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Complete List of Authors:	Onoshima, Daisuke; Nagoya University Uchida, Kentaro; Nagoya University Iida, Tomomine; Takeda Pharmaceutical Co Ltd Shonan Research Center Kojima, Takashi; Takeda Pharmaceutical Co Ltd Shonan Research Center Ikeda, Yukihiro; Takeda Pharmaceutical Co Ltd Takeda Shonan R and D Iwata, Daijiro; AGC Inc Nagasawa, Ikuo; AGC Inc Yukawa, Hiroshi; Nagoya University Baba, Yoshinobu; Nagoya University

Single-cell detection and linear discriminant analysis of bacterial Raman spectra in glass filter microholes

Daisuke Onoshima[1,*], Kentaro Uchida[2], Tomomine Iida[3], Takashi Kojima[3], Yukihiro Ikeda[3], Daijiro Iwata[4], Ikuo Nagawawa[4], Hiroshi Yukawa[1,2,5], and Yoshinobu Baba[1,2,5]

1. Institute of Nano-Life-Systems, Institutes of Innovation for Future Society, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

2. Department of Biomolecular Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan

3. Takeda Pharmaceutical Co., Ltd., 2-26-1, Muraoka-Higashi, Fujisawa, Kanagawa 251-8555, Japan

4. AGC Inc., Suehirocho 1-1, Tsurumi-ku, Yokohama-shi, Kanagawa, 230-0045, Japan

5. Institute for Quantum Life Science, National Institutes for Quantum Science and Technology (QST), Anagawa 4-9-1, Inage-ku, Chiba, 263-8555, Japan

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ABSTRACT: We report a study of micro-Raman spectroscopy towards an optimal approach for single cell measurements for the detection of bacteria by vibrational spectroscopy. The use of glass membrane filters was tested by microfiltration to separate individual bacterial cells. The glass membrane filters were applied to the study of Raman spectral classification analysis. This approach achieved the capture and individual detection of spiked bacterial cells. Linear discriminant analysis (LDA) of Raman spectra measured on glass membrane filters was successfully used to distinguish several bacterial species.

INTRODUCTION

Vibrational spectroscopy is a non-invasive technique that offers the possibility of experiments to access accurate information from the internal composition and surface structure of living bacterial cells [1-3]. While traditional microbiological diagnostics using culture often require weeks to obtain results, spectroscopic methods require minimal sample volume and short measurement time. Furthermore, spectroscopic methods can be applied to almost any microorganism. In recent years, rapid and reliable cellular analysis by infrared absorption and Raman spectroscopy in combination with statistical pattern recognition methods such as hierarchical cluster analysis (HCA) and principal component analysis (PCA) has shown promise [4-8].

Infrared absorption spectroscopy is well suited for probing vibrations that involve changes in dipole moments [9]. For example, CO stretching vibrations of amide bonds in peptides and proteins or organic esters in carbonic acids and lipids produce very strong absorption bands in the infrared spectrum. This has been reported to be useful for probing internal changes associated with bacterial growth and aging processes [2,3].

Raman spectroscopy helps to obtain rich spectral information about the overall chemical composition of bacteria by excitation in the visible wavelength range [10,11]. Recent Raman spectroscopy techniques allow laser light to be focused to the size of a bacterial cell (approximately 1 μm). Raman spectra recorded from single bacterial cells, also called Raman signatures, are used to identify bacteria [12]. This technique is not suitable for localizing bacterial cells from other biological components. Therefore, an appropriate separation protocol is needed to isolate bacterial cells. Various separation strategies have been reported, including density gradient centrifugation [13] and the capture of bacteria by antibodies immobilized on Raman-compatible substrates [14].

Here we demonstrate the use of our microfiltration device for single-cell level separation of bacteria and its application to Raman spectroscopic analysis. To characterize the glass fabricated filter device, we validated the capture and discrimination of bacterial cells and polystyrene (PS) particles of similar size. Discriminant analysis was performed on Raman spectra obtained from different bacterial species.

