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Rapid evaluation of the efficacy of microbial cell removal from fabrics

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Abstract The efficacy of microbial cell removal (EMR) from fabrics is a practically important indicator for the evaluation of cleansers and detergents. EMR is expressed quantitatively by the relative number of viable cells remaining on a fabric swatch after the treatment with these reagents. In order to count the viable cells on the swatch directly and rapidly, we have developed a unique microscopic imaging system with an ultra-deep focusing range. Standard swatches of cotton fabric were inoculated with microorganisms such as *Pseudomonas fluorescence*, *Staphylococcus aureus*, or *Candida albicans*. After the incubation on an agar medium, each swatch was treated with a fluorescent glucose, 2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxyglucose, to stain only viable cells. The images of every cell distributed within the surface layer with no greater than 130 μm thickness could be integrated into one image. Thus visualized cells could be counted automatically by a novel imaging program. Using a pair of cotton swatches ($0.5 \times 1.0\ \mathrm{cm}^2$) inoculated with *C. albicans*, EMR was evaluated quantitatively. Before washing, the total number of viable cells found on the observation area ($3.8 \times 10^{-4}\ \mathrm{cm}^2$) was 288 cells. After washing with a test detergent, no cell (<1) was detected. For this case, EMR was given by the formula: $\log(288/<1) =$ greater than 2.5. The imaging and cell count of a test fabric could be performed within 1 h.

Keywords Ultra-deep focusing range (UDF) fluorescent microscope · Efficacy of microbial cell removal (EMR) · A fluorescent glucose · Viable cell imaging

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Introduction

In recent years, there are many household products on the global market claiming the efficacy of microbial cell removal (EMR) from fabrics and other solid products [2, 6, 10, 12, 14, 23, 27]. The evaluation of EMR is especially important from the viewpoint of laundry treatment evaluation. To evaluate EMR, several guidelines have been issued [9, 24, 25, 26]. These guidelines recommend traditional agar plating and incubation procedures. Practically, however, a more rapid and more direct method is required. To meet this requirement, non-culture rapid methods using fluorescent staining dyes have been proposed and recognized to be potentially applicable to liquid samples. However, they could hardly be applied to solid samples with rough surface.

Recent advances in fluorescent bio-imaging [4, 5] have enabled the visualization of particles with 10 μm in diameter existing at a depth of 1 mm [5]. However, to detect microbial cells with no greater than 0.5 μm, the magnification of the objective lens should be 60× or 100×. The working distance and the in-focus depth of these lenses (VC60×oil, VC100×oil) are 130 μm and a few micrometers, respectively. It is only those cells being located within the in-focus depth 130 μm (i.e., working distance) apart from the lens that can be observed as a clear image. Therefore, our efforts have been focused on the development of a novel microscopic apparatus with an ultra-deep focusing range (UDF) by combining with an automatic Z-scanning system. Confocal microscopy [18, 20, 21] and deconvolution microscopy [17] should satisfy the requirement of deep focal distance in principle. However, it was difficult to modify these commercial models to fit for our specific purpose at reasonable cost. Thus, we intended to construct a novel fluorescent microscopic system with a Z-scanning apparatus and an associated image-editing program.

Another point is the introduction of edible fluorescent probe for the detection of viable cells. For this purpose, the authors synthesized a fluorescent glucose, 2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG) [26]. 2-NBDG was found to be taken only by viable cells of various microorganisms [15, 16] as well as animal cells [28]. Based on these results, we decided to use this fluorescent glucose to visualize viable microbial cells on fabrics. This study reports the performance of the novel microscopic apparatus and thereby evaluation of EMR of cleanser using standard samples of cotton swatches.

Materials and methods

Microbial strains

As test microorganisms, 13 strains of 8 species were selected from ATCC cultures and environmental isolates [3]. They were *Pseudomonas aeruginosa* ATCC15442, *Pseuduomons aeruginosa* environmental isolate, *Burkhordelia cepacia* environmental isolate, *Pseudomonas* sp. environmental isolate (Zinc pyrithione resistant), *Pseudomonas fluorescence* environmental isolate, *Citrobacter freundii* environmental isolate, *Serratia marcescens* environmental isolate, *Staphylococcus aureus* ATCC6538, *Enterobacter gergoviae* environmental isolate, *Klebsiella pneumoniae* 2 environmental isolates, *Candida albicans* ATCC10231, and *Enterobacter cloacae* environmental isolate (HCHO resistant).

