Automatic Mapping of Viable Microbial Cells Distributed in the Surface Layer of Cotton Fabrics

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Received 17 October 2006/Accepted 18 January 2007

Viable microbial cells distributed in a 130 μm thick surface layer of cotton fabrics were stained with a fluorescent glucose, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG), and automatically mapped with an ultra-deep focusing range microscope (UDF) system. The software of the UDF system was upgraded and the number of Candida albicans cells could be counted at a higher precision than before. Bacterial cells of Pseudomonas fluorescens, Serratia marcescens, and Citrobacter freundii, which were smaller than 1-2 μm, were successfully mapped for the first time. These results indicate the practical importance of the present method in the evaluation of the antibacterial properties of fabrics and the efficacy of washing.

Key words: Ultra-deep focusing range (UDF) fluorescent microscope/Fluorescent glucose/Viable cell imaging/Cell deposition on fabrics.

Visualization in situ of viable microbial cells on the surface of fabrics is of practical importance and has been important for the evaluation of antibacterial properties of fabrics (JIS L1902, 2002; Borkow and Gabbay, 2004; Cen et al., 2004) and the efficacy of washing (Petrocci and Clarke, 1969; JIS L0844, 1997; ASTM E2274-03, 2004). One of the key challenges in conducting such an evaluation is to deal with the topology of fabric surfaces which is not flat at the micrometer scale but composed of many fibers to form a complex structure. Microbial cells are deposited on thin fibers or entrapped deeply between fibers. To detect these cells within a deep focusing range simultaneously, confocal microscopy (Roldán et al., 2004; Staudt et al., 2004), deconvolution microscopy (McNally et al., 1999), and other methods (Burton, 2003; Buda et al., 2005) have been proposed and in fact some models based on these principles are commercially available. However it was difficult to modify available models at a reasonable cost to fit our specific resolutional purpose. Thus we developed a novel microscopic system with an UDF system (Fujioka et al., 2006). In combination with the staining of viable cells with a fluorescent glucose derivative, 2-NBDG (Yoshioka et al., 1996; Matsuoka et al., 2003), the UDF system was found to be useful for the rapid evaluation of the efficacy of microbial cell removal (EMR) from fabrics in the specific case of Candida albicans microbes greater than 5 μm.

From a practical viewpoint, however, it is essential to establish a spatial resolution as high as 1-2 μm. In this study, we have critically revised the principal image processing software. As described below, the mapping of C. albicans has been successfully performed with much higher resolution. The mappings of bacterial cells smaller than 1-2 μm are also demonstrated.

Seed cultures of C. albicans ATCC10231, the environmental isolates of Pseudomonas fluorescens,
Serratia marcescens, and Citrobacter freundii were prepared from respective frozen stocks with MICROBANK kit (Pro-lab Diagnostics, Toronto, Canada) and cultured in 1/10 strength Trypticase Soy Broth (1/10 TSB) to approximately 10⁶ cfu/ml. Fabric samples used were Kanakin 3 (JIS L0803, 1998), Cotton knit without a brightener, and Cotton 100 denim. These are differently knitted to form unique textures and certified by the Japan Spinners' Association. The fabric swatches were prepared as 1.0 cm × 1.0 cm squares, wrapped with aluminum foil, autoclaved at 121°C for 15 min, and dried up under sterilized conditions. The synthesis of 2-NBDG was performed following the protocol described elsewhere (Yoshioka et al., 1996).

A 50 μl inoculum of the seed culture containing about 5 × 10⁵ cells of C. albicans was inoculated onto each swatch, and the swatch was placed on Trypticase Soy Agar (TSA) plates. After the incubation at 33°C for 16 h, each swatch was soaked in 9ml saline and vortexed for 5 min to remove most of the microbial cells from the each swatch. Thus we prepared swatch samples on which only small numbers of microbial cells remained. Each swatch was cut into 2 pieces (0.5 cm × 1.0 cm each). One piece (I) was used for the visualization experiment after being stained with 2-NBDG. The other piece (II) was used for the colony count assay only in the case of bacterial cells.

