論文内容の要旨

Elucidating the Origin of the "Raman Spectroscopic Signature of Life" in Yeast Cells
(酵母の「生命のラマン分光指標」の起源の解明)

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The "Raman spectroscopic signature of life" is a very unique Raman band at 1602 cm^{-1} first discovered in *Schizosaccharomyces pombe* around ten years ago. Since its discovery, it has been well established that the signature sharply reflects the metabolic activity of cells. Cells incubated in culture media that contain yeast extract possess stronger 1602 cm^{-1} signature than those incubated without yeast extract while other major Raman signatures remain unchanged [1]. Stress inducers such as H_2O_2 and respiration deficiency achieved by adding KCN to wild type yeast cells or mutation to petite strains also causes the intensity of the 1602 cm^{-1} signature decrease after laser irradiation [1-3]. Two days of anaerobic culture weakens the intensity of the 1602 cm^{-1} signature to less than 1/10 of its original intensity [1]. Further investigations even reported that the 1602 cm^{-1} signature is related to the spontaneous death process of *Saccharomyces cerevisiae* [4]. All these experimental results clearly show that the "Raman spectroscopic signature of life" is a reliable metabolic activity indicator of living yeast cells. However, for the better understanding of the signature behaviour and its further application in biological researches, the molecular origin of this signature must be elucidated first.

In this thesis, I have tried to address many long existing issues on the "Raman spectroscopic signature of life". The Chapter 1 of this thesis is a general introduction of Raman spectroscopy starting from the discovery of the Raman effect by C. V. Raman back in 1928 to its application in biology in the recent years. It also describes the history of the "Raman spectroscopic signature of life" and explains in detail the debates that have showed up in the course of the study. One major

issue on the signature has been whether its intensity is directly related to respiration activity. The assumption that the intensity of the signature represents respiration activity has led to the "ubisemiquinone hypothesis", which assigns the ubisemiquinone radical anion as the origin of the signature. Chapter 2 describes the first systematic study of the 1602 cm^{-1} signature in an isolated organelle. It has confirmed the existence of the signature in the isolated enriched mitochondria fraction from yeasts. Furthermore, the study has clearly showed that the intensity of the signature does not directly represent respiration activity. The mechanism of how the signature intensity decreases after KCN and NaN_3 treatment is also described in the chapter. Chapter 3 describes the first yeast knockout experiment that aimed to reveal the nature of the 1602 cm^{-1} signature. The experimental results on ubiquinone knockout yeasts have indicated that the ubisemiquinone radical anion is not the sole origin of the 1602 cm^{-1} signature. Further studies on haem knockout yeasts have helped us to propose several other possible candidates as the origin of the signature. Chapter 4 combines the techniques mentioned in Chapter 2 and Chapter 3 as well as new experiments on the authentic ergosterol sample and showed that ergosterol is the main origin of the "Raman spectroscopic signature of life". It is the key chapter of the thesis and the chapter title is the same as the thesis title. Chapter 5 is the final chapter of the thesis and concludes the whole work. It also gives perspectives on the application of the signature in biological studies. In the following paragraphs, I will focus on the key content of the thesis and briefly explain how I elucidated the origin of the "Raman spectroscopic signature of life".

The approach I used to elucidate the origin of the "Raman spectroscopic signature of life" was to step by step isolate individual cellular compartments and track the existence of the signature to finally determine its molecular origin. Since respiration deficiency largely affects the behaviour of the signature, it has long been speculated that the signature originates from mitochondria. To verify the speculation, mitochondria were isolated by differential centrifugation from homogenized yeasts. After the last step of the differential centrifugation, the mitochondria rich fraction will settle into the pellet and a white layer of lipid mainly composed of lipid droplets will come to the top of the supernatant. Figure 1 shows the Raman spectra of tetraploid and haploid yeasts as well as the spectra of the isolated organelles taken from the mitochondria rich fraction and the lipid droplet rich fraction of the two strains. It is very clear that the 1602 cm^{-1} signature exists in both the mitochondria rich fraction and the lipid droplet rich fraction. This result strongly suggests that the 1602 cm^{-1} signature belongs to a stable lipid structure in yeasts.

