論文内容の要旨

論文題目：Development of Fast and Quantitative Raman Microspectroscopies and their Application to Living Cells

（高速定量的顕微ラマン分光法の開発と生細胞への応用）

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Raman microspectroscopy, which combines high molecular specificity of Raman spectroscopy and high spatial resolution of optical microscopy, has emerged as a unique and powerful method for *in vivo* and *in situ* living cell studies at the molecular level. Extensive use of this method, however, has been hindered by slow data acquisition speed that is due to small Raman cross-sections of molecules. In this study, I have developed new linear and nonlinear Raman microspectroscopies, multi-focus confocal Raman microspectroscopy and quantitative coherent anti-Stokes Raman scattering (CARS) microspectroscopy for fast vibrational imaging. I have adopted the multi-focus excitation and the image compression techniques to develop a new scheme of spontaneous Raman microspectroscopy, multi-focus confocal Raman microspectroscopy, in order to improve the data acquisition speed without increasing the excitation laser power at the sample. I have combined CARS microspectroscopy with the maximum entropy method (MEM) in order to convert CARS spectra into the $\mathrm{Im}[\chi^{(3)}]$ spectra whose intensities are linear to molecular concentration.

I also paid attention to the absolute quantitative nature of Raman microspectroscopy. The number of Raman scattered photons is strictly proportional to the number of molecules in the focal volume. If the absolute Raman cross-section of a molecule is known, the concentration of this molecule can be directly determined from the observed number of Raman scattered photons. This absolute quantitative character can be a great advantage in molecular imaging, particularly in molecular imaging of living cells. In this study, I have made a challenge to estimate the numbers/concentrations of biomolecules in living cells by determining their absolute Raman cross-sections.

Quantitative CARS microspectroscopy

Figure 1 (a) shows a typical multiplex CARS spectrum obtained from a budding yeast cell. I employ MEM to extract the amplitude and phase of vibrational resonances from the obtained multiplex CARS spectra. The MEM does not require any *a priori* knowledge of the vibrational bands contained but still is able to retrieve the phase information on the third-order nonlinear susceptibility $\chi^{(3)}$, whose imaginary part corresponds to ordinary (spontaneous) Raman spectra. Figure 1 (b) shows the reconstructed $\mathrm{Im}[\chi^{(3)}]$ spectrum of Fig. 1 (a) with MEM. Many vibrational bands in the fingerprint region are clearly found with high signal to noise ratio. It has been confirmed that this spectrum is very similar to the corresponding spontaneous Raman spectrum obtained from a yeast cell. CARS microspectroscopy combined with MEM has realized label-free and quantitative molecular mapping of a single living cell in the fingerprint region (CARS molecular fingerprinting).

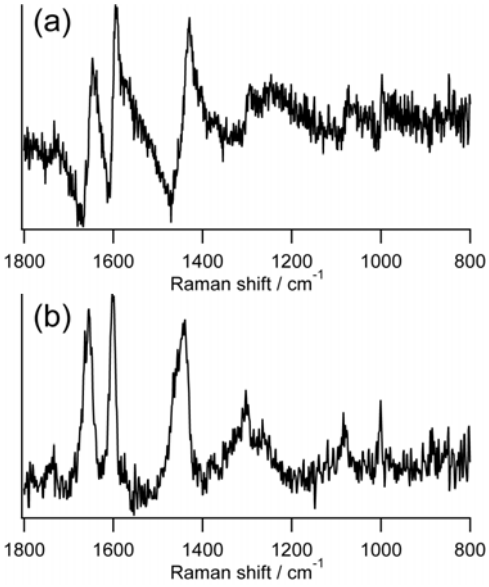

Fig. 1 (a) Typical CARS spectrum in the fingerprint region of a living budding yeast cell. (b) $\mathrm{Im}[\chi^{(3)}]$ spectrum of (a) with MEM.

CARS molecular fingerprinting enables us to obtain label-free and multi-colour images with high speed. Although the fingerprint region is spectrally congested in a living yeast cell, more than 10 vibrationally resonant $\mathrm{Im}[\chi^{(3)}]$ images by MEM have been successfully extracted. This method has been also applied to a time-resolved study of dynamical processes in a single living yeast cell such as rapid cell death by the laser irradiation and the cellular uptake of surfactant molecules.