EXPERIMENTAL

Bacterial culture

Bacillus subtilis (NBRC 3134) and *Staphylococcus epidermidis* (NBRC 100911) were cultured in SCD medium (bioMérieux 51019). *Methylobacterium extorquens* (NBRC 15911) was cultured in R2A medium (NIPRO 12-036). Cultures were grown overnight in culture tubes (Evergreen Scientific 222-2376-080) at 32°C. Staining was performed by incubation in PBS with 10 nM of Hoechst (Thermo Fisher Scientific H1399) for 30 minutes at 32°C. Two centrifugations (x 500 g for 3 min and x 5000 g for 10 min) with sterile water were used for washing prior to testing.

Microfiltration Device

Samples were filtered through a glass membrane filter consisting of tapered-shaped holes. The glass membrane filter was made of quartz processed by laser and wet etching as in previous reports [15]. In summary, micropores were fabricated on AGC's high-speed processing equipment, and debris was

removed by etching followed by SiO_2 thin film coating to reduce the pore size. Pore size reduction was applied to achieve an appropriate size for separating bacterial cells. Quartz was utilized in place of alkali-free glass to minimize autofluorescence and background noise.

The glass membrane filter was inserted into a filter holder (SWINNEX SX0001300). A sample syringe (TERUMO ss-10LZ) was placed upstream of the filter holder. The sample syringe was set in a syringe pump (KD Scientific KDS210). A silicone tube (AS ONE S-3×5) was connected downstream of the filter holder. Samples were prepared by adding PS particles (Polysciences PolyFlor570 or Polybead) or cultured bacteria to 5 mL of ultrapure water; the number of PS particles was calculated based on the reagent data sheet. The number of bacterial cells was calculated based on the fluorescence intensity of the stain and the step dilution.

Micro-Raman Spectroscopy

Raman spectra were measured by a confocal Raman microscope (HORIBA Scientific Xplora Plus) equipped with a 100x objective. The confocal hole size was 300 nm, the slit size was 100 nm, and the grating was 600 g/mm. The laser wavelength was 532 nm, depth of focus was 4.5 μm, and spot diameter was 1 μm. An EMCCD with a cooling temperature of -60°C was used for detection. Quartz glass slides (Matsunami Glass t1.0) or glass film filters were used as supports for spectral measurements. Peaks in the bacterial cell spectrum were attributed according to previous reports [1,7,8,10,16]. Fluorescence images were acquired by specifying the spectral conditions for the dye.

Statistical Analysis

An open-source library for Python (scikit-learn 0.20.1) was used for linear discriminant analysis (LDA). The procedure was adapted from the official documentation. Spectral data were converted to comma-separated value (csv) files and all data sets were compiled.

RESULTS AND DISCUSSION

Capture and individual detection of bacterial cells

Microfiltration was tested using a sample of aqueous solution spiked with PS particles and bacteria (Figure 1a). The glass membrane filter was set in a filter holder and a 5 mL sample was extruded at 1 mL/min. Figure 1b shows an example of a glass membrane filter with 225,625 microholes in a 9.5 mm filter format. The film thickness was 200 μm. The microholes were aligned at a pitch of 20 μm and the

12 μm hole diameter tapered to 0.5 μm in depth. This was the result of narrowing the exit of the microholes by depositing SiO_2 using ion sputtering (Figure 1c).

Figure 2a is a schematic of microfiltration. Through a glass membrane filter, 100,000 each of fluorescent PS particles and *Staphylococcus epidermidis* stained cells were filtered. The glass membrane filter was removed from the filter holder after filtration for micro-Raman spectroscopy. PS particles and bacterial cells on the glass membrane filters were clearly distinguished. Figure 2b shows a typical example of the fluorescence image and Raman spectra detected on the glass membrane filter. Raman spectra of single PS particles and bacterial cells were measured at the position of their respective fluorescent images. The laser power was 20 mW, exposure time was 1.0 s, and the number of integrations was 2. The Raman spectra of the PS particles showed high scattering intensity. This intensity is due to the high density of uniform chemical bonds derived from the polymeric origin of the material. The Raman spectra of bacterial cells showed a strong peak of C-H stretching vibration (2930 cm^{-1}). This peak was not detected in the vacancies of the glass membrane filter.

