changed during cumulative laser illumination on the same spot. Six peaks were selected from the spectra for chemical mapping, which showed distinct cellular features, including the central and peripheral parts of the cytoplasm, cell wall, sheath, and medium residues. Possible chemicals/organelles in these parts and mechanisms were discussed.

Keywords: *Spirulina platensis*, Raman spectroscopy, chemical imaging, cell morphology.

ДВУХМЕРНАЯ И ТРЕХМЕРНАЯ СПЕКТРОСКОПИЯ КОМБИНАЦИОННОГО РАССЕЯНИЯ СВЕТА КЛЕТОК *Spirulina platensis*

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Двухмерная (2D) и трехмерная (3D) спектроскопия комбинационного рассеяния света использована для изучения химического распределения и морфологии клеток водорослей *Spirulina platensis*. Трихомы *Spirulina platensis* легко повреждаются лазерным излучением, если параметры лазера не отрегулированы. Спектры комбинационного рассеяния света имеют признаки флуоресценции, которая меняется при кумулятивном лазерном освещении на том же пятне. Из спектров для химического картирования отобраны шесть пиков, характеризующих различные клеточные особенности, включая центральную и периферическую части цитоплазмы, клеточную стенку, оболочку и остатки среды. Обсуждаются возможные химические вещества/органеллы в этих частях и механизмы.

Ключевые слова: *Spirulina platensis*, спектроскопия комбинационного рассеяния света, химическая визуализация, морфология клеток.

Introduction. *Spirulina*, which belongs to the ancient group of cyanobacteria that existed on earth since the beginning of life, has nowadays become one of the most well-known and versatile algae to mankind. Its most prominent feature is the high content of protein, occupying 50–70% of its total dry weight and including all the necessary amino acids [1], which makes it an ideal plant protein source for human beings. In addi-

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tion, *Spirulina* contains a great variety of other prophylactic and pharmaceutical nutrients, such as vitamins, minerals, trace elements, fatty acids, antioxidants, carotenoids, and several bioactive compounds, which remain to be investigated [2]. Given its high nutritional value, *Spirulina* was awarded various titles, such as “Mankind’s best health product,” “Most ideal food for tomorrow,” and “One of the best protein sources” [3], and it has found its great spectrum of applications in food and supplements [4], animal feed [5], biofuel [6], cosmetics [7], wastewater treatment [8], pharmaceuticals [9], bacteriostat [10], and so on.

To maximize the production of this valuable biotic resource, scientists have carried out numerous studies to investigate the influence of cultivation conditions, such as illumination, temperature, pH, nutrient medium, carbon source, and so on, on *Spirulina* growth [11]. As a result, how *Spirulina* will change in different environments and the corresponding underlying mechanisms must be studied. Numerous biochemical properties of *Spirulina*, including optical density, quantum yield, fatty acids, pigments, and antioxidants, have been measured to monitor its growth status [12–14] – specifically, the cellular morphology and sub-cellular structural alternations have been recorded in response to environmental changes in light intensity, ultraviolet irradiance, temperature, nutrient concentration, and so on [15–19]. However, the biochemical property measurements usually used culture medium or filtered *Spirulina* mass as samples, during which individual cell information was lost. Although transmission electron microscopy (TEM) can visualize the structural changes in *Spirulina* cells during cultivation, its sample pretreatment process is relatively troublesome, and it cannot offer the chemical information of cells. For further understanding of the essential principle of *Spirulina* growth, the in-situ chemical imaging information of *Spirulina* cells, which has rarely been investigated to date, must be acquired.

Among various chemical imaging techniques, Raman spectroscopic imaging, which uses light scattering to probe the chemical composition of a sample, has attracted great attention and prevailed in a great number of research areas, including forensic judging [20, 21], drug differentiation [22], pesticide movement [23], chemical mapping of macroscopic plants [24], microscopic cells [25, 26], and so on. This method is famed for its noninvasive, nondestructive, water-compatible features and its minimum sample pretreatment, easy operation, and so on [27], which make it especially suitable for the chemical mapping of biological living cells. A number of studies have been carried out to investigate the spectroscopic and two-dimensional (2D) distribution features of bioactive content in algae; however, a 3D imaging study of *Spirulina* cells is still scarce [28–30]. Considering all of its analytical merits, Raman spectroscopic imaging should be a viable tool to obtain the chemical mapping information of *Spirulina* cells.

