論文内容の要旨

論文題目　Development of 1064 nm Excited Multichannel Raman Microspectrometer and its Application to *in vivo* Structure/Function Analysis of Cyanobacteria
（1064 nm 近赤外マルチチャンネル顕微ラマン分光計の製作と
シアノバクテリアの構造・機能解析への応用）

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Introduction

Molecular level investigation of cellular processes is of great importance in understanding life. Raman microspectroscopy is one of the most informative methods for *in vivo* studies of living cells, providing sub-μm space-resolved molecular information without any sample pretreatment. However, this method is not readily applicable to photosensitive / photolabile organisms because of the following two difficulties. One is that such organisms often emit strong autofluorescence that easily interferes with the Raman measurements. The other difficulty is that photolabile systems are easily photodamaged by visible excitation. Some photosensitive organisms emit strong fluorescence and/or are photodamaged even with near-infrared (NIR) excitation at ~800 nm. For the purpose of further reducing the fluorescence interference, I have newly developed a 1064 nm deep NIR Raman system with the sensitivity high enough for *in vivo* measurements of single living cells.

This new apparatus has been applied to the structure/function analysis of photosynthetic pigments in cyanobacterial cells, which are prototype model organisms for photosynthesis research. Because cyanobacteria contain several kinds of photosynthetic pigments, they emit strong autofluorescence even with ~800 nm excitation. By using 1064 nm deep NIR excitation, it has become possible to obtain high S/N Raman spectra from carotenoid, chlorophyll a and phycobilins. Thus, their distribution, structure and function analysis within a single cell become possible under physiological conditions.

1064 nm excited multichannel Raman microspectrometer (Chapter 2)

When constructing a Raman microspectrometer with deep NIR excitation, the detection efficiency is a peculiar problem. A CCD camera, which is widely used in the visible excitation Raman system, cannot be used in the deep NIR region because of its low quantum efficiency. I here use an InP/InGaAsP NIR image intensifier (NIR-II), whose quantum efficiency is approximately 4 % in the 900 – 1400 nm wavelength region, which covers the overall fingerprint region when excited at 1064 nm.

The developed apparatus is shown in Fugure 1. By using electrical gating synchronized with a Q-switched Nd:YAG laser (1064 nm, 30 ns pulse, 10 kHz), the thermal noise from the NIR-II is considerably lowered. The laser beam is focused on the sample with a ×100/ 1.3 NA microscope objective. A 50 μm cross slit is used as the entrance slit of a polychromator as well as the confocal pin hole, in order to reduce the number of optics used. The lateral spatial resolution of 0.7 μm and the depth spatial resolution of 3.1 μm are obtained. Because of the multichannel detection with NIR-II, approximately 1000 cm^{-1} wavenumber region is simultaneously detected with 10 cm^{-1} spectral resolution.

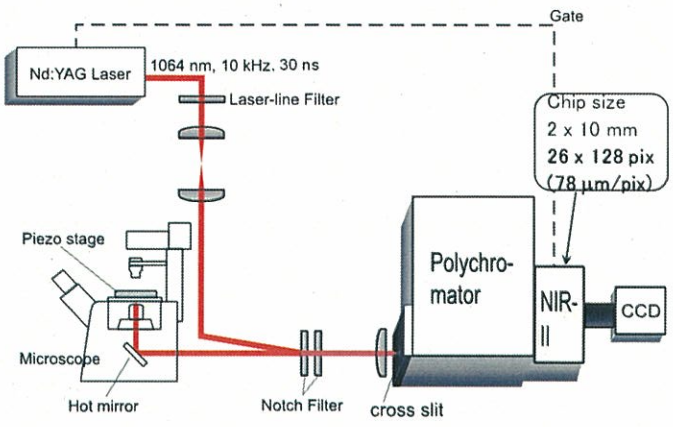

Figure 1. Schematic diagram of 1064 nm excited multichannel Raman spectrometer

Deep NIR Raman measurements of cyanobacteria (Chapter 3)

As the sample, I here use cyanobacteria *Thermosynechococcus elongatus* (*T. elongatus*), which is a well-known model organism for the photosynthesis research. For the preparation of sample, *T. elongatus* cells are fixed between a slide glass and a cover glass by controlling the amount of sample. For avoiding the photodamage to the sample, laser power is lowered to less than 1 mW, typically 0.5 mW.