Each strain was revived from the frozen stock with MICROBANK kit (Pro-lab Diagnostics, Toronto, Canada) and precultured on Trypticase Soy Agar (TSA) plates (BBL Company, Franklin Lakes, NJ) for 24 h at 33°C. After that, the respective inocula were suspended in 0.9% saline solution as ca. 10^6 colony forming unit (cfu)/ml and used as seed cultures.

Chemicals

A commercially available reagent, Ariel Bleach Plus® (Procter & Gamble Far East, Inc.) was used to demonstrate EMR test. 2NBDG was prepared according to the protocol described in [16]. Other reagents were of commercially available analytical grade.

Fabric substrates and inoculation thereon

Test fabric was Kanakin No.3 described in JIS L0803 [7] as a standard cotton fabric to be used for a color fastness test after staining. The fabric swatches were prepared as 1.0 cm×1.0 cm square size, wrapped with aluminum foil, autoclaved at 121°C for 15 min, dried up under a sterilized condition. A 50 μl seed culture was inoculated onto each of fabric swatches. Then, the swatches were placed on TSA plates and incubated for 48 h at 33°C. Immediately after the incubation, each of these swatches was cut into two pieces (0.5 cm×1.0 cm each). One piece was used for the visualization experiment with 2NBDG and another was used for the colony count assay.

Treatment of fabric swatches with 2NBDG

2NBDG was used for the visualization of viable microbial cells on the inoculated fabric swatches. A 200 μl aliquot of 12 μM 2NBDG was placed on one fabric swatch (0.5 cm×1.0 cm) and incubated at 33°C for 10 min. After the incubation, the fabric swatch was treated with 50 μl of 30% HCHO solution for 1 min in order to fix the microorganisms. Immediately after the fixing, the fabric swatch was soaked into 0.9% saline solution for 5 min, 2 times, and centrifuged in an Ultrafree-MC centrifugal filter device (0.22 μm pore size, 0.5 ml size, Millipore Co.) for 30 s at 6,000 rpm to eliminate extracellular 2NBDG.

Construction of a novel UDF fluorescent microscope

The novel apparatus constructed in this study is shown in Fig. 1. The fundamental system of a novel UDF fluorescent microscope was ECLIPSE 80*i* system (NIKON Inc., Tokyo) with a *Z*-axis auto tuning system. As a fluorescence objective lens system, CFI Plan Apo VC60×oil (WD 130 μm) or VC100×oil (WD 130 μm) (NIKON Inc.) was used with a BV-2A fluorescence filter (excitation 400–440 nm, emission 470 nm). The image detection device was KRI-100K (KOGAKU Inc., Osaka). A Lumina Vision 2.20 Bio-imaging analysis program and a WinROOF automated macro program (Mitani Trading Inc., Fukui) were optimized for our purpose and installed on Windows XP system.

Observation with an UDF fluorescent microscope

A test swatch was attached on an Adhesive Durotak slide glass (Dermatologic Lab & Supply, Inc., Council

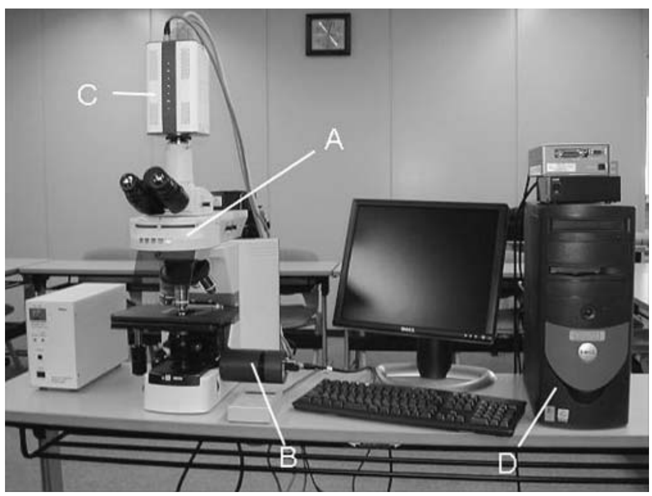

Fig. 1 UDF fluorescent microscope. *A* microscope, *B* *Z*-axis auto tuning system, *C* image detection device and *D* computer

Bluffs, IA) and observed with an UDF fluorescent microscope. The images of six square spots (each spot $78 \times 82\ \mu m^2$) were analyzed and total number of cells was estimated.