Discrimination of bacterial spectrum

A portion of the sample containing bacterial cells was dropped onto a quartz glass slide and Raman spectra were measured after the droplets dried. *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Methylobacterium extorquens* were used as samples. Samples were measured 20 times each. The laser power was 25 mW, exposure time was 7.5 s, and the number of integrations was 2.

Figure 3a shows the results of normalization of the averaged Raman spectra. The standard for normalization was the peak intensity of the C-H stretching vibration. Multiple overlapping peaks were detected in the fingerprint region (700-1800 cm^{-1}). Some peaks were derived from amide bonds and aromatics in the protein, while others were weak peaks that could not be recognized alone. The Raman spectra of *Bacillus subtilis* and *Staphylococcus epidermidis* could not be distinguished by peak attribution. The Raman spectra of *Methylobacterium extorquens* showed peaks of relatively high intensity. *Methylobacterium extorquens* is a photosynthetic red bacterium. The cells contain carotenoid pigments, which have a resonance Raman enhancing effect [17]. The background of the support was detected as a SiO_2 peak (464 cm^{-1}), distinct from the fingerprint region [18].

Figure 3b shows the results of LDA for all the data of the Raman spectra in Figure 3a. LDA is one of the feature extraction methods. This method looks for the dimension (or axis) that best separates the classes of data and allows classification analysis by dimensionality reduction [19]. Dimensionality reduction is a tool that reduces multidimensional data to low-dimensional data with as little information

loss as possible. Raman spectra are treated as multi-dimensional vector data. The Raman spectra were dropped into a two-dimensional plot with the number of classes discriminated as 3. *Bacillus subtilis* and *Staphylococcus epidermidis* were clearly distinguished by LDA. Some plots were cross-classified. *Methylobacterium extorquens* showed less overlap of plots within classes. The amount of carotenoid pigments in *Methylobacterium extorquens* is variable. This variability probably affects the LDA calculations.

Microfiltration of the samples on glass membrane filters was applied for subsequent discriminant analysis of Raman spectra. PS particles, *Staphylococcus epidermidis*, and *Methylobacterium extorquens* were used as samples. Samples were measured 20 times each. The laser power was 50 mW, exposure time was 2.0 s, and the number of integrations was 2.

Figure 4a is a typical example of the Raman spectra detected on the glass membrane filter. The background noise for *Staphylococcus epidermidis* and *Methylobacterium extorquens* was reduced. This is presumably due to the effect of trapping bacterial cells in the microholes. Figure 4b shows the results of LDA for all the data of the Raman spectra in Figure 4a. The Raman spectra were dropped into a 3-dimensional plot with a class number discrimination of 4. PS particles, bacterial cells and vacuoles were clearly distinguished by LDA. Staphylococcus epidermidis and Methylobacterium extorquens were also distinguished by LDA. Reduction of background noise resulted in greater overlap of plots within classes.

Application to rapid microbial methods

Bacterial cells prior to testing were transferred to sterile water and placed in a nutrient-limited state for this experiment. This is intended for future aseptic testing. Bacterial growth goes through a logarithmic growth phase, followed by a stationary phase, and then a death phase [20]. Except under ideal conditions in the culture medium, many bacteria in the environment are in a starvation-like state. For example, sterility tests in factories and hospitals find bacteria contaminated in purified water [21]. *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Methylobacterium extorquens* are typical examples of bacterial species found in industrial environment [22].

Raman spectroscopy has attracted much attention in recent years as a technique applicable to rapid microbial methods [23]. Our microfiltration device can be configured to scale up the sample throughput. This is particularly useful when testing low bioburden samples in large volumes, such as water for pharmaceutical use. This allows for comparative testing with conventional methods. A faster test system could be developed by constructing a protocol to screen the microholes of glass membrane filters prior to measuring Raman spectra.