The new deep NIR excited microspectrometer enables *in vivo* Rman measurement of single living cyanobacterial cells. Figure 2 shows the comparison between the Raman spectra of *T. elongatus* cells measured with 785 nm excitation and that measured with

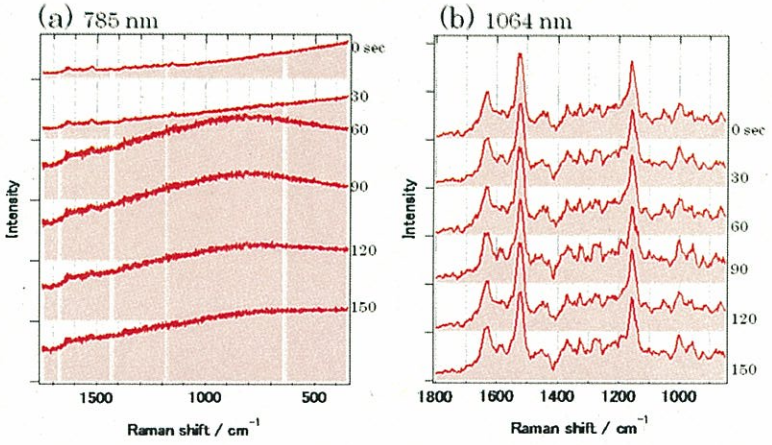

Figure 2. 785 nm (a), 1064 nm (b) excited Raman spectra of *T. elongatus*.

1064 nm excitation. With 785 nm excitation, fluorescence from photosynthetic pigments strongly interferes. It is also seen that, after the start of laser irradiation, the spectral profile of fluorescence background changes with time, indicating that the cell is photodamaged. On the other hand, 1064 nm excitation drastically decrease the autofluorescence and shows several distinct Raman bands. Furthermore, the spectra do not show time dependence, indicating that the photodamage or photobleaching does not occur. The present deep NIR excitation is suitable for the Raman measurement of photo-labile living cells.

Raman band assignments (Chapter 4)

For the purpose of band assignments, Raman spectrum of *T. elongatus* is compared with those of three photosynthetic pigments, β-carotene, chlorophyll a and phycobilin (Figure 3). From the comparison, Raman bands at 1008, 1157 and 1523 cm^{-1} are assigned to carotenoids. The 1225, 1325 cm^{-1} bands and 1279, 1369, 1586, 1635 cm^{-1} bands are assigned to chlorophyll a and phycobilin, respectively. In this way, almost all bands are assignable to the three types of pigments. These three photosynthetic pigments are selectively observed in the 1064 nm excited Raman measurements of cyanobacteria, because of the pre-resonance Raman effect due to their strong absorptions in the visible region.

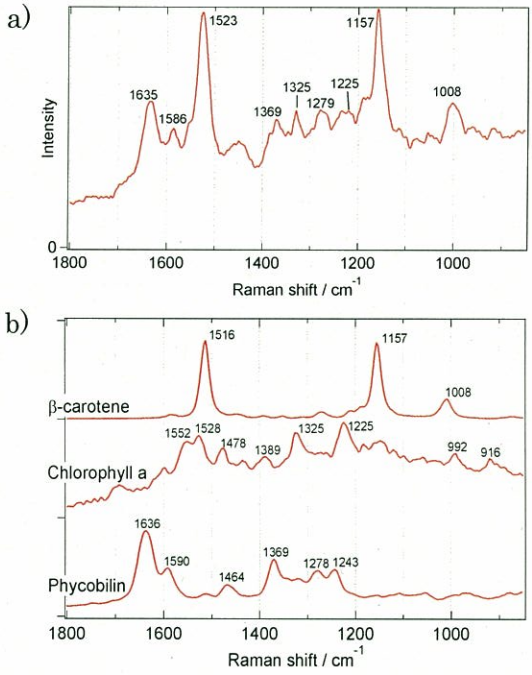

Figure 3. Raman spectra of *T. elongatus* (a) and photosynthetic pigments (b).

