Visualization of Cells at the Solid-microbe Interface Using Confocal Reflection Microscopy With Index-matching Materials

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Research Article

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Abstract

Herein, we demonstrated that the use of index-matching materials (IMM) allows direct visualization of microbial cells maintained at a solid-liquid interface through confocal reflection microscopy (CRM). The RI mismatch induces a background reflection at the solid-liquid interface, which dwarfs the reflection signals from the cells and results in low-contrast images. We found that the IMM sufficiently suppressed the background reflection at the solid-liquid interface, facilitating the imaging of microbes at the solid surface using CRM. Further, we succeeded in temporal imaging of initial biofilms directly colonizing the IMM with CRM in a tag free fashion, and thus, it is highly advantageous for probing the dynamics of biofilm formation, along with visualization of environmental organisms and newly isolated bacteria, for which transformation methods are difficult to establish.

Introduction

Confocal reflection microscopy (CRM) has been applied to visualize the three-dimensional distribution of intact microbiological samples. Distinct from the better-known fluorescence confocal laser scanning microscopy, CRM detects incident light scattered by opaque samples, thereby eliminating the need for epifluorescent tagging\textsuperscript{1,2}. CRM, thus, offers a unique solution to analyze the three-dimensional structure of microbial communities such as microcolonies, biofilms, and biofouling-communities in a tag-free fashion\textsuperscript{3,4}.

CRM imaging of cells directly adhered to a solid surface however, is often hindered by the refractive index (RI) mismatch between the solid and the liquid phases. Many bacteria exist in a surface-associated aggregate referred as biofilm rather than planktonic state, and bacterial adhesion to the surfaces is a key process for understanding the dynamics of biofilm formation\textsuperscript{5,6}. Microscopic studies of biofilm formation typically involve maintaining biofilms on a glass surface for optical access\textsuperscript{5,6,7}. RI mismatch at the glass-medium interface causes a strong background reflection of incident light, which dwarfs the reflection signals of cells. For example, the RI of the coverslip and culture mediums such as Luria-Bertani (LB) medium and M9 minimal medium are around 1.5 and 1.3, respectively\textsuperscript{8,9}. This difference between the two RIs is large enough to cause background reflection at the interface, and the CRM image at the glass-liquid interface thus suffers from poor contrast\textsuperscript{3}.

Here, we demonstrate the use of index-matching materials (IMM) to allow for the direct visualization of microbial cells maintained at a solid-liquid interface using CRM. We examined the effectiveness of two different materials, as IMM\textsuperscript{s}, namely fluorinated polymers and hydrogels. We overlaid the IMM\textsuperscript{s} on the glass surface and showed that both materials sufficiently canceled the background reflection at the solid-liquid interface. We demonstrated that, with the aid of IMM\textsuperscript{s}, CRM can be used to visualize the entire biofilm structure including the parts directly interacting with the substrate surface, without the use of any tags.

Results
**IMMs cancel background reflection.** The use of an IMM successfully canceled the background reflection at the interface of water and solid surfaces. Figure 1 shows the placement of the IMM and the experimental set-up, where the solid surface with a paint mark is illuminated using a laser and reflected light is detected as a signal in an inverted confocal system. We measured the level of background signals at the solid–liquid interface, with and without fluorinated acrylic polymer (MY-133-EA; My Polymers Ltd., RI=1.333) overlaid on glass as an IMM. Figure 2a and 2b show the Z-stack images near the interface without and with the MY-133-EA, respectively. The use of the MY-133-EA makes the paint mark clearly visible, while it is hardly visible without the MY-133-EA owing to the strong background reflection along the Z-axis from the interface. Figure 2c shows the average signal intensities of the field of view along the Z-axis with and without MY-133-EA. Without the MY-133-EA, a strong peak of background signal appears at the interface. This signal is almost eliminated with the use of MY-133-EA. These results confirm that the IMMs chosen for this experiment can counter the background reflection at the solid-liquid interface.

**Cell imaging at the interface between liquid and IMM.** IMMs allowed the clear visualization of *Schizosaccharomyces pombe* cells directly resting on glass using CRM (Fig. 3). We obtained Z-stack images of cells on the glass or on the IMM and represented them as 3D projections of the cells. The cells on the glass were hardly imaged in the range of -0.5–2.0 mm, along the Z-axis, from the interface (Fig. 3a), and the resulting 3D projections were far from the actual cell morphologies (Fig. 3b). In contrast, cells imaged using MY-133-EA were successfully imaged without background reflection (Fig. 3c), and thus, the resulting 3D projections clearly resembled cell morphologies (Fig. 3d). Similar 3D projections were also achieved using other IMMs, including an acrylamide-based hydrogel and an agar hydrogel (Fig. 4). These results indicate that a lower background reflection with the use of IMMs overlaid on the glass results in better visualization of the cells with CRM.

**Temporal monitoring of initial biofilms.** We temporally monitored the initial biofilm formation on the IMM using CRM (Fig. 5). We obtained Z-stack images of the initial biofilms on MY-133-EA at predetermined time points, up to 11 h, and reconstructed them into 3D projections of biofilm formation. The 3D projections successfully represented the initial biofilm formation process, where cells adhered to the solid phase (Fig. 5a), grew (Fig. 5b), and formed further microcolonies (Fig. 5c, d).

**Discussion**

In the present study, we imaged microbiological samples on solid surfaces using CRM, by eliminating the RI mismatch between the liquid and solid phases. This was achieved using IMMs as the solid phase. We found that the IMMs sufficiently suppressed background reflection at the liquid–solid interface, resulting in high contrast images of cells directly on the solid phase.