We investigated the applicability of the Raman imaging technique to acquire the chemical distribution information of *S. platensis* cells. Alternations of cell morphology and Raman spectra were observed under different laser illumination intensities and durations. The 2D and 3D spectroscopic images of *Spirulina* cells were obtained, showing different cell structures. Possible chemical substances in these structures were also discussed.

Materials and methods. NaHCO_3 , NaNO_3 , NaCl , K_2HPO_4 , K_2SO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, EDTA, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 2\text{H}_2\text{O}$, H_3BO_3 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ were bought from Xilong Chemical Co., Ltd. (analytical grade, Guangdong, China), and primary water was prepared by a water purifier (UPH-II-20T, Sichuan YOUPU Ultrapure Technology Co., Ltd., China).

The *S. platensis* was obtained from the Soil and Fertilizer Institute of the Chinese Academy of Agricultural Sciences and carefully maintained in a medium that was slightly different from Zarrouk’s Medium [3], the composition of which is shown in Table 1. The medium was sterilized before use, loaded in a 2.5-L flask, and subjected to intermittent illumination of 14 h:10 h light/dark cycle at $80 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

TABLE 1. Concentration of Various Nutrients in the *Spirulina* Cultivation Medium Used in this Study

Composites	Concentration, g/L	Composites	Concentration, g/L
NaHCO_3	10.00	$\text{FeSO}_4 \cdot 2\text{H}_2\text{O}$	0.02
K_2HPO_4	0.50	H_3BO_3	2.80
NaNO_3	2.50	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.80
K_2SO_4	1.00	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.22
NaCl	0.50	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.05
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.20	$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	4.4
EDTA	0.04	$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.01
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.02		

Given that the *S. platensis* trichomes tend to aggregate, the medium liquid retrieved as a sample should be diluted before instrumental measurement if a single trichome was to be analyzed. Meanwhile, dilution can decrease the concentration of nutrients and other substances in the medium and, hence, reduce crystallization when the water surrounding the trichome evaporates. In this study, a 10- μ L medium was pipetted from the flask and diluted to a 5-mL centrifuge tube with primary water to a total volume of 1 mL. After mixing by simple shaking of the centrifuge tube, several drops were pipetted onto a glass slide for rough examination of the presence of *Spirulina* trichome by a normal optical microscope (US3, Shenzhen Aikexue Digital Technology Co., Ltd., China). A circular mark was made by a marker pen around the drop, in which a single *Spirulina* trichome existed, to record its location on the slide. Afterwards, the slide was delivered into a confocal Raman microscope (DXR, Thermo Fisher Scientific, USA) for Raman spectroscopic analysis.

The instrument was carefully aligned and calibrated before measurement. When the glass slide was fixed onto the moving stage in the instrument, the 10 $\times$ objective was first used to locate the *Spirulina* trichome of interest, and the 100 $\times$ objective was ultimately utilized to maximize the imaging resolution. For 2D imaging, the area of interest was selected and scanned with a pre-set step size. For 3D imaging, successive mappings were carried out by keeping the *x-y* area constant and changing the *z* position quantitatively via the software. The spectrum data of each sampled point were recorded for further processing. The instrumental parameters during Raman spectroscopic analysis measurement are follows: laser wavelength 633 nm, laser power 0.1 W, estimated spot size 0.7 μ m, aperture 25- μ m pinhole, grating 600 lines/mm, spectrum range 50–3500 cm^{-1} , collect exposure time 0.1 s, and sample exposures 2 times.

All the spectrum data were processed via a self-developed LabVIEW program to construct 3D and 4D data (including the spatial positioning coordinates and spectroscopic data of a particular Raman shift) for 2D and 3D chemical mapping via Surfer 9 and Voxler 3 (Golden Software Inc.), respectively.

Results and discussion. Given that the study of Raman spectroscopic study of *S. platensis* has been seldomly carried out to date, we first investigated the *Spirulina* and its Raman spectrum after laser excitation. Figure 1 shows the morphology of *Spirulina* under laser illumination of different powers and lasting times. The transparent solid substance surrounding the trichomes was originally the nutrient salts and other chemicals in the culture medium which crystallized when the water evaporated during the sample preparation process. The cells were easily altered by laser excitation. Figure 1b shows that the trichome was seriously