Multi-focus confocal Raman microspectrometer

The developed apparatus is schematically shown in Fig. 2. It consists of an inverted microscope, a micro-lens array, a pinhole array, a fiber-bundle and a multichannel spectrometer. The micro-lens array is used to split the illuminating laser into beamlets such that the objective lens produces a pattern of 8 x 8 independent foci at the sample. The role of the pinhole array is to improve the axial spatial resolution and to enhance the ratio of the Raman signal to the background by the confocal effect. This multi-confocal configuration is indispensable for obtaining high contrast images of living cells in which weak Raman signals are easily overwhelmed by the strong background arising from the

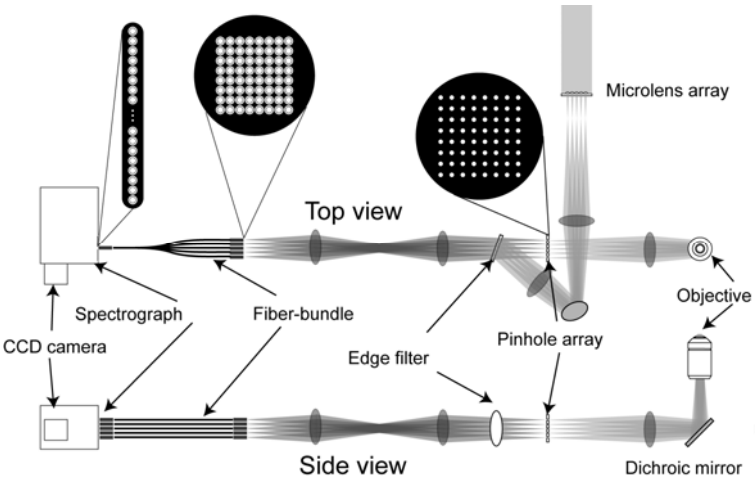

Fig. 2 Schematic of multi-focus confocal Raman microspectrometer

cover glass and the cell culturing medium. The 64 fiber-bundle is arranged in a 8 x 8 rectangular pattern at the collection end and a 1 x 64 linear stack at the detection end, which is connected to the entrance slit of the spectrograph directly. Using this fiber-bundle, a 3-D (2-D spatial and spectral) data cube is converted into a 2-D data array, which can be detected simultaneously by a 2-D CCD camera. The lateral and axial spatial resolutions of the system are determined to be 300 nm and 1 μm, respectively. In the present setup, 48 spectra from 48 points (X: 8 points Y: 6 points) are detectable with one CCD exposure.

Multi-vibratioal mode Raman images obtained at 1446 cm^{-1} (Fig. 3 (a)), 1583 cm^{-1} (Fig. 3 (b)), 1602 cm^{-1} (Fig. 3 (c)) and 1655 cm^{-1} (Fig. 3 (d)) are shown in Figs. 3. The Raman bands at 1446 cm^{-1}, 1583 cm^{-1} and 1655 cm^{-1} are assigned to the CH bend, the porphyrin in-plane C=C stretch mode of the porphyrin skeleton and the superposition of the *cis*-C=C stretch of unsaturated lipid chains and the amide I mode of proteins, respectively. The Raman band at 1602 cm^{-1} is called the "Raman spectroscopic signature of life". These images show similarity and variation, originating from distributions of different chemical species giving rise to different Raman bands. In the experiment, the total laser power at the sample is ~70 mW, so that the laser power at each focus is approximately 1 mW. It is obvious that a ~ 70 mW single focus totally destructs living cells. The distribution of the total laser power into multiple foci significantly reduces the photodamage. The overall measurement time is 20 sec and the images are reconstructed from 768 Raman spectra. This is equivalent to less than 25 msec for measuring one Raman spectrum. It is not possible to obtain a high S/N Raman spectrum in such a short exposure time by conventional Raman microspectroscopy.

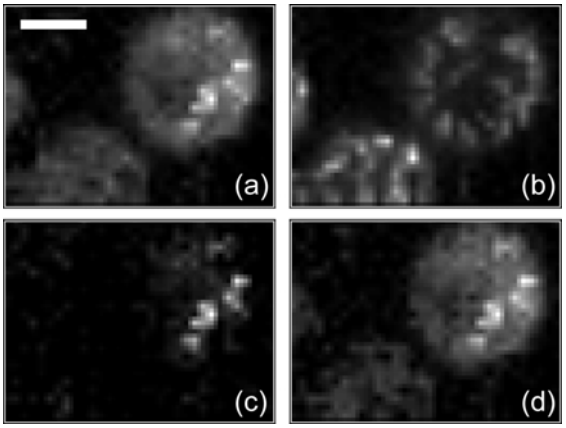

Fig. 3 Raman images of budding yeast cells of the Raman bands at (a) 1446 cm^{-1}, (b) 1583 cm^{-1}, (c) 1602 cm^{-1}, (d) 1655 cm^{-1}. The total acquisition time is approximately 20 sec. The scale bar is 4 μm.

Absolute quantitative imaging by Raman microspectroscopy

Raman signal intensity is strictly proportional to the number of molecules in the focal volume. The number of Raman scatted photons can be expressed as follows:

(Number of Raman scattered photons in unit time [/s])
= (Absolute Raman differential cross-section of molecule [cm^2 / sr])
x (Photon flux density of the incident light [/s cm^2]) x (Number of molecules in the focal volume)
x (Collecting solid angle [sr]) x (Detection efficiency [%]).

