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In Vivo Resonance Raman Detection of Ferrous Cytochrome *c* from Mitochondria of Single Living Yeast Cells

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Raman spectra of mitochondria in living budding yeast cells (zygote of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*) have been recorded with 532 nm excitation. Strong and sharp Raman bands are observed at 1584, 1315, 1129, 749, and 601 cm^{-1}, in addition to known phospholipid bands. These Raman bands are not observed in the corresponding Raman spectrum of mitochondria obtained with 632.8 nm excitation. Their peak positions and relative intensities agree excellently with the reported resonance-enhanced Raman bands of ferrous cytochrome *c*, which has a Q-band absorption at 520 nm. We have thus succeeded in detecting ferrous cytochrome *c*, one of the key intermediates in the electron-transport chain in mitochondria, in vivo without any pretreatment. In vivo resonance Raman detection of ferrous cytochrome *c* opens up new possibilities for real-time and quantitative tracing of respiration dynamics in mitochondria in a single living cell.

In the last decade, Raman microspectroscopy has been proved to be a powerful tool for studying living cells in vivo and at the molecular level. We have applied this method to yeast, one of the most basic model systems for eukaryote cells and reported several new findings related to single cell molecular dynamics.[1–5] Our goal is to establish a new physicochemical means for in vivo, real-time, and quantitative monitoring of cellular bioactivities. We have already reported the finding of a strong unknown Raman band at 1602 cm^{-1} from mitochondria of living yeast cells and have shown that the intensity of this band sharply reflects the metabolic activity of mitochondria. We call this band the "Raman spectroscopic signature of life".[2,3] In the most recent paper, we have suggested that this 1602 cm^{-1} band is a resonance-enhanced band from ubisemiqionone, an intermediate in the electron-transport chain in mitochondria.[6] In the present study, we examine the resonance effect in the Raman spectra of yeast cell mitochondria using two different excitation wavelengths, 632.8 and 532 nm. In previous studies, we used the 632.8 nm line in order to avoid fluorescence and photodamage. If a shorter wavelength is used for excitation, we have a good chance for the resonance Raman detection of other new species that have electronic absorptions in the visible region. We report in the following the observation of the resonance Raman spectrum of ferrous cytochrome *c* from mitochondria in a single living budding yeast cell.

Raman spectra of living yeast cells were obtained by two Raman microspectrometers with different excitation wavelengths. The details of the apparatus are described in our previous reports.[1–5] We used two different lasers for Raman excitation; the 632.8 nm laser line of a He-Ne laser and the 532 nm line of a Nd:YVO_4 laser. The other components, microscopes, spectrometers, and detectors were optimized for the detection of the fingerprint region (1000–1800 cm^{-1}) for each excitation wavelength. Accumulation time was 60 s for the 532 nm excitation and 100 s for the 632.8 nm. The excitation laser power at the sample point was less than 1 mW for 532 nm excitation and 6 mW for 632.8 nm excitation.

Cells (zygote of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*) were grown at 30 °C under aerobic conditions in YPD medium overnight. YPD medium is a complete medium for budding yeast, which contains 1% yeast extract (w/v), 2% polypeptone (w/v), and 2% D-glucose (w/v). The cells in the stationary phase were used. They were mounted between slide and cover glasses for Raman measurements under a microscope. The thickness of the sample was nearly the same as the diameter of a single budding yeast cell, so that the cells were immobilized between the slide and cover glasses. The sample was sealed with vaseline in order to prevent water evaporation during the experiments. The Raman spectral measurements were carried out at room temperature (25 °C).

The Raman spectra of mitochondria in single living budding yeast cells excited at 532 nm (spectrum 1A) and at 632.8 nm (spectrum 1B) are compared in Figure 1. The spectrum 1B is an average of 10 spectra from mitochondria of 10 different cells. It shows the Raman spectrum of mitochondria that we have already reported and assigned. Raman bands at 1655, 1445, 1302, 1260, and 1080 cm^{-1} are assigned to the bands of phospholipids, which are the dominant components of mitochondria membrane. In addition to these phospholipid bands, the "Raman spectroscopic signature of life" is observed at 1602 cm^{-1}. In the spectrum 1A, several Raman bands that are absent in the spectrum 1B do appear. In particular, five bands are distinct at 1583, 1315, 1130, 749, and 601 cm^{-1}. Considering the fact these bands are absent in the 632.8 nm spectrum, they are likely to be enhanced by the resonance Raman effect. Thus, these bands are assigned to a molecular species that has an electronic absorption around 532 nm. A number of yeast cells also showed the same five bands with the same relative intensities. Therefore, they are considered to derive from a single molecular species.

The difference spectrum between the two spectra in Figure 1 (Figure 1A − Figure 1B) is shown in Figure 2A. In this difference spectrum, several weak Raman bands are observed at 1498, 1395, 1362, 1172, 923, 690, and 481 cm^{-1}, in addition to the five strong bands described above. The peak at 1583 cm^{-1} has a shoulder due to the 1602 cm^{-1} band. It indicates that the intensity of the 1602 cm^{-1} band increases as the excitation wavelength changes from 632.8 to 532 nm. The possibility of resonance Raman enhancement of the 1602 cm^{-1} band has already been discussed.[2–6] Here, we focus on the new