Potential applications of this method include inspection tools for food raw materials and products [24]. Food companies would be able to easily analyze the source of microbial contamination and predict the growth of microorganisms at the manufacturing site of their products. This would provide a new scientific basis for measures to improve the food sanitation environment. In addition, this method could be applied to environmental monitoring by obtaining not only measurement data at a certain point in time, but also continuous measurement data.

CONCLUSIONS

In this paper, we present a method for discriminating bacteria using a glass membrane filter and micro-Raman spectroscopy. By isolating single cells in microholes and measuring Raman spectra, the distinction of bacterial species by LDA was verified. This method is expected to be a tool for rapid identification of bacteria during aseptic testing.

The glass membrane filters in this paper are processed by SiO_2 deposition using ion sputtering. The size and shape of the microholes are designed to be more flexible. Compared to filters for cell filtration reported in previous studies [15,25], this study presents the value of using smaller size and higher density microholes. The precise microhole design allows for adaptation to instrument configurations with scaled-up sample throughput and optimized cell capture success rates. While commercial filters made of resin are cost-effective, the glass membrane filters in this paper are reusable and have high chemical durability.

AUTHOR INFORMATION

Corresponding Author

* E-mail: onoshima-d@nanobio.nagoya-u.ac.jp

Author Contributions

Concept and study design: Daisuke Onoshima, Kentaro Uchida, Tomomine Iida, and Takashi Kojima. Data acquisition: Daisuke Onoshima, Kentaro Uchida and Daijiro Iwata. Drafting of the manuscript: Daisuke Onoshima and Kentaro Uchida. Critical revision of the manuscript for important intellectual content: Tomomine Iida, Yukihiro Ikeda, Takashi Kojima, Daijiro Iwata,

Ikuo Nagawawa, Hiroshi Yukawa, and Yoshinobu Baba. All the authors approved the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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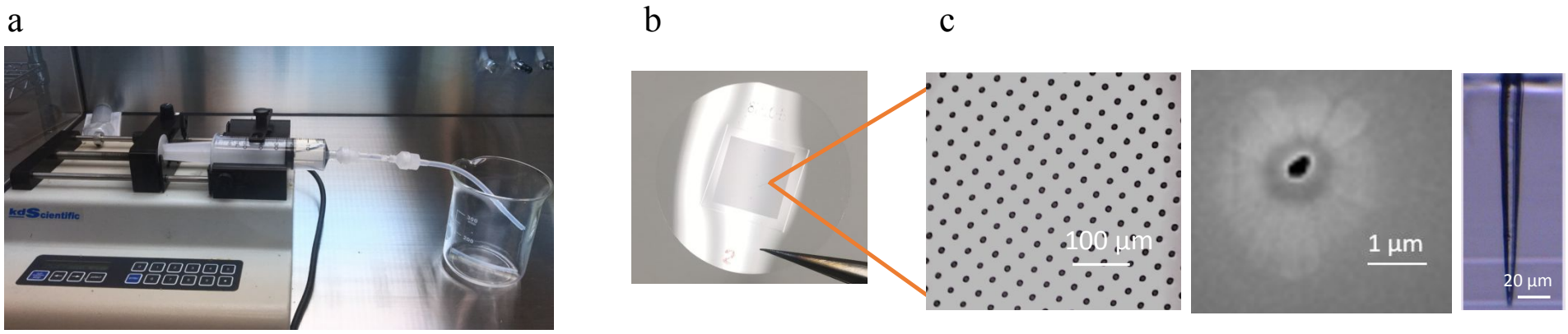

Figure 1. Microfiltration system for bacterial cells. (a) Photograph of the filtration device. (b) Photograph of 9.5 mm filter. (c) Microscopic image of the filter. Planar view of multiple and single hole followed by cross section of single hole.