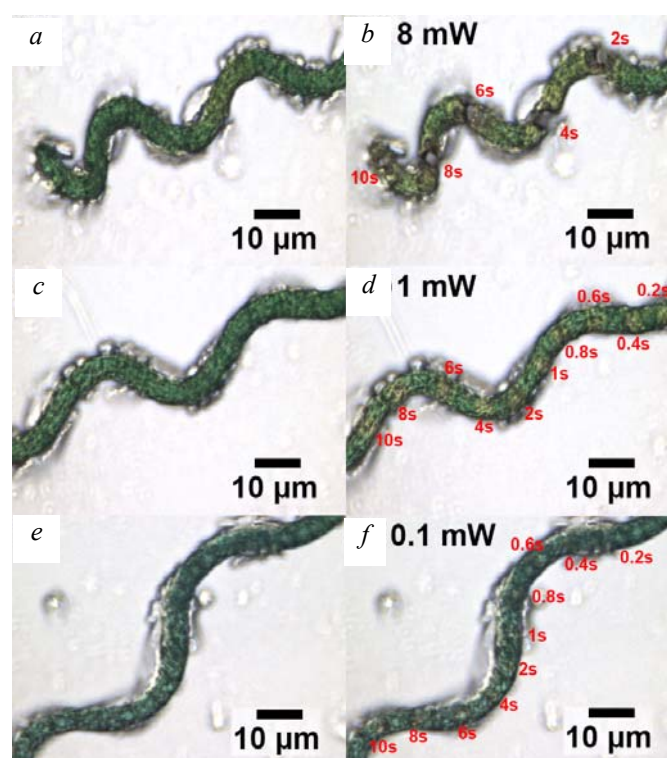


Fig. 1. Morphological changes in a trichome segment under different irradiance powers and durations: (a) and (b) 8 mW, 2–10 s; (c) and (d) 1 mW, 0.2–10 s; (e) and (f) 0.1 mW, 0.2–10 s.

ablated under 8-mW laser illumination. The area illuminated was completely penetrated by the laser, with the green color of the surrounding part having turned shallower. Considering the estimated laser focal spot diameter of $0.7\ \mu\text{m}$, the laser irradiance can be as high as $2.08 \times 10^6\ \text{W} \cdot \text{cm}^{-2}$, which was high enough to reach the ablation threshold [31], especially when using a continuous laser. In addition, the ablation hole was considerably larger than the estimated laser spot size. Studies have shown that lasers with a pulse width longer than the “thermal relaxation time” of the sample, which is typically lower than 1 ms, will result in a wider thermal injury zone than the focal spot size [32], which may be the explanation for the phenomenon observed here. To further explore the suitable laser power, we lowered the laser power down to 1 mW and obtained the result shown in Fig. 1d. Still, under the shortest illumination time of 0.2 s, the trichome was damaged, as indicated by the yellow regions that were originally green. The total illumination time cannot be adjusted any lower than 0.2 s because of the instrumental settings (a minimum of 0.1 s collects the exposure time and two sample exposures). Consequently, we adjusted the laser power to the lowest settable level of 0.1 mW and investigated the effect. As shown in Fig. 1f, the trichome again showed slight signs of damage after more than 1 s of illumination. However, when the illumination time was shorter than 1 s, no evident morphological change occurred on the trichome. This phenomenon implied that a cumulative damage effect occurred during the laser excitation process, and no more than 1 s of total exposure time should be applied to the *Spirulina* cell. To minimize this “memory effect,” we selected the lowest option of 0.2 s for the acquisition of Raman spectra in this study.

In addition to the morphological condition, the Raman spectrum of *S. platensis* was investigated to determine how it will change under different laser conditions on the same sampling spots (the sampling points were selected to be the innermost part of cells). As depicted in Fig. 2, all the peaks were wide and strong, which indicated that they were more related to the fluorescent feature of substances in *S. platensis*. Under 0.1-mW laser illumination, the spectrum peaked at about 50 (peak 1), 3500 (peak 2), and $2650\ \text{cm}^{-1}$ (peak 3) in the first 0.2 s. When the illumination lasted for 1 s, peak 1 blue-shifted, its signal decreased, and the latter two peaks disappeared. Then, two new peaks emerged at about $2360\ \text{cm}^{-1}$ (peak 4) and $770\ \text{cm}^{-1}$ (peak 5). With the ongoing laser illumination, peak 1 continued to blue-shift, and its signal declined until its disappearance; peak 5 slightly red-shifted and stabilized at about $640\ \text{cm}^{-1}$; peak 4 underwent a long red-shift together with the decrease in its signal and finally stabilized at about $1430\ \text{cm}^{-1}$, which may belong to a new group of substances and will be further verified in the next section (peak 6). On the other hand, in the first 0.2 s of 1-mW laser illumination, the same three peaks, namely, 50 (peak 1'), 3500 (peak 2'), and $2770\ \text{cm}^{-1}$ (peak 3'), occurred. Afterward, these peaks quickly disappeared together with the advent of $2290\ \text{cm}^{-1}$ (peak 4') and $640\ \text{cm}^{-1}$ (peak 5') in 1 s, the same as those observed under 0.1-mW laser illumination. In contrast, as the illumination progressed, peak 5' blue-shifted quickly while its signal increased, peak 4' red-shifted, and its signal decreased considerably faster than that under the 0.1-mW laser illumination. The two peaks converged and stabilized at about $1300\ \text{cm}^{-1}$ (peak 6').