Cell collection from fabric swatches for colony count method

A test swatch was soaked in a 5 ml cell collection medium being composed of Modified Letheen Broth (MLB, BBL Co.), 1.5% Tween 80 (Wako Co., Tokyo), and 0.93% soybean lecithin (Wako Co.), which was prepared in a 15 mmϕ glass tube and autoclaved at 121°C for 15 min beforehand. The swatch in the tube was agitated for 5 min with a sonic device and for 5 min with a vortex device successively. We checked beforehand the influence of ultra sound on the cell viability and found it no lethal level (Data not shown). One milliliter aliquot of the cell collection medium was tenfold diluted with 9 ml 0.9% saline solution in glass test tubes. After the stepwise dilutions, the individual diluents were pour-plated on MLAT [(Modified Letheen Agar (MLA, BBL Co.) containing 1.5% Tween 80], which was autoclaved at 121°C for 15 min and maintained as molten condition at 47°C beforehand. Then, the MLAT plates were solidified, inverted, and incubated at 33°C for 72 h. After the incubation, the colonies that appeared on the plates were counted.

Washing treatment condition for microbial cell removal

The microbial cell removal from test swatches was performed according to the test method of ASTM E2274-03 [19]. Briefly, a strip of cotton swatch, Kanakin no. 3, was wound around the three horizontal extensions of a stainless steel spindle with sufficient tension. Then the test swatch was inserted in the laps of the wound cloth. The test swatches were washed with a detergent solution (1.0 g/l) in a 500 ml glass jar (8.1 cmϕ × 12.8 cm) with agitation at 60 rpm/min. This washing condition was originally proposed for the color fastness test [8] and later confirmed to be a proper condition for bacteria removal test from various fabric samples (unpublished data). After the washing, the number of cells remaining on the test swatches was counted. Half the test swatches were applied to the 2-NBDG staining and its visualization process. The rest swatches were applied to the cell collection and successive colony count method as described above.

Comparison of the developed method and conventional agar plate colony count method

To demonstrate the feasibility of the developed method, test swatches inoculated with *C. albicans* ATCC 10231 were assayed by both methods. After the washing treatment with a detergent, each of test swatches was cut into two pieces (0.5 cm × 1.0 cm each). One piece was used for the developed method involving 2NBDG staining and the other was used for the colony count method. As the control, test swatches without washing were assayed by both methods in the same manner. The EMR determined by both methods were compared.

Results

Fluorescent images of yeast cells obtained with the UDF fluorescent microscope and an ordinary microscope

Fabric swatches inoculated with *C. albicans* ATCC10231 were observed with an ordinary fluorescent microscope (Nikon X2-F) and the present UDF fluorescent microscope. The image obtained with an ordinary microscope could hardly give us a clear image of microbial cells (Fig. 2a). In contrast, the UDF fluorescent microscope could give us a bright and clear image of microbial cells being distributed on rough surface of the swatch (Fig. 2b). This indicates that the developed system is feasible for the direct detection of microbial cells with several micrometers in diameter being distributed in UDF (see Discussion about the detail).

The general non-specific fluorescence over the test swatches observed is speculated as a residue of extra cellular 2NBDG, and also trace level of florescence derived from the fabric components. Practically, however, the present fluorescent background is a permissible level,