This equation indicates that the number/concentration of molecules in the focal volume can be estimated if the absolute Raman cross-section is known. The absolute Raman cross-section is a molecular property, which is a specific constant for a Raman band of a molecule and independent of experimental conditions except for the excitation wavelength. In order to determine absolute Raman cross-sections of biomolecules, polarized Raman measurements have been performed by using a confocal Raman microspectometer, whose polarization

characteristics and the wavelength dependence of sensitivity are accurately calibrated. From the observed polarized Raman spectra, the parallel and perpendicular components of the absolute Raman cross-sections of several biomolecules are determined with accuracies of 20 %.

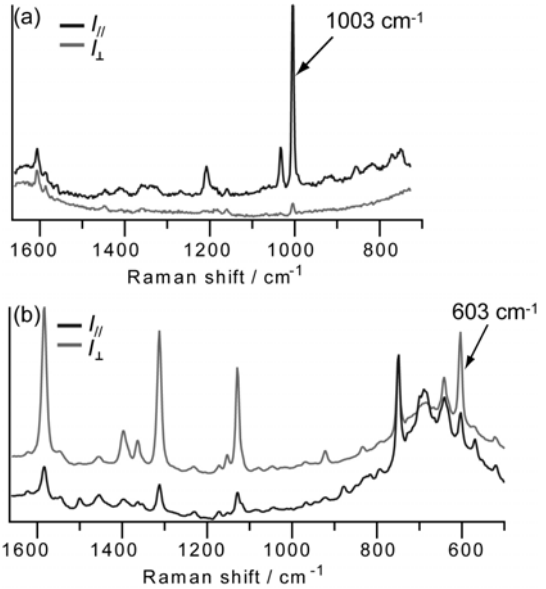

Fig. 4 The parallel ($I_{//}$) and perpendicular ($I_{\perp}$) Raman spectra of phenylalanine (a) and ferrous cyt. *c* solutions (b).

Figures 4 show the parallel and perpendicular Raman spectra of phenylalanine and cytochrome *c* reduced form (ferrous cyt. c) aqueous solutions. Phenylalanine has a marker band at 1003 cm^{-1} and ferrous cyt. *c* at 603 cm^{-1}. From these polarized spectra, the parallel and perpendicular components of the absolute Raman cross-section of the 1003 cm^{-1} Raman band are determined to be 0.96 and 0.05 x 10^{-29} [cm^2/molecule sr], respectively. Those of the 603 cm^{-1} band of ferrous cyt. c are determined to be 2.5 and 4.6 x 10^{-26} [cm^2/molecule sr]. The absolute Raman cross-section of ferrous cyt. *c* is more than 1000 times larger than that of phenylalanine due to the resonance Raman effect, which enables sensitive and specific detection of ferrous cyt. *c*.

Using these values, the concentration of the phenylalanine residue in proteins and that of ferrous cyt. *c* are estimated in a cell (L929 NCTC) as shown in Figs. 5. The distribution of the phenylalanine residue, which is known as an indicator of protein abundance in a cell, is rather homogeneous inside the cell with 40~70 mM concentrations. On the other hand, that of ferrous cyt. *c* is highly localized at the marginal area of the cell, most probably reflecting the distribution of mitochondria in the cell. The estimated concentration of ferrous cyt. *c* in mitochondria is typically 10~30 μM. The concentration of phenylalanine in mitochondria is 50~70 mM, while that in the other area is about 40 mM. As far as the author is aware, these absolute numbers of concentrations have never been estimated with any other existing methods of molecular imaging.

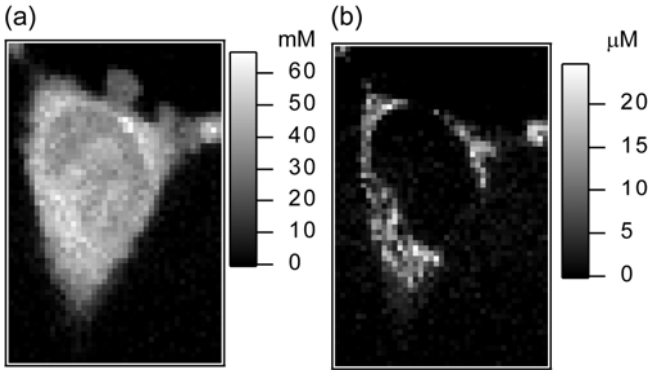

Fig. 5 The molecular concentration images of a L929 (NCTC) cell. (a) phenylalanine, (b) ferrous cyt. *c*

New linear and nonlinear Raman microspectroscopic systems have been constructed. Raman imaging speed has been improved to be more than 10 times faster than that of the conventional confocal Raman microspectroscopy. Absolute quantitative imaging of a living cell is also demonstrated. The Raman cross-sections of the protein and ferrous cyt. c marker bands have been determined and their concentrations in a L929 NCTC cell estimated *in situ* without staining. A new potential of Raman microspectrosopy is thus shown for fast and absolutely quantitative molecular imaging