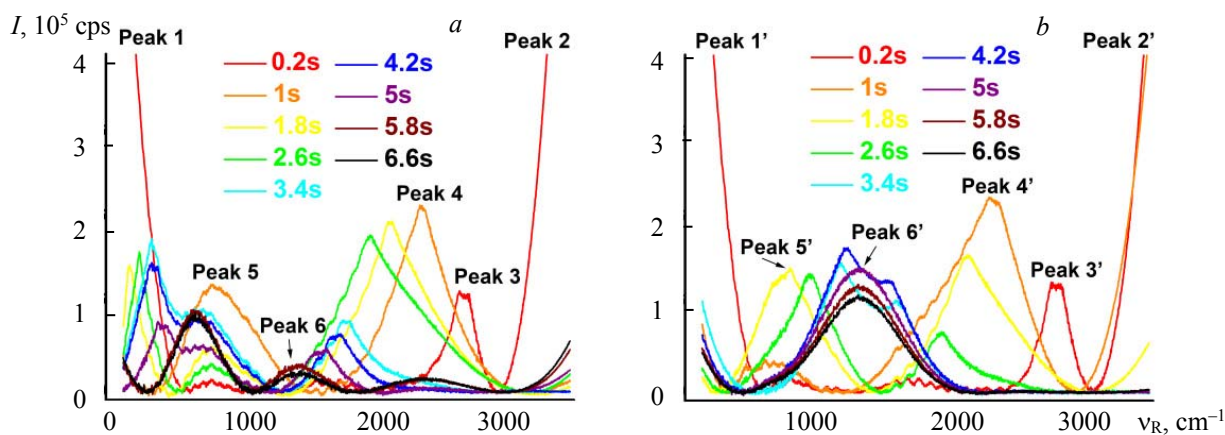


Fig. 2. Distinct Raman spectra under different laser irradiance powers and durations: (a) 0.1 mW, 0.2–6.6 s; (b) 1 mW, 0.2–6.6 s.

Two reasons may be accounted for this alternation. First, the cumulative laser irradiances altered the trichome morphology (Fig. 1f), probably causing the irradiated cell to shrink due to water content evaporation. This condition will result in changes in the sampled position from the core of the cell to the outer part, leading to the disappearance of peaks representing substances in the cytoplasm (peaks 1–3) and the emergence of peaks of the chemicals in the outer layer of the cell or the crystal (peaks 4–6). Second, the relatively intense laser irradiance will decompose or change the structure of certain chemicals, causing the red- and blue-shifting of the peaks. Peaks 1' and 5' shifted and diminished notably faster than peaks 1 and 5, which implied that these chemicals might have decomposed during laser irradiance, which was accelerated by one order of magnitude stronger laser illumination.

As discussed earlier, the Raman peaks of *S. platensis* were wide and shifted during laser illumination. Figure 3a shows the selected spectra during the 2D imaging of a cross-section of a cell. The peaks were “moving around” and greatly overlapped with each other. To construct 2D and 3D imaging datasets, we determined the “right” peak positions from which the signal data can be extracted. Our solution was to sum the spectra from all the sampled points during chemical imaging and obtain the local maxima as the peaks to be further processed. In this study, two 3D and two 2D mappings were carried out, the normalized summed spectra of which are shown in Fig. 3b. From the spectra, we selected six peaks (135, 3500, 2664, 2275, 615, and 1520 cm^{-1}) for further processing, which correlated to peaks 1–6 in Fig. 2a, implying that these peaks may actually originate from different parts of a *Spirulina* cell.