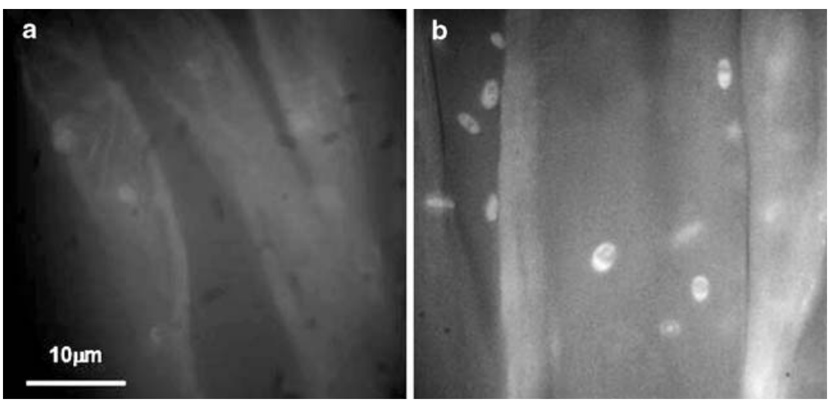

Fig. 2 Fluorescent images of a fabric swatch inoculated with *C. albicans* ATCC10231 taken with an ordinary microscope (**a**) and the UDF fluorescent microscope (**b**)

because viable cells are fluorescent enough to be distinguished from dead cells and non-biological particles.

Images of bacterial cells on fabric swatches

The images of bacterial cells smaller than *C. albicans* were obtained with the UDF fluorescent microscope. A typical image of *P. fluorescens* environmental isolate that was inoculated on a fabric swatch is shown in Fig. 3. Many fluorescent spots with rod shape were speculated as single-cells of *P. fluorescens*. Non-specific fluorescence exists over the whole area of the swatch but this could be eliminated by a proper imaging program as described below. Figure 4 shows an image obtained with a swatch inoculated with *P. aeruginosa* ATCC 15442. In the magnified inset, respective single cells can be recognized. In this image, cells are more densely distributed than Fig. 3. In the case of *S. aureus* ATCC6538, the typical coccal shape could be clearly recognized as shown in Fig. 5 together with its magnified inset.

In the same manner, nine other species and strains (*B. cepacia* environmental isolate, *Pseudomonas* sp. environmental isolate (Zinc pyrithione resistant), *P. aeruginosa* environmental isolate, *C. freundii* environmental isolate, *S. marcescens* environmental isolate, *E. gergoviae* environmental isolate, *K. pneumoniae 2* environmental isolates, and *E. cloacae* environmental isolate (HCHO resistant)) could be visualized (Data not shown).

In spite of asperity of the fabric surface, microbial cells widely distributing throughout the fabric surface could be recognized.

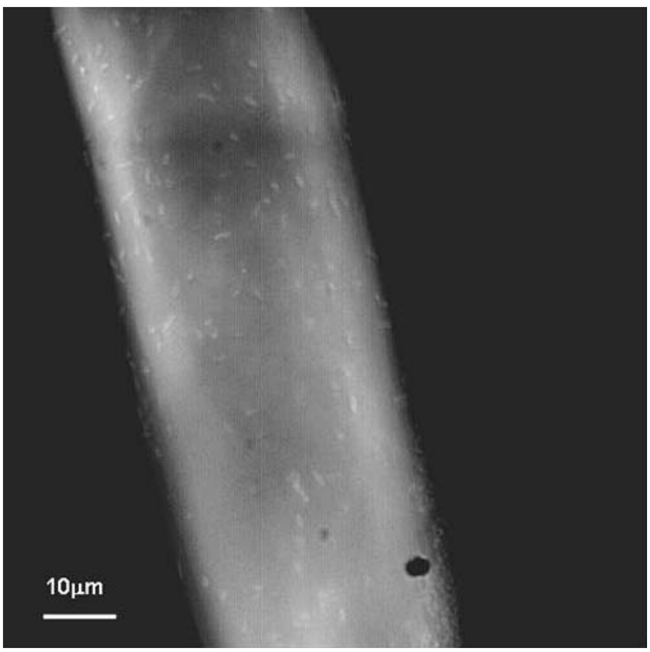

Fig. 3 Fluorescent image of a fabric swatch inoculated with *P. fluorescens* environmental isolate taken with the UDF fluorescent microscope