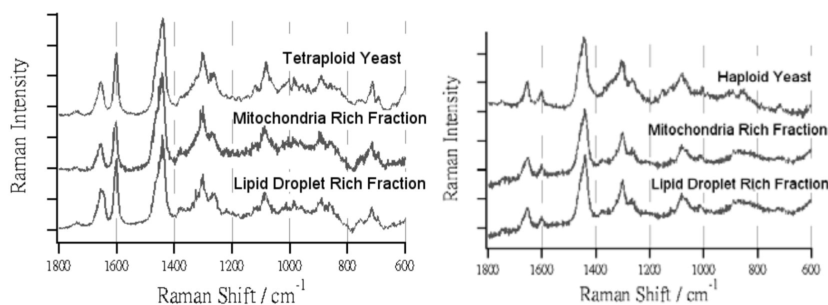


Figure 1. Left: Raman spectra of tetraploid yeast (top) and the mitochondria rich (middle) and the lipid droplet rich (bottom) fractions isolated from tetraploid yeast cells. Right: Raman spectra of haploid yeast (top) and the mitochondria rich (middle) and lipid droplet rich (bottom) fractions isolated from haploid yeast cells.

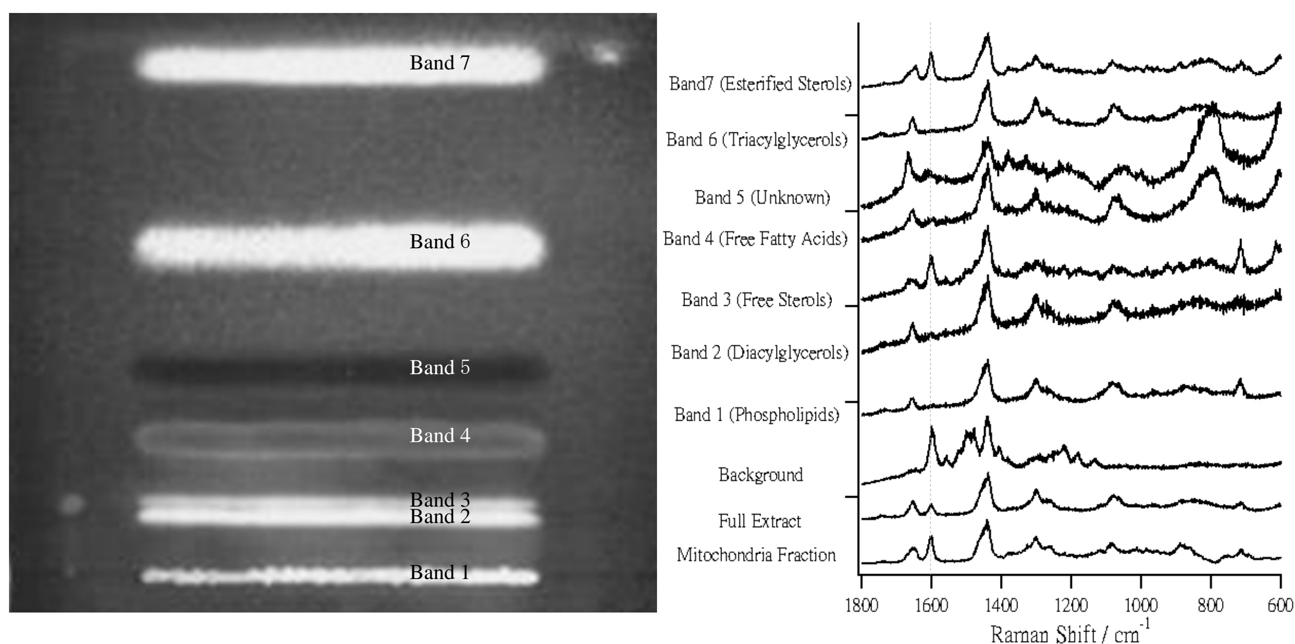
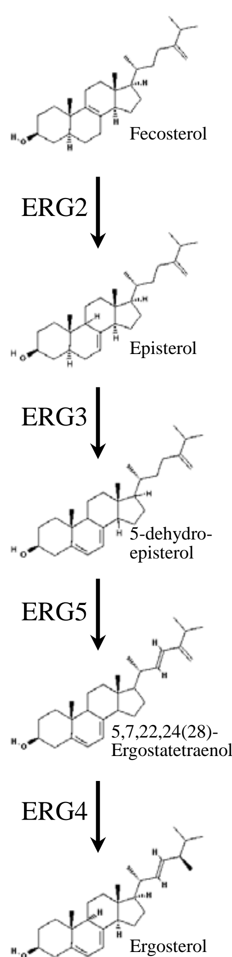


Figure 2. Left: The TLC pattern of lipids extracted from the mitochondria rich fraction. The standard on the left of the plate is free sterol and the standard on the right is esterified sterol. Right: The Raman spectra and molecular content of each TLC bands respectively and the spectra of the dark background of the TLC plate (Background), the total lipid extract of the mitochondria rich fraction (Full Extract) and the organelles taken directly from the mitochondria rich fraction (Mitochondria Fraction). The grey dash line indicates 1602 cm^{-1} Raman shift.