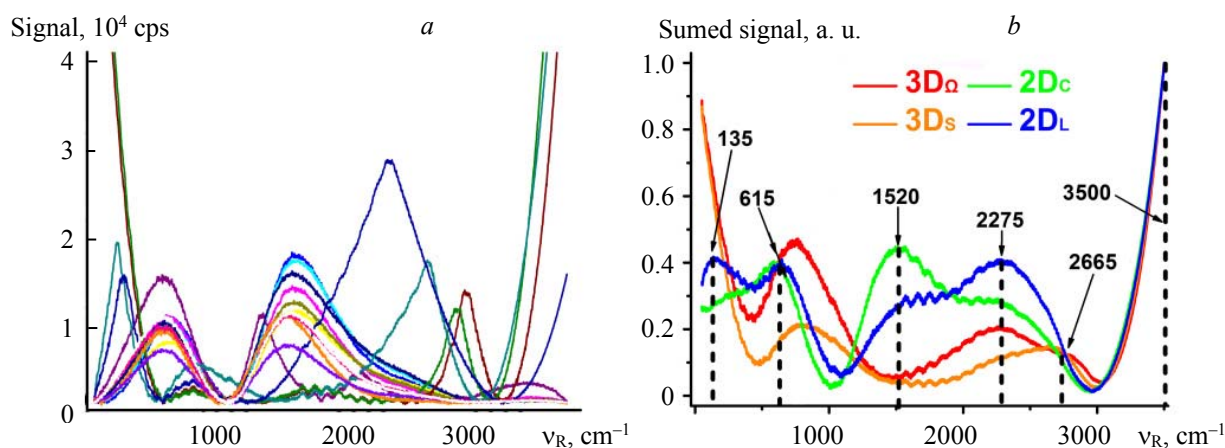


Fig. 3. (a) Selected spectra from a 2D imaging experiment; (b) Summed spectra of the 2D and 3D imaging experiments in this study.

2D imaging of an *S. platensis* cell. Before chemical imaging, the stability of the instrument was tested. Polystyrene, which had been utilized as the routine calibration sample, was tested every 1 h, and nine Raman spectra were obtained (Fig. 4). The Raman signal changed slightly within 8 h, verifying that the instrument was steady and reliable enough to carry out the imaging experiment in this study. Afterward, we carried out two 2D Raman spectroscopic mappings on the same *S. platensis* trichome, one for a longitudinal section ($2D_L$, $20 \times 15 \mu\text{m}$, $1 \mu\text{m}$ step) and the other for a cross-section ($2D_C$, $10 \times 15 \mu\text{m}$, $1 \mu\text{m}$ step), as shown in Fig. 5a. As shown by the general trend in Fig. 5b (5h) to Fig. 5g (5m) (peaks 1–6), the location of corresponding chemicals gradually moved outward from the inner part of the trichome. According to the distribution, peaks 1–3 belonged to chemicals within the cytoplasm. Substances represented by peak 1 were highly concentrated in the core part of the cell (Fig. 5h), and their segmental distribution in different *Spirulina* cells can be viewed in Fig. 5b. Thus, the peak might have originated from polyphosphate granules which commonly occupy the central part of *Spirulina* cells [33]. These granules feature diameters within $0.5\text{--}1 \mu\text{m}$ and contain about 50% of the total amount of phosphorous in the cell, which can easily emit fluorescence light after laser excitation and result in the high fluorescent signal in the Raman spectra obtained in this study. Figure 5i, j shows that the substances indicated by peaks 1 and 2 surrounded the cell core and were situated in different parts of the cell. As shown in Fig. 5d, peak 3 showed a considerably more prevailing distribution along the whole sampled trichome than peaks 1 and 2. The TEM images of *Spirulina* cells revealed that the central polyphosphate granules were surrounded by photosynthetic thylakoid membranes [33].