Raman imaging of photosynthetic pigments in *T. elongatus* (Chpater 4 and 5)

Chemical specificity shown above enables the Raman imaging of photosynthetic pigments distributions inside the cyanobacterial cells. For the Raman mapping experiment, the *T. elongatus* cell is translated by a piezoelectric stage horizontally with a step of 0.3 μm. At each sample point, Raman spectra are measured in 10 seconds. After using singular value decomposition in order to reduce the noise, the Raman images are constructed from the band intensity at each sample point. As shown in Figure 4, distribution information of each photosynthetic pigment is clearly visualized.

Images belonging to the same pigments show identical distribution patterns, supporting the band assignments given above. It is obvious that the distributions of the three pigments are distinct from one another. In particular, carotenoids show a quite different distribution from those of chlorophyll a and phycobilins.

It is also found that the C=C stretch band of carotenoids shows peak shift from 1526 cm^{-1} to 1520 cm^{-1}

(Fig. 5a), suggesting their structural variance within the cell. This peak shift is closely correlated with the variance of intensity ratio of carotenoid / phycobilins (Fig 5b, c). In the photosynthesis processes, phycobilins play a role of light harvesting antenna, whereas carotenoids are thought to play a role of antioxidant and photoprotection. This finding indicates that the localization of structurally different carotenoids is likely to be related to the photoprotective process of cyanobacteria

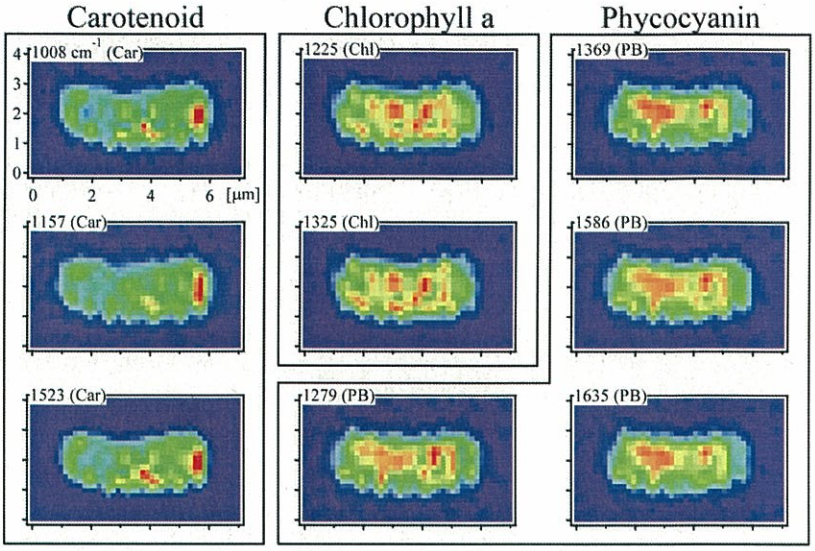

Figure 4. Raman mapping images of each photosynthetic pigments obtained from a *T. elongatus* single living cell.

(a) Car C=C str. (b) PB / Car (c)

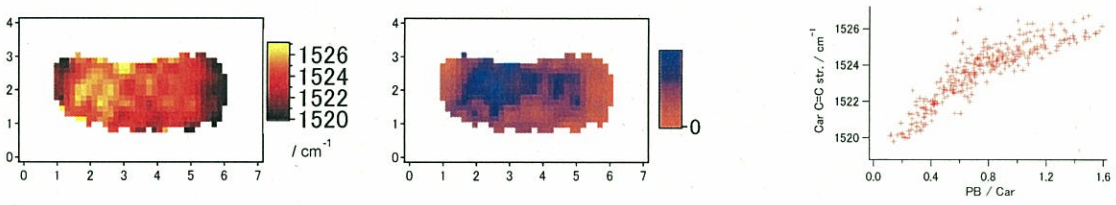

Figure 5. (a) Peak position mapping image of carotenoid C=C stretch band and (b) intensity ratio mapping image of phycobilin / carotenoid. (c) Peak posision of carotenoid C=C stretch band is plotted against intensity ratio phycobilin / carotenoid.

Conclusion

A newly constructed 1064 nm NIR excited Raman microspectrometer using InP/InGaAsP image intensifier is described. In the measurement of a living cyanobacterial cell, bypassing the interference from autofluorescence, varied distribution of photosynthetic pigments, along with the structural variance of carotenoids, are visualized. A new possibility is thus shown for *in vivo* and molecular level studies of photosynthesis systems.