Since the 1602 cm^{-1} signature is likely to originate from a stable lipid structure, the next step is to isolate the stable cellular lipid components by thin layer chromatography (TLC) and check which molecule the signature comes from. Figure 2 shows the TLC pattern of lipids extracted from the mitochondria rich fraction and the Raman spectra of each TLC bands respectively. It is very clear that the 1602 cm^{-1} signature appears in the free sterol and esterified sterol TLC bands while all other bands do not show the existence of the 1602 cm^{-1} signature. Although a Raman band around 1602 cm^{-1} is seen in the background spectrum, which is likely to be the primuline dye evenly sprayed on the TLC plate for the visualization of lipid bands under ultraviolet irradiation, other major Raman signatures in the background are not seen in the spectra of the TLC bands, suggesting that the Raman spectra of the TLC bands are essentially background-free. This experimental result indicates that the origin of the “Raman spectroscopic signature of life” is most likely the major sterol structure in yeasts. Since the major sterol structure in yeasts is ergosterol, it is clear at this point that ergosterol is an important contributor of the 1602 cm^{-1} signature in yeast cells.

Despite knowing ergosterol as an important contributor of the signature, the exact molecular vibration that gives rise to the signature still remains unclear. Since isolation techniques are no longer useful at this point, other strategies are needed to resolve the problem. Here, I performed a series of experiments on yeast knockout strains impaired in the ergosterol synthesis pathway to elucidate this “exact” origin of the 1602 cm^{-1} signature. Figure 3 shows the final four steps of the ergosterol synthesis pathway and the spectra of wild type and knockout yeasts that are impaired in the four steps respectively. The Raman spectra clearly show that yeasts knocked out upstream of

the ERG3 gene does not have the 1602 cm^{-1} signature and those knocked out downstream of the formation of 5-dehydroepisterol, including wild type yeast, show the signature. This experimental result strongly supports the statement that the 1602 cm^{-1} signature originates from the conjugated 5,7 diene structure because only the sterol structures downstream of 5-dehydroepisterol in the synthesis pathway have the 5.7 diene conjugation. Therefore, the essential meaning of the data set is that yeasts which could not synthesize the 5,7 diene conjugation structure do not have the 1602 cm^{-1} signature; while all those that could synthesize have the signature.



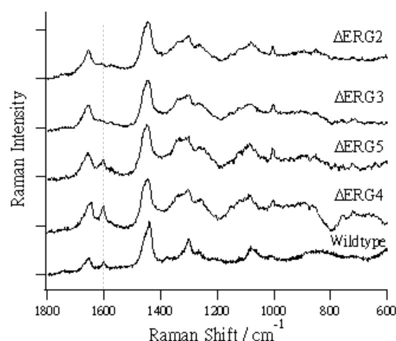


Figure 3. Left: The final four steps of the ergosterol biosynthesis pathway. ERG2 ~ ERG5 are the genes that encode the proteins responsible for catalyzing the reaction shown in the pathway. The structures are retrieved from PubChem. Up: The Raman spectra of the wild type and knockout yeasts. The grey dash line indicates 1602 cm^{-1} Raman shift.

The experimental results so far have led to the conclusion that the conjugated 5,7 diene structure in ergosterol is an important contributor of the “Raman spectroscopic signature of life”. However, the sterol spectra in Figure 2 showed multiple strong bands other than the 1602 cm^{-1} signature, which is contradictory to previous observations that the signature exists as a single strong band. It is because the samples measured in Figure 2 are in their solid phase and the Raman spectrum of ergosterol is very different in solid phase and solution phase. Figure 4 shows the Raman spectra of ergosterol dissolved in chloroform and the spectrum of the depleting component when NaN_3 treated mitochondria are exposed under laser irradiation. The ergosterol spectrum here shows a single strong Raman signature around 1602 cm^{-1} and many similarities could be found between the two spectra. All these data supported the idea that the conjugated 5,7 diene structure in sterols (especially ergosterol in yeasts) is an important contributor of the “Raman spectroscopic signature of life”.

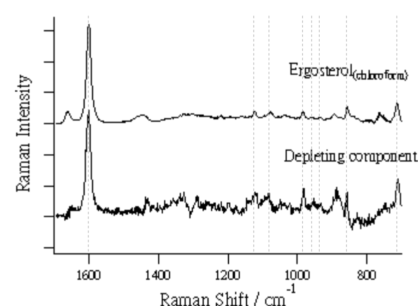


Figure 4. The Raman spectra of ergosterol dissolved in chloroform (up) and the depleting component observed *in vivo* while the 1602 cm^{-1} signature weakens. The chloroform background is already subtracted from the upper spectrum. The grey lines indicate similar spectral patterns between the two spectra.

References:

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