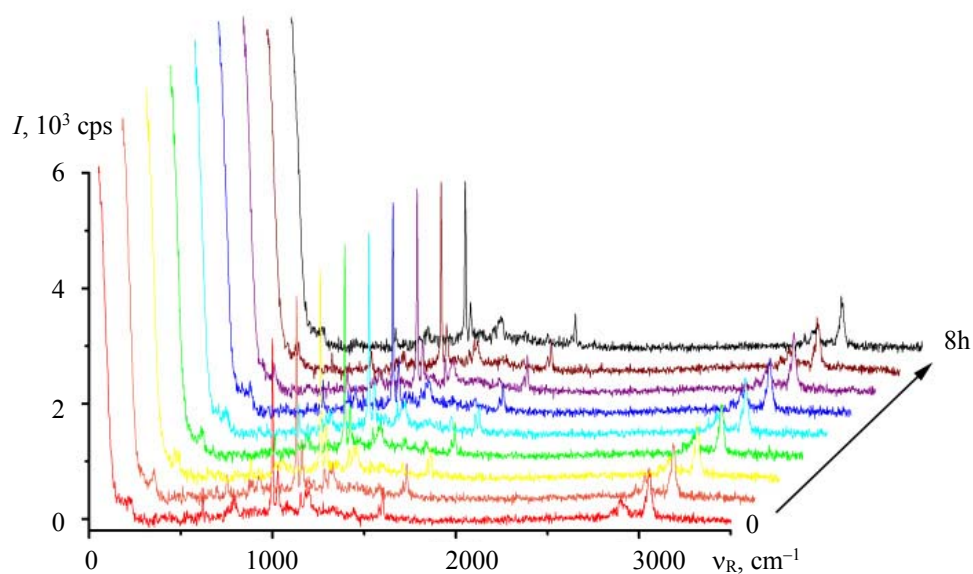


Fig. 4. Signal stability testing results of the confocal Raman microscope used in this study. Nine Raman spectra of polystyrene were acquired every 1 h under the instrumental parameters shown in Table 2.