The conditions of 2-NBDG staining were as follows. A 400 μl aliquot of 12 μM 2-NBDG was placed on the fabric swatch piece (I). After incubation at 33°C for 10 min, the remaining aqueous liquid was removed by Ultrafree-MC centrifuging treatment (6000 rpm × 30s). After that, a 100 μl of 30% formaldehyde (HCHO) solution was added with a pipette on the swatch and incubated at 33°C for 1 min in order to fix the microbial cells. Immediately after that, the swatch was soaked in 9 ml saline for 5 min and centrifuged (6000 rpm × 30 s) to remove extracellular 2-NBDG. This washing with saline was repeated 2 times and microscopic observation with the UDF system was performed.

Previously we often encountered the image of a C. albicans cell indicated by an arrow in Fig. 1. In such a case, the single-cell emitted intense fluorescence at both ends and consequently was recognized as 2 cells in the automatic mapping. Such an image was due to a large vacuole that could hardly be stained by 2-NBDG. The increase in the spatial resolution, however, has enabled the recognition of such a case as a single-cell. Typical cases are observed at 4 positions in Fig. 2-Aa. These spots could be successfully registered as single-cells, respectively, as No. 1, 2, 5, and 8 in Fig. 2-Ab. A similar case is also observed in Fig. 2-Ba and registered as No. 5.

As may be observed in the Figs. 2-Ab, 2-Bb, and 2-
Cb, it is noticed that every fluorescent spot looks equally clear in outline and similar in size, though every cell does not necessarily exist in the same depth. The UDF system can integrate microscopic images from the surface to 130 μm depth at maximum (Fig. 3-D). Therefore the mapping data include the information of the depth of each cell. Based on these data, approximate positions of respective cells are shown in Figs. 3-A, 3-B, and 3-C. Such data are useful to estimate the degree of cell invasion into fabric matrices of different physical properties as well as their removal by washing.

Next is the automatic mapping of bacterial cells smaller than 2-3 μm. The objective lens was ~100 APO to zoom into the bacterial cell. In the case of Fig. 4-Aa, many fluorescent spots could be observed with similar fluorescent intensities. Thus every spot could be mapped as a light spot of similar size by adjusting the threshold level for the binarization at an appropriate level (Fig. 4-Ab). In the other two cases, only one cell was recognized as a light spot (Figs. 4-Ba, 4-Ca). According to the properly adjusted threshold level and the criteria for single-cell size, only this spot could be registered as a bacterial cell (Fig. 4-Bb, 4-Cb).

Practically, it is necessary to confirm the quantitative relation between the cell numbers determined by the present method and by the conventional colony count method. However, the challenge of statistics regarding sample size still remained. In fact the area that was analyzed by the present method was too small to be compared to the colony count method. This problem will be resolved by the future development of an automatic scanning system for a fabric swatch of a much larger area.

Since only one cell is detected in Fig. 4-B and 4-C respectively, it may be necessary to confirm by the colony count method that bacterial cells were actually remaining on/in the fabric swatch. The other halves (swatch piece (II)) used for Fig. 4 were assayed for viable cells according to the following protocol. The swatch piece (II) was immersed in 9 ml of 1/10 TSB and vortexed for 5 min and then taken out from the 1/10 TSB. A 0.5 ml aliquot of the 1/10 TSB
(suspension A) was mixed with TSA and poured in a dish for culturing at 33°C for 72 h. Since the cell concentration in the suspension A was thought to be markedly small, suspension A was also incubated at 33°C for another successive 48 h to increase it. A 100 μl aliquot of the resulting suspension (suspension B) was spread on a TSA plate and incubated at 33°C for 24 h to count the colony number. As a result, after the incubation, no colony growth was observed on the TSA plates of suspension A. On the other hand, some growth was observed on the plates of suspension B (Fig.4-B: 44 cfu/plate, Fig.4-C: 55 cfu/plate). This supports the idea that the amounts of the residual levels of bacteria are very low.

In conclusion, the UDF system has been upgraded so that it may count automatically the cell number of C. albicans as well as smaller bacterial cells at a higher precision than before. The present results suggest the importance of the further development of a practical version of the UDF system.

Acknowledgments

This research was supported by the Microbial Visualization Community of Practice of the Procter & Gamble Company. The authors would like to thank Dr. P. Geis and Dr. S. Donaldson of the Procter & Gamble Company for holding constructive discussions with us and for reviewing the manuscript. We also thank Mr. Tottori of Kogaku Inc. for his support for and input into the microscopic system designs. One of the authors, H. Matsuoka, acknowledges support from Grant-in-aid for Scientific Research for the Promotion of Safety and Security of Foods, The Ministry of Health, Labor, and Welfare.

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