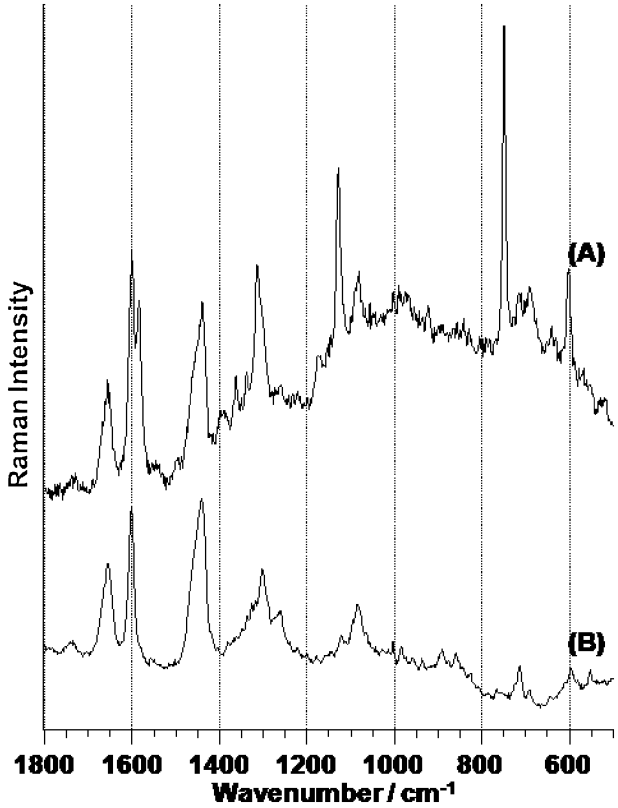

Figure 1. (A) Space-resolved Raman spectrum of mitochondria of a single living budding yeast cell excited at 532 nm and (B) that excited at 632.8 nm.

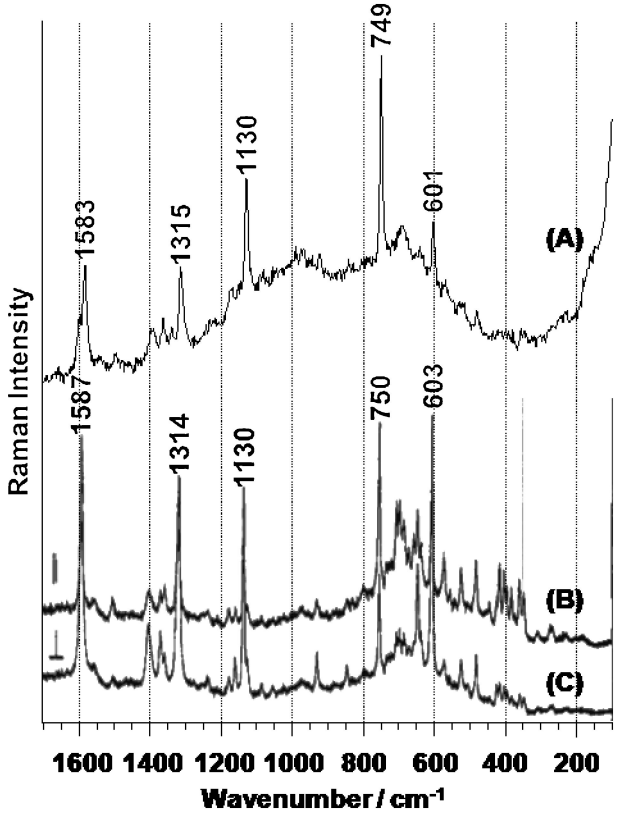

Figure 2. (A) Difference spectrum (Figure 1A − Figure 1B), (B) polarized resonance Raman spectrum (∥) of ferrous cytochrome *c* excited at 530.9 nm, and (C) polarized resonance Raman spectrum (⊥) of ferrous cytochrome *c* excited at 530.9 nm. The spectra in Figures 2B and 2C are reproduced from Figure 3 of ref 7.

bands that emerge with 532 nm excitation. In Figure 2, two polarized resonance Raman spectra of ferrous cytochrome *c* excited with 530.9 nm laser light are reproduced from Figure 3 of a paper by Hu et al.[7] The reported Raman bands of ferrous cytochrome *c* show an excellent agreement with those in the difference spectrum in Figure 2A, both in peak positions and relative intensities. We, therefore, conclude that these additional Raman bands in the 532 nm excited spectra originate from ferrous cytochrome *c* acting in mitochondria.

The ferrous state of cytochrome *c* is known to have visible absorption bands at 416 nm (B-band) and at 520 and 550 nm (Q-bands).[7] The 532 nm laser line is in a rigorous resonance with one of the Q-bands. This resonance condition is considered to facilitate the strong enhancement of the ferrous cytochrome *c* Raman bands in Figure 1A. The absence of the ferrous cytochrome *c* bands in the 632.8 nm spectrum is also well accounted for with this resonance condition. The in vivo resonance Raman detection of the ferrous state (and not of the ferric state) of cytochrome *c* has an important meaning. Cytochrome *c* is located in the intermembrane space bound to the innermembrane of mitochondria. The complex III (cytochrome *c* reductase) provides electrons to cytochrome *c* to induce the transition to the ferrous state. The complex IV (cytochrome *c* oxidase) then picks up the electrons from cytochrome *c* to put it back to the ferric state. Therefore, the detection of the ferrous state of cytochrome *c* means that Raman microspectroscopy can quantitatively monitor the electron flow in the electron-transport chain of mitochondria in a single living yeast cell.

In the course of the present study, we have noticed that the intensities of the ferrous cytochrome *c* bands relative to those of phospholipid bands fluctuate markedly cell to cell. It is highly likely that this fluctuation reflects rapid dynamics of mitochondrial activity like respiration oscillation.[8] Time- and space-resolved measurements of the ferrous cytochrome *c* Raman bands are now in progress.

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