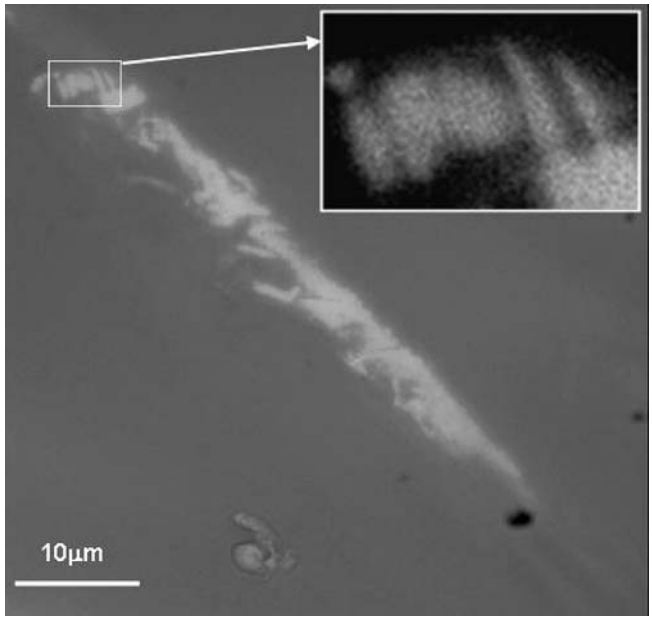

Fig. 4 Fluorescent image of a fabric swatch inoculated with *P. aeruginosa* ATCC15442 taken with the UDF fluorescent microscope

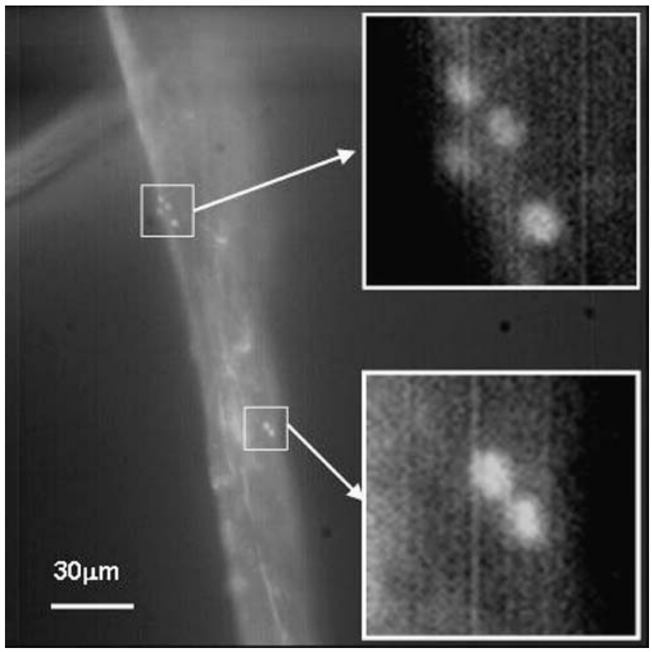

Fig.5 Fluorescent image of a fabric swatch inoculated with *S. aureus* ATCC6538 taken with the UDF fluorescent microscope

Observation of floating of microbial cells in damp-dried fabrics

In the course of this study, we have often observed the floating cells in damp-dried fabrics. Such water in damp-dried fabrics should contain salts and detergents. Their concentrations should be sufficiently low just after washing but they might be unexpectedly concentrated during successive drying process. Consequently such a residual solution should be toxic to cells. The present method is based on the viable cell counting and therefore its possible influence on the un-removed viable cells should be considered.

To demonstrate that the developed method can detect viable cells even in such an environment, a damp-dried fabric was observed. A series of photographs from a to i of Fig. 6 were taken at every 1 s. If these photographs are carefully observed, it may be recognized that fluorescent spots are fluctuating during this observation period. In order to depict the cell fluctuation, one target cell was circled. In fact, the fluorescent spots were continually fluctuating in the microscopic view. Some spots disappeared probably because they moved to out-of-focus plane.

Demonstration of EMR evaluation

In order to demonstrate the evaluation of EMR of a laundry detergent, fabric swatches inoculated with *C. albicans* ATCC 10231 were observed before and after the washing treatment with the test detergent. Before the washing, viable cells on the swatch were observed as depicted in Fig. 7a. In contrast, after the washing, no fluorescent cell was observed (Fig. 7b). Therefore the EMR can potentially be evaluated.

In order to express EMR quantitatively, the image analysis program was optimized so that the non-specific fluorescent area may be discriminated from microbial spots. Figure 8 shows a typical result obtained after the image processing. The fluorescent spots in Fig. 8a were speculated as microbial cells. In this case, there are 39 cells. After the washing, no cell was detected (Fig. 8d). These analyses were repeated as triplicates.

