Probing the metabolic heterogeneity of live *Euglena gracilis* with stimulated Raman scattering microscopy

**Raman spectral analysis of paramylon**

While the Raman spectrum of lipids is well known [16], that of polysaccharides in microalgae has not been reported to date. To obtain and analyze the Raman spectrum of paramylon, we used our SRS microscope with 91 spectral points between 2800 and 3100 cm\(^{-1}\) to measure paramylon granules extracted from *E. gracilis* (provided by euglena Co. Ltd.) suspended in water. Supplementary Fig. 2a shows a typical SRS image of the paramylon granules. The figure shows that the size of the paramylon granules ranges from 0.5 to 2 \(\mu\)m. Supplementary Fig. 2b shows the measured SRS spectrum of the paramylon granules. The spectrum was obtained by spatially averaging the SRS spectra of paramylon granules in the image. A notable spectral peak is evident at a wavenumber of 2910 cm\(^{-1}\), presumably due to CH\(_2\) antisymmetric stretching. Because this peak is unique to paramylon and is not shared with lipids or chlorophyll, we chose this wavenumber as one of the four Raman spectral points to separate paramylon from other constituents within the cell and identify the paramylon content of motile *E. gracilis*.

**Image analysis and distribution analysis**

As a pre-processing step, we denoised the raw SRS images with total variation regularization [S1] and low-pass filtering and removed the lock-in signal offset from them, which were then used to create intracellular metabolite images and cell masks. Metabolite imaging of *E. gracilis* is possible, considering that the measured hyperspectral SRS data \(d_j\) at the \(j^{th}\) pixel is decomposed using the spectral bases \(s_i\) of the \(i^{th}\) constituent (lipids, paramylon, chlorophyll, and others), that is, \(d_j = \sum_i c_{ji} s_i\), where the coefficients \(c_{ji}\) are the concentrations of the corresponding constituents. Through pseudo matrix inversion using the four spectral bases shown in Fig. 1c and Fig. 1d, we obtained the spatial distributions of the four chemical constituents from each SRS image of *E. gracilis*. We measured 108 *E. gracilis* cells from the three cultures (before and 2 and 5 days after the application of nitrogen-deficiency stress) to sufficiently observe the group characteristics. Supplementary Fig. 3a, Supplementary Fig. 3b, and Supplementary Fig. 3c show all the metabolite images of the 324 cells taken from the three cultures. Compared to the nitrogen-sufficient (Day-0) group, the increased amounts of paramylon and lipids as well as the decreased amount of chlorophyll are evident in the Day-2 and Day-5 nitrogen-deficient groups. Furthermore, by virtue of our SRS microscope’s fast imaging capability, the obtained images exhibit the spatial distributions of metabolites without motion artifacts. We applied cell masks to obtain these images in order to suppress the background noise in each image and hence to evaluate the amount of each intracellular constituents with high accuracy and precision. The cell masks are generated by the following procedure. We first binarized the averaged image of the four spectral images using a threshold value
and then iteratively applied dilation and erosion operations to connect the segmented areas within a cell. Furthermore, we deleted unnaturally small objects in the binarized images to generate the cell masks. Supplementary Fig. 3d, Supplementary Fig. 3e, and Supplementary Fig. 3f show the cell masks, which are in good agreement with the metabolite images of the corresponding cells shown in Supplementary Fig. 3a, Supplementary Fig. 3b, and Supplementary Fig. 3c. Using the metabolite images shown in Supplementary Fig. 3a, Supplementary Fig. 3b, and Supplementary Fig. 3c, we examined changes in the distributions of the cellular constituents by calculating the density \( Q_i \) of the \( i \)-th constituent (i.e., lipids, chlorophyll, and paramylon) in the masked cellular area \( A \) as \( Q_i = (\sum_{j=\mathcal{A}} c_{ji})/A \) for each cell (Fig. 2b). The amount of each constituent in single cells without dividing it by the cellular area was examined and is shown in Supplementary Fig. 4, which exhibits similar traits to Fig. 2d.

**SRS metabolite imaging of other microalgae that contain different polysaccharides**

Supplementary Fig. 6a shows our SRS microscope’s capability of imaging different polysaccharides from paramylon. Here we used purified agarose, curdlan, and glucose in their powder forms. To obtain the spectrum of starch, we measured flour purchased from a grocery store. The figure shows that these polysaccharides have similar, but distinguishable spectral shapes, firmly proving our system’s potential for decomposing these polysaccharides within microalgal cells. To show our system’s label-free metabolite imaging capability of other living microalgae that produce different polysaccharides, we examined three other types of microalgae (\textit{Hamakko caudatus}, \textit{Gungnir kasakii}, and \textit{Gloeomonas anomalipyrenoides}) which produce starch instead of paramylon. The strains of \textit{Hamakko caudatus} KzCl-4-1 (NIES-2293), \textit{Gungnir kasakii} IwCl-1, and \textit{Gloeomonas anomalipyrenoides} HkCl-5-3 (NIES-3640), were provided by Dr. Takashi Nakada at Keio University. The strains NIES-2293 and NIES-3640 are also available at the Microbial Culture Collection, NIES. They were measured after 5 days of cultivation in AF-6–N. Details of the AF-6 culture used in this work can be found in Refs. S2 and S3. Supplementary Fig. 6b shows differential interference contrast optical microscopic images of these microalgae, in which starch and lipids are difficult to distinguish. By analyzing these cells, we found multiple SRS spectra corresponding to different constituents (Supplementary Fig. 6c, Supplementary Fig. 6d, and Supplementary Fig. 6e). Importantly, starch exhibits an SRS spectral peak at about 2910 cm\(^{-1}\) similar to paramylon, but has a broader spectral width. By using these obtained spectral bases, we were able to decompose the metabolite images of these cells (Supplementary Fig. 6c, Supplementary Fig. 6d, Supplementary Fig. 6e) using both full 91 and reduced 4 spectral points, showing our system’s capability of label-free metabolite imaging of various types of microalgae.

