論文の内容の要旨

論文題目：Resonance Raman Microspectroscopy of Single Living Cells

(単一生細胞の顕微共鳴ラマン分光)

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Introduction

Physicochemical studies of single living cells are essential for molecular level in-depth understanding of life. Time- and space-resolved Raman spectroscopy, which provides detailed information on molecular structures and dynamics, is a promising powerful method for *in vivo* studies of living cells. Raman spectra of lipids, proteins and nucleic acids in living cells have been studied with the excitation in the 600 ~ 1000 nm wavelength range, which suffers less from the interference by the cell autofluorescence. Shorter wavelength excitation in the 400 ~ 600 nm range increases the risk of fluorescence interference but, at the same time, facilitates resonance Raman spectroscopy of hem enzymes that have strong electronic absorption bands in the visible region. Resonance Raman spectroscopy has much higher sensitivity and selectivity than ordinary (non-resonant) Raman spectroscopy, because of more than 1000 times resonance enhancement of the observed Raman band intensities.

In the present study, I have constructed a multi-wavelength excitation Raman microspectrometer using seven different laser lines in the wavelength range of 450 ~ 650 nm. The apparatus has been applied to single living cells of budding yeast, which is known as a good model of eukaryotic species. Raman spectra with different excitation wavelengths have been measured from the same spot of the same cell in order to examine the resonance Raman effect in living cells. It has been shown that multi-wavelength excitation Raman microspectroscopy enables us to image the spatial distributions of two different hem enzymes that have different resonance conditions.

Multi-wavelength excitation Raman microspectrometer

The constructed system is schematically shown in Figure 1. An Ar-Kr ion laser is used for a multi-line excitation light source in addition to a He-Ne ion laser and a laser diode green laser. The ion laser is suitable for a laser microscopic system because of its high-quality beam pattern. Seven wavelengths, 457.9, 488, 514.5, 532, 568.1, 632.8 and 647.1 nm, are available for Raman excitation in this system. A prism beam splitter (reflectance: 25 %, transmittance: 75 %) is used for introducing the excitation laser beam into the microscope. The laser beam is focused by a X100/ 1.40 N.A. oil immersion objective lens. The backward Raman scattering is collected by the same objective, passed through the beam splitter and notch/dichroic filters, and analyzed with a polychromator equipped with a CCD camera. The polychromator has a cross slit that acts as a confocal pin hole as well as an entrance slit. The use of the cross slit reduces the number of optics and therefore markedly increases the efficiency of the collecting optics. Detection efficiency becomes more critical for shorter wavelength excitation experiments, in which lowest possible excitation laser power is used for avoiding the sample damage. The spatial resolution, which depends on the excitation wavelength, is 0.3 μm in the lateral direction and 1.8 μm in the axial direction with the 532 nm line.

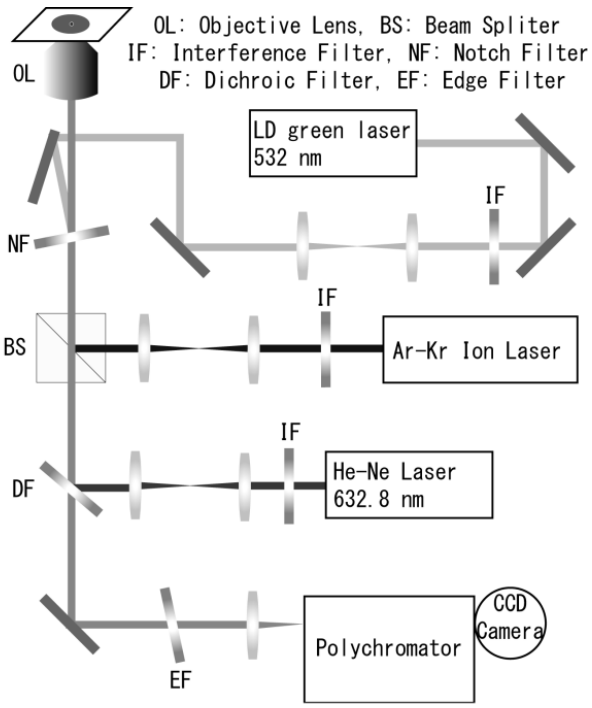

Figure 1. Schematic diagram of the constructed multi-wavelength excitation Raman spectrometer.

