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Chemotaxonomic Raman Spectroscopy Investigation of Ascomycetes and Zygomycetes

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Enormous diversity exists in fungi, which cause epidemic diseases and infections by producing mycotoxins.[1] Conventional fungal identification methods involve elaborate observations and time-consuming measurements of macroscopic and microscopic morphologies. The drawbacks with these methods include the need for an experienced investigator and the length of time taken to process the data. More recently, bioinformatic methods based on sequencing parts of the genome have been used to analyze fungi or fungal extracts by introducing chemotaxonomical markers.[2,3] It is necessary to introduce a novel monitoring method that can accurately and efficiently identify fungal species. Spectroscopic analysis, which requires little sample preparation procedures, would meet the purpose of rapid analysis or identification of fungi.[4]

Raman spectroscopy is a molecular fingerprinting technique with several benefits and advantages such as non-destructive illumination with a quick response time and facile sample preparation.[6,7] Only tiny sample amounts are required to obtain the Raman spectra to make this method suitable to monitor living organisms. Narrow spectral bandwidths and a low water background in Raman spectroscopy make this method well suited for the investigation of biological samples in the presence of complex, water-containing, and time-varying compositions. With the attachment of a microscope, a small number of single spores from fungi can be studied using micro-Raman spectroscopy.[8,9] Previous works on chemotaxonomic identification of fungi have mainly focused on unicellular yeasts.[10-14] In this work we have performed a Raman spectroscopic study of the two different fungal species: ascomycetes and zygomycetes in order to test rapid chemotaxonomic identification by means of a spectroscopic technique.

Figure 1 shows our experimental set-up for the study of fungal species. This design is almost identical to study the mammalian cells using gold nanoparticles (NPs).[15] Since NPs were not introduced to generate surface-enhanced Raman scattering effects, it took more time and effort to obtain Raman signals for this study. By taking an advantage of z-depth scanning using a confocal Raman spectrometer, the interior and outside of fungal cells could be differentially monitored for this study. It was beneficial to introduce dark-field microscopy (DFM) to monitor the fungal cells. Although we also checked the bright images, it seems more conspicuous after introducing DFM images of fungal species.

Since the sizes of fungal cells are smaller than those of mammalian cells, it was not easy to locate fungal cells in this study. To study fungal species, we first attempted to observe Raman spectra using portable Raman spectrometer without much success. It seems that a confocal set-up may be required to observe Raman signals at the single spore level. By introducing confocal Raman spectrometer, we could manage to obtain proper Raman signals as reported previously.[8,9] We first tested Raman spectra of the yeast *Saccharomyces cerevisiae* to fail to observe strong features as reported previously.[10-14] Although not shown here, we could find characteristic peaks from yeast using a confocal Raman spectrometer excited at 532 nm. Due to their smaller sizes and weak intensities without NP enhancements, we could not observe much stronger Raman signals from *Saccharomyces cerevisiae* using our DFM/Raman spectrometer. We tested approximately 10 fungal species to obtain Raman spectra from several samples using confocal Raman spectrometers excited at either 532 nm or 633 nm. We observed Raman peaks from several fungal species including *Aspergillus fumigates*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium aculeatum*, *Penicillium herquei*, and *Penicillium glabrum*. We performed Raman spectroscopy for both hyphae and single fungal cells. It has to be admitted that spectral features varied depending on the spectral data acquisition points. It was not certain whether Raman spectra vary depend-

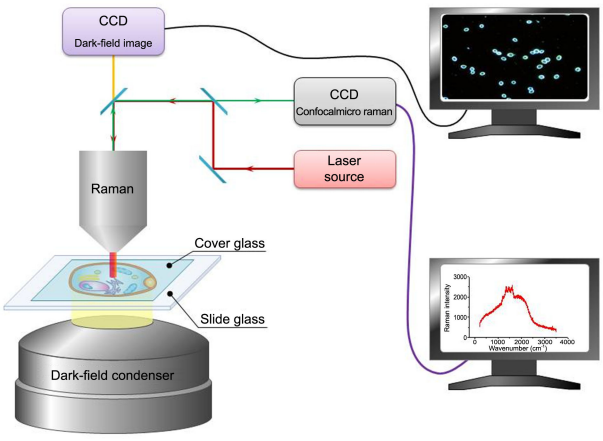

Figure 1. Schematic diagram of fungal cell measurements using a DFM-equipped confocal Raman spectrometer.