**Evaluation of cross phase modulation**

Cross phase modulation (XPM), a nonlinear optical effect in which one wavelength of light affects the phase of another wavelength of light through the optical Kerr effect, is known to cause artifacts in SRS images and can hence reduce the accuracy of metabolite mapping and quantification in our analysis. We evaluated the
level of the XPM signal compared to the SRS signal level from the 91 spectral images of a static *E. gracilis* cell from 2800 to 3100 cm\(^{-1}\) in Supplementary Figure 7. The image at 2910 cm\(^{-1}\) is shown in Supplementary Figure 7a while the cross-sectional plot along the yellow broken line is shown in Supplementary Figure 7b. The estimated XPM signal level is also shown in Supplementary Figure 7b. To estimate the XPM signal level, we analyzed the SRS spectrum of water obtained by averaging the spectra in the rectangular area in Supplementary Figure 7a. The water spectrum was corrected for the wavelength dependence of our Stokes beam power and is plotted in Supplementary Figure 7c. The spectrum shows that the SRS signal increase starts at 3000 cm\(^{-1}\), which is characteristic to water. We determined the XPM signal level as the level of the polynomial fit of the water spectrum at 2800 cm\(^{-1}\), considering that water shows a negligible SRS signal at 2800 cm\(^{-1}\). The paramylon spectrum, averaged over 5 \(\times\) 5 pixels near the position indicated by the arrow in Supplementary Figure 7a, is also shown in the figure and exhibits a significantly higher peak signal than the XPM level. As seen in these figures, the XPM signal level is well below the noise level of the detected lock-in amplifier signal, thus confirming that the effect of XPM is negligible in our measurement. Other than XPM, two-photon absorption (TPA) is also known to cause artifacts in SRS images. However, assuming that TPA mainly occurs in chlorophyll in the case of *E. gracilis*, we assigned the origin of all TPA signals to chlorophyll in our spectral decomposition of metabolites by utilizing the “flat” wavelength dependence of the TPA spectrum (as seen in the chlorophyll plot in Figure 1c).

**Correlation between 2D and 3D metabolite images**

To show that the acquisition of 2D metabolite images is a good indicator of the amounts of intracellular metabolites, we made a comparison in the quantified amount between the 2D and 3D metabolite images. By taking advantage of our system’s fast imaging capability at 27 2D metabolite image frames per second, we examined live *E. gracilis* cells in 3D as shown in Supplementary Figure 8. 3D scanning was conducted by moving the piezoelectric transducer on the sample stage at 27 \(\mu\)m/s in the axial (z) direction to achieve a step size of 1 \(\mu\)m (Supplementary Video 2). Supplementary Figure 8a shows 2D images in the central plane of all the examined cells in 3D. We verified strong correlations between the quantified amounts of the intracellular metabolites (paramylon, chlorophyll, and lipids) in 2D and 3D as shown in Supplementary Figure 8b in which the error bars represent standard deviations calculated from the central 20% portions of the 3D cellular image stacks.