The resonance Raman effect in a single living cell

A tetraploid (zygote of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*) strain of budding yeast cultured in a synthetic complete medium (glucose 2 % w/v, yeast nitrogen base w/o amino acids 0.67 % w/v, amino acids mixture drops 0.2 % w/v) has been used for studying the resonance Raman effect in a single living cell. Figure 2 shows the Raman spectra measured from the same spot (star in the inset of Fig. 2) of the same yeast cell excited with the 632.8, 532 and 457.9 nm lines. Laser power at the sample point is 7.1, 0.6 and 0.3 mW in the 632.8, 532 and 457.9 nm excitation, respectively. Although these three spectra are obtained from the same spot in cytosol, their features are completely different because of the resonance Raman effect. In the 632 nm excitation spectrum, Raman bands of proteins (1003 cm^{-1}) and nucleic acids (785 cm^{-1}) are dominant with no resonance enhancement of other Raman bands. On the other hand, resonance Raman bands of

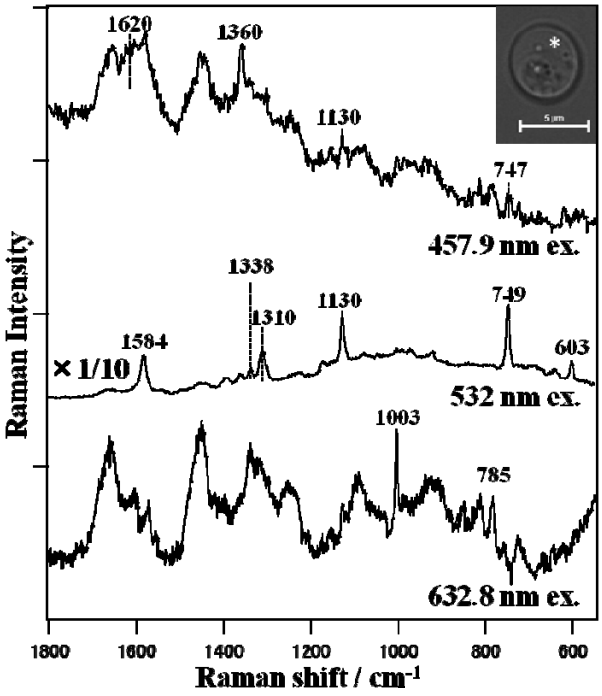

Figure 2. Raman spectra of a single yeast cell excited by 632.8, 532 and 457.9 nm at the same spot of the same yeast cell (star in the inset).

cytochromes dominates the 532 and 457.9 nm excitation spectra. In the 532 nm excitation spectrum, strongly enhanced bands are ascribed to cytochrome *b* and *c*. The Raman bands at 749, 1130, ~1310 and 1584 cm^{-1} are common to cytochrome species. The 603 cm^{-1} band is characteristic to cytochrome *c* and the 1338 cm^{-1} band to cytochrome *b*. Different resonance Raman bands are obtained at 747, 1130, 1360, 1588 and ~1620 cm^{-1} in the 457.9 nm excitation spectrum. Although these bands are common to cytochrome species, the intensity pattern suggests that the main contributor is cytochrome c oxidase that has a strong Soret absorption at ~445 nm[1]. The Resonance Raman effect in living cells enables highly selective detection of hem enzymes.

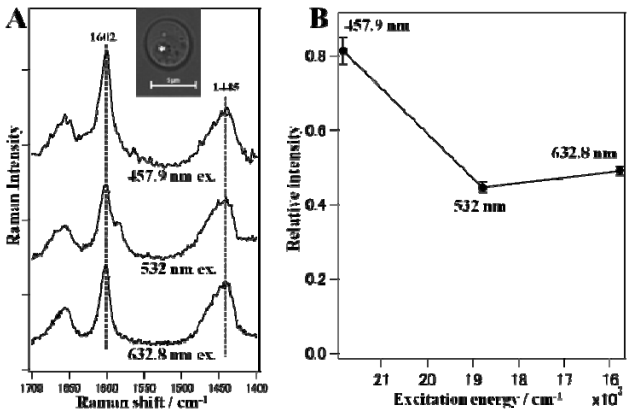

Figure 3. (A); Space-resolved Raman spectra from the same spot of a living budding yeast cell (star in the inset) with 632.8, 532 and 457.9 nm excitation; (B) excitation profile of the 1602 cm^{-1} band. The 1445 cm^{-1} band of lipids is used as an internal intensity standard.