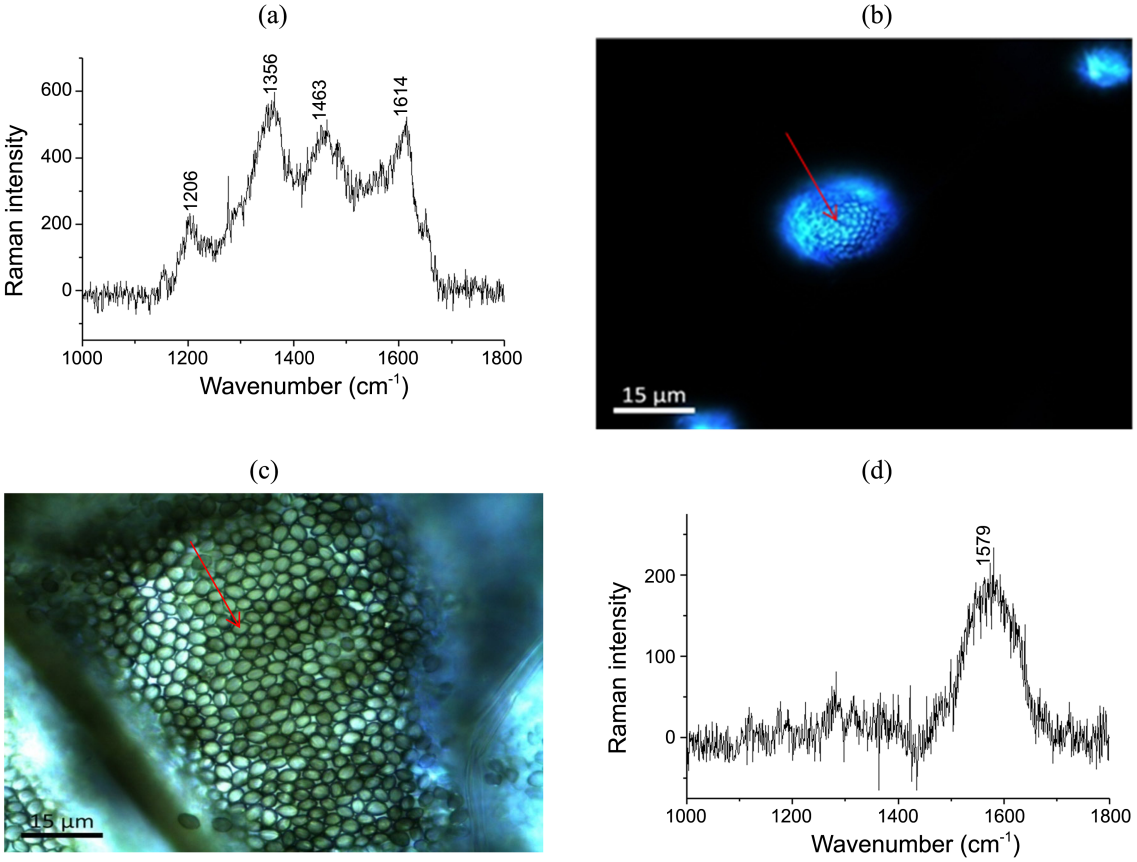

Figure 2. (a) DFM and (b) Raman spectra of ascomycete *Penicillium funiculosum*. (c) DFM and (d) Raman spectra of zygomycete *Rhizopus oryzae*. The scale bars in the DFM images are 15 mm.

ing on the cell division processes. Although we could indentify characteristic peaks as previously reported,[8,9] further investigation seems necessary to apply Raman spectroscopy to obtain consistent spectral data.