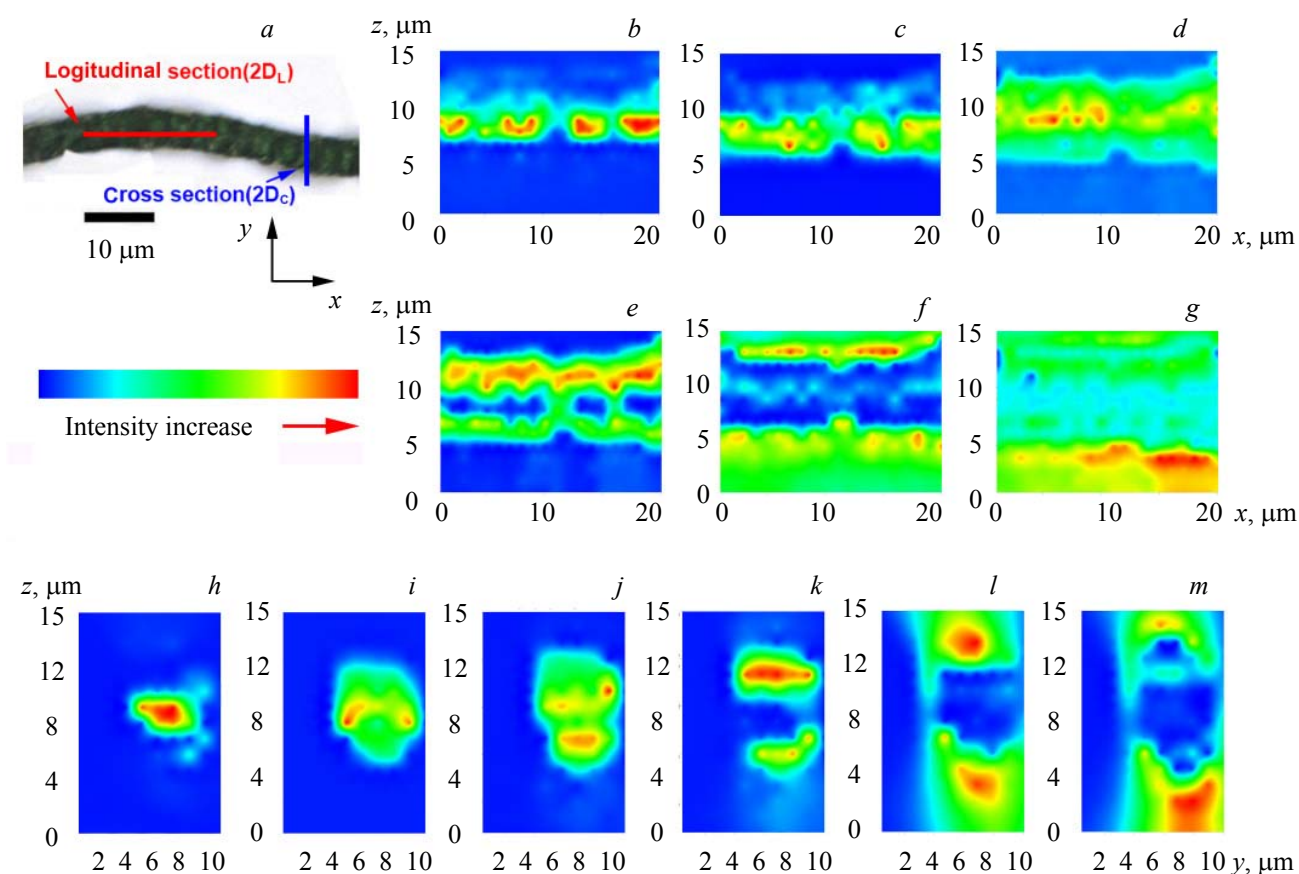


Fig. 5. 2D imaging of a longitudinal section (2D_L) and a cross-section (2D_C) of a trichome. (a) Trichome image and sections; (b) to (g) 2D_L mappings obtained at 135, 3500, 2665, 2275, 615, and 1520 cm⁻¹; (h) to (m), 2D_C mappings of the same peaks.

These cells fill the whole *Spirulina* cell and contain almost all the cellular chlorophyll and carotenoids, which are responsible for the photosynthetic process and emit fluorescence upon laser excitation. This finding strongly suggests that peak 3 could have originated from photosynthetic thylakoid membranes; however, peak 2 may have several origins. First, it might have come from phycobilisomes, which are highly molecular aggregates of fluorescent-active phycocyanin, allophycocyanin, and phycoerythrin and closely attached to photosynthetic thylakoid membranes. They act as light-harvesting antennae and transfer the absorbed energy to chlorophyll a in the photosynthetic thylakoid membranes [17]. Secondly, peak 2 could have been from cyanophycin granules, which are normally located in the periphery of the cell, irregularly shaped with a diameter of about 0.5 μm , and can serve as sites for respiration [17, 33]. Lastly, this peak might have originated from nucleoplasm, in which cylindrical and polyhedral bodies exist with suitable sizes for sampling (at the micrometer level) [17].

For peak 4, its imaging result in Fig. 5e shows the outline of the trichome, in which the cross wall (septum) and cavities enclosed in the cell envelopes can be observed. Moreover, the “spots” in Fig. 5b can be ideally fitted into the cavities in Fig. 5e, which implies that peak 4 came from the peptide-glycan cell wall. The septum is an ingrowth form of the outer cell wall, which only differs in the number of constituent peptide-glycan layers [34]. As shown in Fig. 5f,g,l, m, peaks 5 and 6 belonged to substances outside the *Spirulina* cell wall given that the high signal area was located farther from the central part of the cell than that of peak 4. However, both peaks showed wide distributions outside the trichome and spread out of the 2D imaging boundary. Thus, we were unable to determine the differences between them from this aspect. To view the distribution in a broader space, we carried out 3D imaging experiments, which will be illustrated in the next section. The discussions in this section corroborated the hypothesis proposed in the next section – that is, cumulative laser illumination can cause *Spirulina* cells to shrink, leading to the disappearance of peaks 1–3 and the successive advent of peaks 4–6.

3D imaging of two *S. platensis* trichomes. An entire Ω -shaped trichome ($3D_{\Omega}$, $50 \times 50 \times 25 \mu\text{m}$, $2\text{-}\mu\text{m}$ step) and a segment of a straight trichome ($3D_S$, $20 \times 10 \times 10 \mu\text{m}$, $1\text{-}\mu\text{m}$ step) were subject to 3D Raman spectroscopic imaging experiments herein, and the results are shown in Fig. 6. For better visualization, the specific X-Y-Z coordinates were omitted. The steric shapes of the trichomes were mapped out. Peak 1 arose from the innermost part of the trichomes (Fig. 6b,i). Figure 6c,d reveals the segmental distribution feature