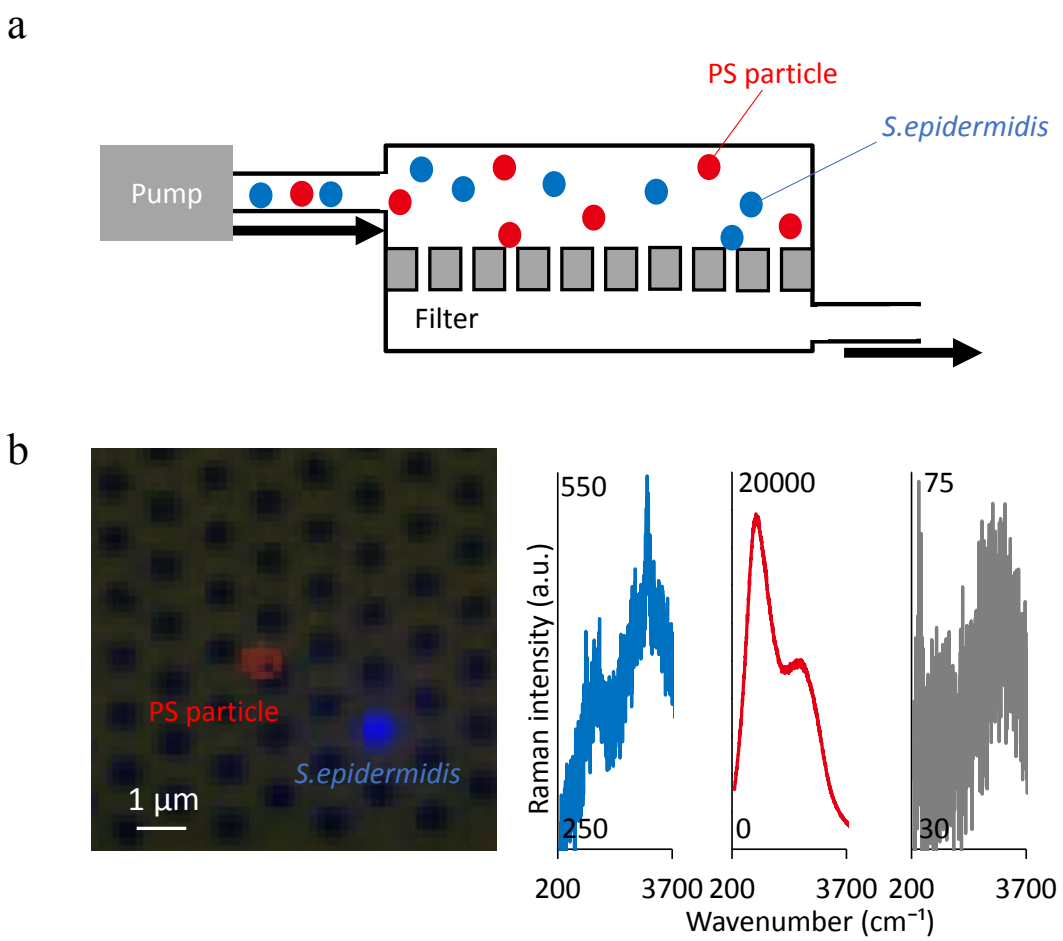

Figure 2. Capture and individual detection of microholes. (a) Schematic of microfiltration. (b) Fluorescence image and Raman spectra detected on the glass membrane filter. The fluorescence image was merged with the brightfield image. Blue Raman spectrum indicates Staphylococcus epidermidis, red Raman spectrum indicates PS particle, and gray Raman spectrum indicates filter hole.