For chemotaxonomic identification of fungi, Raman spectra of the two fungal species were compared in this study. Figure 2(a) and (b) shows the DFM image and the corresponding Raman spectrum of the ascomycete *Penicillum funiculosum*. The strong bands at 1206, 1356, 1463 and 1612 cm^{-1} were observed, consistent with a previous report.[8] Although the band at 1614 cm^{-1} was ascribed to the ergosterol peak of the fungal cell walls,[13] its broad features could be ascribed to the chitin peaks.[8] In fact this band at 1614 cm^{-1} was reportedly quite sensitive to the process of cell divisions. On the other hand, the two broad bands at 1463 and 1356 cm^{-1} were assigned to the deformation band of methyl groups in phospholipids and the beta glucan peaks of the fungal cell walls, respectively. The band at 1206 cm^{-1} can be assigned to phospholipid peaks.[8] Figure 2(c) and (d) shows the DFM image and corresponding Raman spectrum of zygomycete *Rhizopus oryzae*. The strong broad band centered at 1579 cm^{-1} was ascribed to the mixed modes of the amide II and chitin peaks.[8] We checked several points of the samples and found spectral differences between the two fungal species. Despite the broad features, it seems possible to apply Raman spectroscopy for chemotaxonomic identification. The DFM images of the fungi were useful to find the cells without using any highly luminescent metal nanoparticles for this study. Since the DFM set-up in Figure 1 is mainly geared for large mammalian cells, it seems necessary to improve the resolution in monitoring smaller fungal species. As illustrated in Figure 2(a) and (c), we could not exactly differentiate the two fungal species only by microscopic images. After applying Raman spectroscopy, chemotaxonomic identification was possible. This fungal identification was in line with the recent study.[8] It has to be mentioned that we observed a group of fungal cells in Figure 2 instead of single pore or cell. As seen in the scale bars of Figure 2(a) and (c), the fungal cells appeared to be as small as 5 μm. We plan to improve our experimental configuration to aim at monitoring smaller fungal cells at the single cell level.

Most Raman bands could be attributed to specific components of the fungal cell walls. In agreement with the biological role of fungal spores, high amounts of lipids were observed along with carbohydrates. Different types of phospholipids and polysaccharides are expected to be detected in the Raman spectra of fungal species. These differences may be due to dissimilar saccharides, lipids and some minor compounds depending on fungal species. These compounds in cell walls provide specific biomarkers. It has to be mentioned that the Raman spectra of yeast cells reportedly

change depending on the cell division. Since broad spectral bands may cause the exact assignments problematic, we plan to apply the deconvolution analysis. We have attempted to resolve the spectral features of more than twenty fungal species and find spectral differences. Our preliminary results however demonstrate that Raman spectroscopic tools are useful for the chemotaxonomic analysis of zygomycetes and ascomycetes. We plan to continue to perform Raman spectroscopic analysis by optimizing the DFM and Raman data acquisition conditions and improving the sampling procedures.

Experimental

Penicillium funiculosum (ATCC13216) and *Rhizopus oryzae* (ATCC11145) were used in this study. These strains were purchased from the Korean Culture Center of Mircroorganisms (Seoul, Korea). *P. funiculosum* and *R. oryzae* were cultured on potato dextrose agar (PDA). Dark-field microscopy (DFM) was used to monitor the fungal cells.[16] The fungal cells were mixed with a fluorescent mounting medium (DAKO) and mounted on slide glass of the microscope. They were sandwiched with a cover glass for DFM-Raman measurements. To avoid the release of fungal cells into the air, the edges of the cover glass were painted with another mounting medium as shown in Figure 1. In order to avoid leaking of fungal species into laboratory air, special care was taken to seal the sample parts, differently from the case of mammalian cells. Fungi were monitored using DFM with a Leica DL LM upright microscope and a high-resolution CytoViva 150 adapter. Raman spectra were obtained using a Raman confocal system model 1000 spectrometer (Renishaw) equipped with an integral microscope (Leica DM LM). Spontaneous Raman scattering was detected with 180^{o} geometry using a peltier cooled (–70 °C) CCD camera (400 × 600 pixels). An appropriate holographic supernotch filter was set in the spectrometer for 632.8 nm from a 20 mW air-cooled HeNe laser (Melles Griots Model 25 LHP 928) with the plasma line rejection filter. The integration time for a single spectrum acquisition was usually 10 s. We obtained Raman spectra without any further accumulation.

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