The pre-resonance effect of the "Raman spectroscopic signature of life" in yeast cells

The "Raman spectroscopic signature of life" is a strong Raman band at 1602 cm^{-1} that sharply reflects the bioactivity of a living yeast cell. The origin of this 1602 cm^{-1} band still remains controversial with an issue of possible resonance Raman enhancement. The multi-wavelength excitation Raman microspectrometer can measure the 1602 cm^{-1} band intensities excited with different wavelengths. Figure 3 (A) shows the Raman spectra obtained from the same spot (star in the inset of Fig. 3) of a living yeast cell excited at 632.8, 532 and 457.9 nm. All spectra show a strong band at 1602 cm^{-1}. Figure 3 (B) shows the excitation profile (plot of Raman intensity vs excitation energy) of the 1602 cm^{-1} band. The small intensity enhancement on going from 632.8 nm to 457.9 nm is consistent with the pre-resonance Raman effect of the 1602 cm^{-1} band with an electronic absorption lying in the ultraviolet region. The multi-wavelength excitation Raman system enables us to study the pre-resonance Raman effect in a single living cell as well.

Excitation-wavelength specific Raman imaging

Spatial distributions of Raman intensities recorded with the three different excitation wavelengths have been measured by moving the sample with a piezoelectric stage on the microscope with a step of 300 nm. A singular value decomposition analysis has been used in order to increase the signal to noise ratio. Figure 4 shows the Raman images thus obtained. The images constructed by the 1445 cm^{-1}

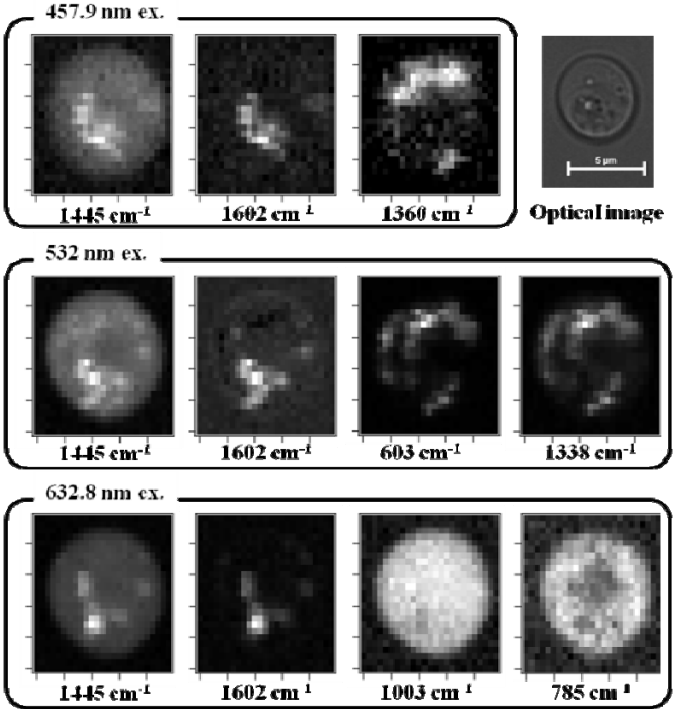

Figure 4. Raman images of a living yeast cell with 632.8, 532 and 457.9 nm excitation. Optical microscope image of the same cell is also shown.

band (C-H bend) of lipids, which are non-resonant with 450 ~ 650 nm excitation, show similar distribution pattern for the three excitation wavelengths. The 1602 cm^{-1} band image shows the localization of the molecular species giving rise to this band, indicating that its spatial distribution does not appreciably change during the measurement. The 632.8 nm image at 785 cm^{-1} shows the spatial distribution of nucleic acids and that at 1003 cm^{-1} the distribution of proteins. The 603, 1338 cm^{-1} images obtained with 532 nm and the 1360 cm^{-1} one with 457.9 nm are ascribed to cytochrome *c*, cytochrome *b* and cytochrome *c* oxidase (with possible minor contributions from other cytochromes), respectively. Raman images of molecular species having different resonance conditions are now obtainable from the same single living cell with the use of the constructed multi-wavelength excitation Raman microspectrometer.

Conclusion

A multi-wavelength excitation Raman microspectrometer with seven different laser lines in the wavelength range of 450 ~ 650 nm has been newly constructed. The apparatus enables the comparative studies of space-resolved Raman spectra obtained from the same spot of the same living cell but with different excitation wavelengths. It facilitates the detailed analysis of the resonance effect to make Raman spectroscopy of living cells still more informative.

[1]T. Yonetani, *J. Biol. Chem.*, 1960, 235 ,845-852