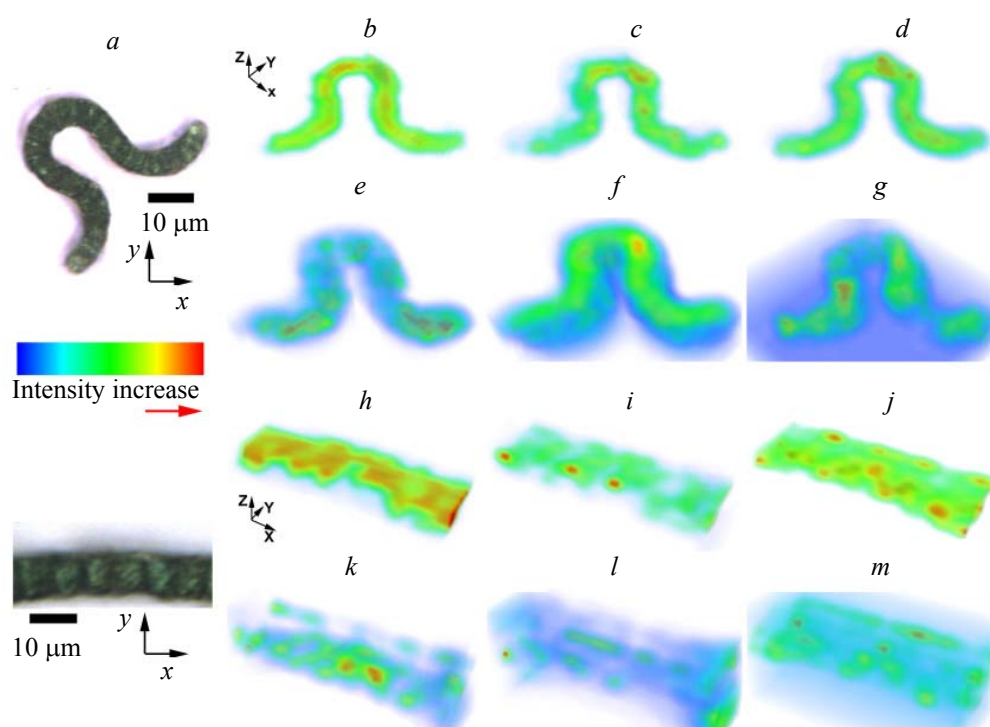


Fig. 6. 3D imaging of a Ω -shaped ($3D_{\Omega}$) and a straight trichome segment ($3D_S$). (a) and (h) Trichome images; (b) to (g) $3D_{\Omega}$ mappings obtained at 135, 3500, 2664, 2275, 615, and 1520 cm^{-1} ; (i) to (n), $3D_S$ mappings of the same peaks.

of peaks 2 and 3, and Fig. 6j,k shows that the trichome was more teeming with substances from peak 3 than those from peak 2. As for peak 4, its distribution featured a Ω -shaped hull in which the 3D images of peaks 1–3 can be filled. Peaks 5 and 6 showed a more exterior distribution than the rest of the peaks. All these observations are consistent with those in the following section. In addition, we can view the difference between the distributions of peaks 5 and 6. Figure 6f,m (peak 5) shows a fluff shell that can enclose the images in Fig. 6e,l (cell wall). On the other hand, substances generating peak 6 also formed a shell; however, the rest of the imaged space contained small amounts of such substances, represented by the shadow “fog” in Fig. 6g,n. According to TEM records of *S. platensis* trichomes, a diffuent fibrillar net-like sheath, which is excreted through pores on the cell wall, existed [35]. According to this description, peak 5 possibly originated from the shell. On the other hand, as the substance represented by peak 6 was mostly located on the cell wall and existed everywhere in the sampled space around the trichome, they might be the crystals and residues of the diluted culture medium generated during water evaporation in the sample preparation process (Fig. 1).

Conclusions. We investigated the changes in the morphology and Raman spectrum of *Spirulina platensis* cells under different laser conditions. The excitation laser evidently causing damage to the illuminated area of the *Spirulina* trichome was adjusted to 0.1-mW laser power and a total exposure time that was shorter than 1 s. Cumulative laser irradiance on the same sampling spot altered the Raman spectrum, which featured more fluorescent signal and caused the successive disappearance and appearance of different peaks, the possible reason for which has already been discussed. Six peaks were selected for two 2D and two 3D imaging, which revealed distinct features of different parts of the *Spirulina* trichome, including the central and peripheral part of the cytoplasm, cell wall, shell, and culture medium residue. Possible substances and organelles in these parts have also been discussed.

The chemicals discussed in the study were deduced from their distribution feature in the cell without accurate qualification and precise quantification, which will be the object of our next endeavor. We intend for this study to be a preliminary investigation of both the applicability of Raman imaging technique to study the cellular chemical process, which remains to be explored, and the morphological alternation of algae during cultivation in addition to TEM technology.

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