RI-mismatch at the solid–liquid interface often induces undesired refraction or reflection in various optical devices such as particles used in a particle imaging velocimetry, optical lenses, and optical fibers. This issue can be eliminated by reducing the difference in RI between the liquid and solid phases using index-matched combinations, such as pairs of immersion oil and glass, mixture oil and epoxy, and p-
cymene and polymethyl methacrylate\textsuperscript{10-12}. Similar to this approach, the use of IMMs was effective in suppressing background reflection (Fig. 2). Thus, we successfully obtained high-contras images of cells, even at the solid surface, using CRM (Fig. 3-5). Quarter-wavelength anti-reflection (AR) coating is another general method used to avoid reflection at the solid–liquid interface. However, the AR coating is not suitable for CRM, which involves the usage of various excitation wavelengths depending on the sample because the AR effect directly depends on the combination of the coating thickness and wavelength of light, unless complex structures such as multilayered, graded, and moth-eye structures are involved\textsuperscript{13,14}.

There are some limitations to this study. First, the solid phase is limited to IMMs. The main types of IMMs available so far are fluorinated polymers and hydrogels\textsuperscript{15,16}. Second, the thicknesses of the IMMs should be greater than the Z-resolution of the confocal systems. This prevents any leakage of reflection signals from the bottom to the top surfaces of the IMMs. Third, IMMs must be of high optical transparency to avoid any loss in the intensity of the incident laser and resultant signals.

CRM is used for temporal imaging of microbial communities in a tag-free fashion\textsuperscript{3}. In this study, we successfully imaged microbial samples on a solid surface (Fig. 3,4) and temporally imaged the initial biofilm formation process (Fig. 5). This temporal imaging at the solid surface is highly advantageous for understanding the dynamics of biofilm formation by microbes for which transformation methods are difficult to establish. Thus, a potential application of our study is to probe how the dynamics of biofilm formation change depending on the chemical and physical properties of the solid surfaces.

**Methods**

**Synthesis of IMM layer.** We synthesized a layer of each IMM onto glass substrates, including a glass slip (Matsunami Glass Ind., Ltd., Japan, RI=1.5255) and a glass bottom dish (AGC Techno Glass CO., LTD., Japan). An MY-133-EA (MY Polymers Ltd., Israel) layer was prepared by photocuring, wherein we spin-coated the MY-133-EA solution on the glass substrates at 4000 rpm for 30 s, followed by photo-initiated curing with a 365-nm lamp under an atmosphere of nitrogen to generate a layer of ~20-mm thick. Poly(N-hydroxymethyl acrylamide) hydrogel was prepared by chemical gelation\textsuperscript{16}. A pre-gel aqueous solution consisting of 8 wt% N-hydroxymethyl acrylamide (Tokyo Chemical Industry Co., Ltd., Japan), N, N'-Methylenebisacrylamide (Tokyo Chemical Industry Co., Ltd., Japan), N, N, N, N'-tetramethylethylenediamine (Tokyo Chemical Industry Co., Ltd., Japan), ammonium peroxodisulfate (Tokyo Chemical Industry Co., Ltd., Japan), and distilled water, was poured into a silicon frame (SYLGARD\textsuperscript{TM} 184 silicone elastomer; Dow Corning Tray Co., Ltd., Japan) adhered to a glass bottom dish. The frame was sealed with a plate and incubated at room temperature (25°C) for gelation. Agar gel was prepared by physical gelation\textsuperscript{16}. The agar (0.8 wv%, Nacalai Tesque, Inc., Japan) was dissolved in PBS buffer (Fujifilm Wako Pure Chemical Co., Japan) while heating. A droplet of agar solution (150 mL) was placed on a coverslip and covered with another coverslip, followed by cooling at room temperature for gelation to make a layer of even ~0.5-mm thick. One side of coverslip was then slide off gently.
**Strains and culture conditions.** The strains used in this study were *Schizosaccharomyces pombe* JY1 and *Pseudomonas aeruginosa* PAO1. S. pombe JY1 was cultured in yeast extract-peptone-dextrose (YPD) medium (BD Bioscience, USA) while shaking (190 rpm) overnight at 30°C. *P. aeruginosa* PAO1 was cultured in LB medium (Nacalai tesque, Inc., Japan) while shaking (190 rpm) overnight at 37°C. For biofilm formation, the culture of *P. aeruginosa* PAO1 was inoculated into fresh LB medium supplemented with 100 mM KNO$_3$ (Fujifilm Wako Pure Chemical Co., Japan) to adjust the optical density of the medium at 600 nm of 0.01 and was placed in a 25-μL frame-seal™ incubation chamber (Bio-Rad Laboratories, Inc., USA) to adhere to the MY-133-EA layer. The chamber was sealed with silicon resin and incubated at 37 °C under aerobic conditions.

**Experimental set up.** Figure 1 illustrates the experimental setup. The sample was illuminated with a 561-nm continuous wave laser. The reflected light passed through a half mirror and 1.2 Airy-unit (AU) pinhole and was detected with a photomultiplier tube in the inverted confocal system (NIKON A1; NIKON Solutions Co., Ltd., Japan). The average signal intensities in a field of view, except for the region of paint mark, were calculated by processing the image using a custom MATLAB (MathWorks, Inc., USA) routine. We reconstructed 3D projections from Z-stack images using NIS elements software (NIKON Solutions Co., Ltd., Japan).

**Declarations**

**Data availability**

The data generated and analysed during the current study will be made available from the corresponding author on reasonable request.

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**Author contributions**

Y. Y. and C.O. designed the experiments. C.O. and T. H. performed the experiments and drafted the manuscript. All authors have reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.
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**Figures**

**Figure 1**

Experimental set up. (a) Conventional type. (b) Present type. There is no RI mismatch between the solid and liquid phases.

**Figure 2**

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Figure 3

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Figure 4

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Figure 5

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