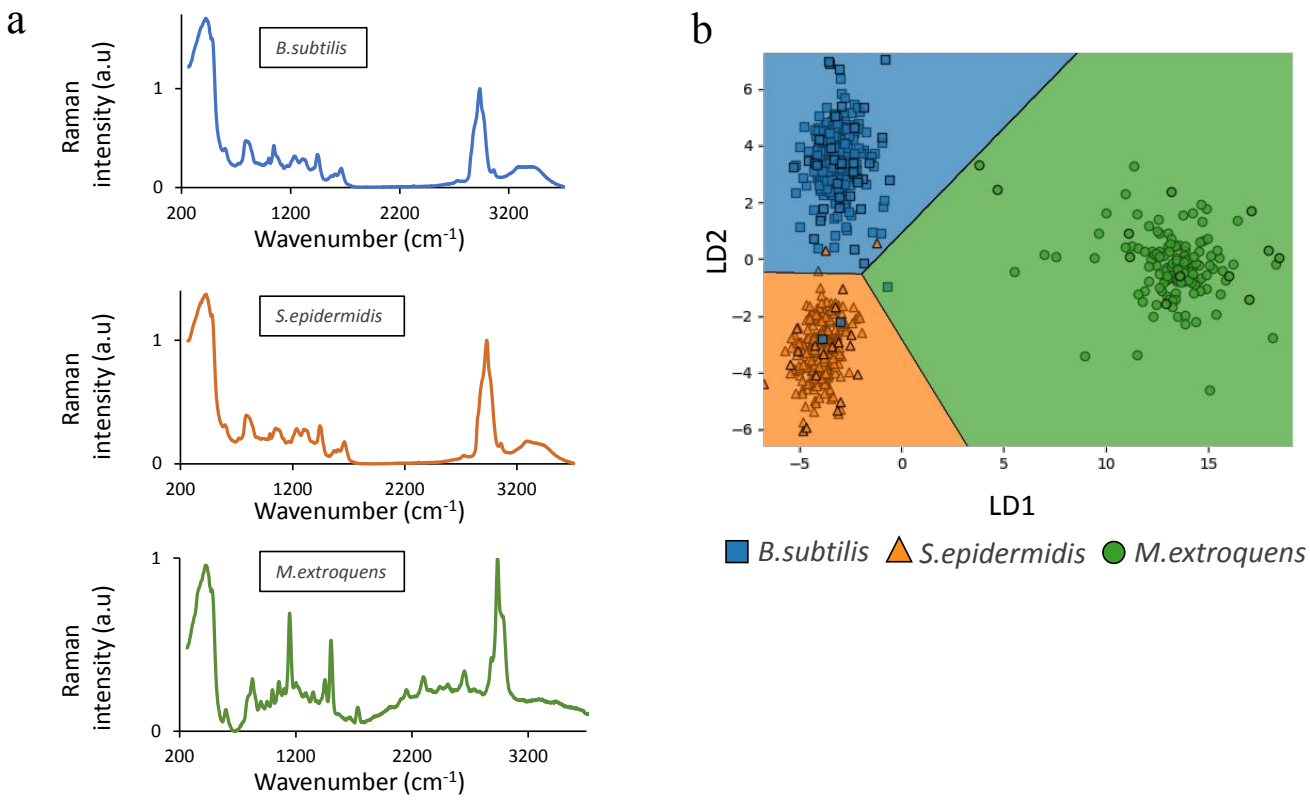

Figure 3. Discriminant analysis of bacterial cells. (a) Average Raman spectra of bacterial cells. (b) LDA results for Raman spectra of bacterial cells.