**References**


Supplementary Figure 1 | Detailed schematic of the SRS microscope. Two picosecond pulse lasers are used to perform SRS imaging: a Ti:sapphire pulse laser (Coherent, Mira 900D) and a home-built Yb fiber pulse laser which generate synchronized pump and Stokes pulse trains at pulse repetition rates of 76 and 38 MHz, respectively. The wavelength of the pump laser is 790 nm while that of the Stokes laser is tunable from 1015 to 1045 nm. The two laser beams are overlapped both spatially and temporally and are focused onto the target cell via the first objective lens (60×, NA 1.2, water). The SRS process occurs within the focused volume of the combined beam inside the target cell, resulting in the transfer of the Stokes-induced intensity modulation to the pump beam. The transmitted pump beam is detected by the Si photodetector via the second objective lens (60×, NA 1.2, water) while the transmitted Stokes beam is removed by an optical short-pass filter. The photodetector signal is demodulated by the lock-in amplifier at 38 MHz to obtain the SRS signal. A 2D SRS image is obtained by scanning the combined beam over the target cell with the resonant scanner at 8 kHz and the orthogonally oriented galvanometric scanner and then digitally mapping the Raman signal into a 2D matrix. A multicolor image stack is obtained by changing the wavelength of the Stokes laser in a frame-by-frame manner.
Supplementary Figure 2 | Multicolor SRS analysis of paramylon. a, Representative SRS image averaged over 91 spectral images of paramylon granules suspended in water. We tested more than 3 replicates of the sample. b, Measured SRS spectrum of paramylon. The size of the scale bar is 10 μm.
Supplementary Figure 3 | Metabolite images and cell masks of 108 measured E. gracilis cells. a, Metabolite images of the E. gracilis cells in the Day-0 group. b, Metabolite images of the E. gracilis cells in the Day-2 group. c, Metabolite images of the E. gracilis cells in the Day-5 group. Lipids, paramylon, and chlorophyll are shown in cyan hot, red, and green. d-f, Masks of the measured E. gracilis cells shown in a-c. The size of the scale bars in a – f is 60 µm.
Supplementary Figure 4 | Scatter plots of intracellular constituents of three groups of 108 cells without normalization by cellular area. These plots have similar traits to those in Fig. 2d. The blue, red, and green colors in the figure represent the nitrogen-sufficient (Day-0) cells, and Day-2 and Day-5 nitrogen-deficient cells, respectively.
Supplementary Figure 5 | Means and standard deviations of the measured cell contents that were chosen for statistical analyses with relative standard deviations of less than 0.1. We extracted 78, 106, and 108 cell images from the three groups Day-0, Day-2, and Day-5, respectively.
**Supplementary Figure 6** | **SRS metabolite imaging of green microalgae with cell walls.**  

**a.** Representative SRS spectra of different polysaccharides. We measured more than 3 replicates of each compound.  

**b.** Differential interference contrast optical microscope images of *Hamakko caudatus*, *Gungnir kasakii*, and *Gloeomonas anomalipyrenoides*.  

**c-e.** SRS spectral bases and metabolite images of *Hamakko caudatus*, *Gungnir kasakii*, and *Gloeomonas anomalipyrenoides*, respectively, using 91 and 4 spectral points. The spectra were extracted from the points indicated by the yellow arrows in the corresponding cell images. The spectra shown in blue and red indicate lipids and starch, respectively. Chlorophyll is shown in green in **e** while the origin of the spectra in magenta and green (found mostly outside of the cells) in **d** is unknown. A polynomial fit was used to alleviate the noise that appears in the chlorophyll spectrum in **e** and is also shown therein. In **c – e**, we tested about 15 cells. Representative results are shown. The size of the scale bars in **b – e** is 10 µm.
Supplementary Figure 7 | Evaluation of XPM. **a,** SRS image of a static *E. gracilis* cell at 2910 cm\(^{-1}\). The SRS images were taken at 91 different spectral points. The size of the scale bar is 10 \(\mu\)m. **b,** Cross-sectional plot along the yellow broken line in **a.** The estimated XPM signal level shown as a blue curve appears well below the noise level of our lock-in amplifier (LIA) signal. **c,** Corrected spectra of water and paramylon for the wavelength dependence of our Stokes beam power. The paramylon spectrum was obtained by averaging the spectra over 5 \(\times\) 5 pixels near the position indicated by the arrow in **a** while the water spectrum was obtained by averaging the spectra in the yellow rectangular region in **a.** We estimated the XPM signal level using the polynomial fitting curve of the water spectrum. The data in **a** – **c** are representative results from 3 tested replicates.
Supplementary Figure 8 | Correlations between 2D and 3D metabolite images of single E. gracilis cells. 

a, 2D cross-sectional images of E. gracilis cells in their central plane which were taken in 3D and used to evaluate the differences between the amounts of intracellular metabolites quantified in 2D and 3D. The constituents shown in red, green, and blue represent paramylon, chlorophyll, and lipids, respectively. The size of the scale bar is 10 μm. 

b, Comparison in the quantified metabolite amounts (paramylon, chlorophyll, and lipids) between 2D and 3D. The error bars represent the standard deviations of the amounts in several 2D cross-sectional images of the cells’ central 20% portion. The red broken lines are least-square linear fits with the constraint to pass through the origin. Shown in a and b are all the 9 tested cells.
Supplementary Video 1 | Comparison in SRS imaging of motile *E. gracilis* in motion between frame rates of 27 fps (top) and 6.75 fps (bottom). The video is repeated 5 times for clarity. The fast dynamical motion of *E. gracilis* shows the need for high-speed SRS imaging capability in order to obtain the motion-artifact-free Raman spectrum of the cell. The constituents shown in red, green, and blue represent paramylon, chlorophyll, and lipids, respectively. The field of view is 60 µm (horizontal) × 30 µm (vertical).
Supplementary Video 2 | 3D metabolite imaging of *E. gracilis*. The colors (blue, red, green) indicate the presence of lipids, paramylon, and chlorophyll. The size of the bounding box is 60 µm (horizontal) × 30 µm (vertical) × 18 µm (axial). The 3D image acquisition was performed within 0.67 s.