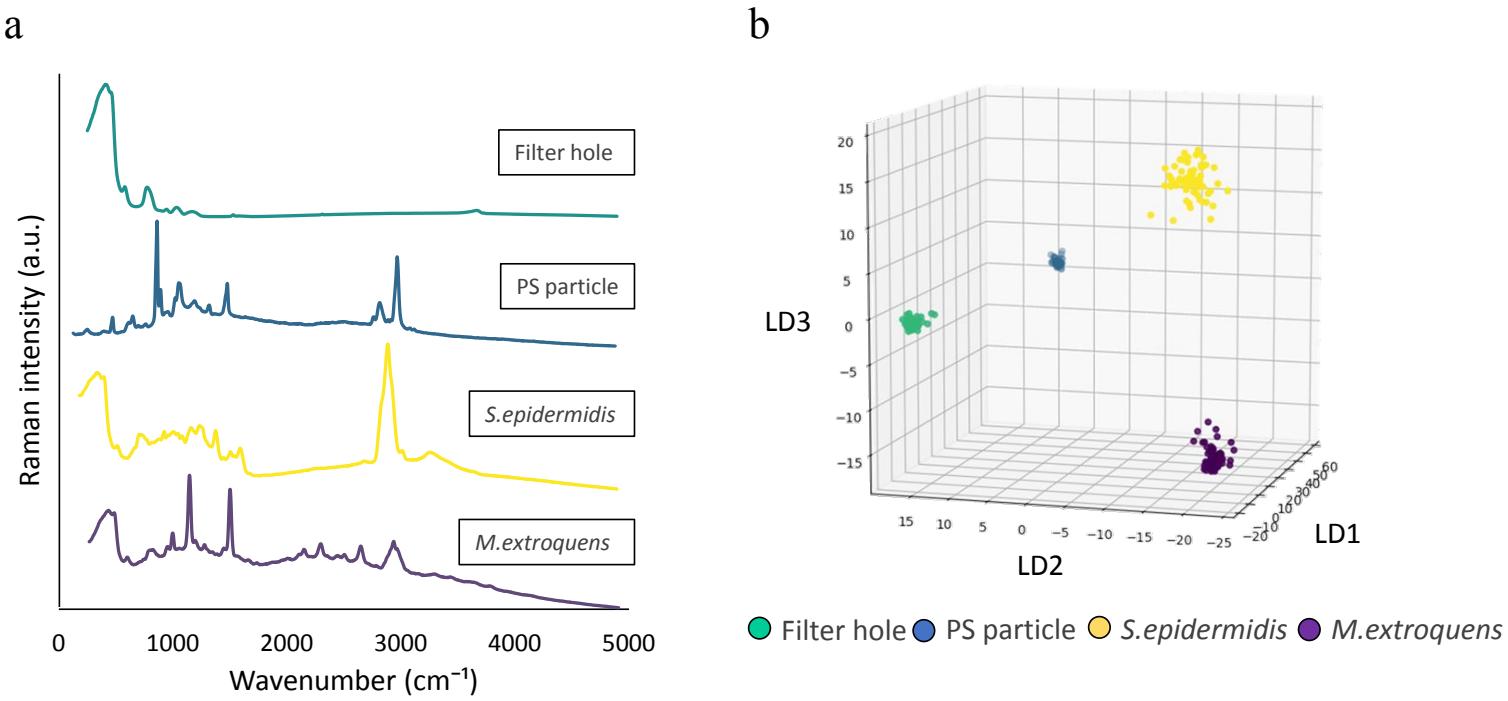

Figure 4. Discriminant analysis of microfiltration. (a) Pattern of Raman spectra detected on the glass membrane filter. (b) LDA results for Raman spectra detected on the glass membrane filter.

Data availability statement for:
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1. Institute of Nano-Life-Systems, Institutes of Innovation for Future Society, Nagoya University, Fu-ro-cho, Chikusa-ku, Nagoya 464-8601, Japan
2. Department of Biomolecular Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan
3. Takeda Pharmaceutical Co., Ltd., 2-26-1, Muraoka-Higashi, Fujisawa, Kanagawa 251-8555, Japan
4. AGC Inc., Suehirocho 1-1, Tsurumi-ku, Yokohama-shi, Kanagawa, 230-0045, Japan
5. Institute for Quantum Life Science, National Institutes for Quantum Science and Technology (QST), Anagawa 4-9-1, Inage-ku, Chiba, 263-8555, Japan

*Corresponding author
E-mail: onoshima-d@nanobio.nagoya-u.ac.jp

Availability of data:

The data that support the findings of this study are available from the corresponding author, [D.O.], upon reasonable request.