Using another pair of test swatches, EMRs determined by the developed method and the conventional colony count method were compared. In the developed method, six square spots (each square $6.4\times10^{-5}\,cm^2$, total $3.8\times10^{-4}\,cm^2$) depicted in Fig. 9 were analyzed. Before the washing treatment with a detergent, the total number of viable cells detected in these six squares was 288 cells. After the washing, no cell was detected, indicating the total number of cells was smaller than 1. Therefore EMR may be determined as $\log[(288)/(<1)]=$ greater than 2.5. On the other hand, in the colony count method, the number of cells on the whole surface of the swatch ($0.5\times1.0\,cm^2$) before the washing was 2×10^6 cfu. After the washing, it decreased to be 4×10^2 cfu. Therefore, EMR may be determined as $\log[(2\times10^6)/(4\times10^2)]=3.7$. Both results are consistent with each other under the present definition of EMR.

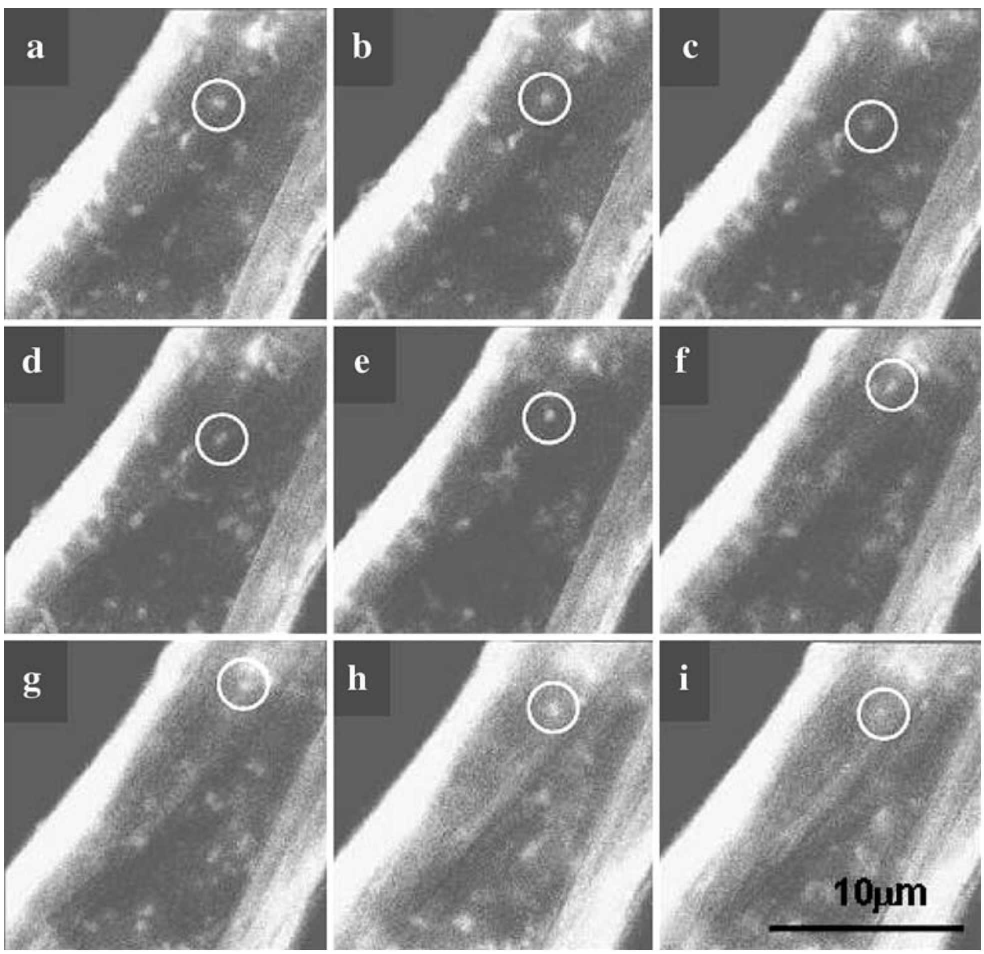

Fig. 6 Real time images of floating cells of *P. fluorescens* environmental isolate on a damp-dry fabric swatch. Photographs **a**–**i** were taken at every 1 s

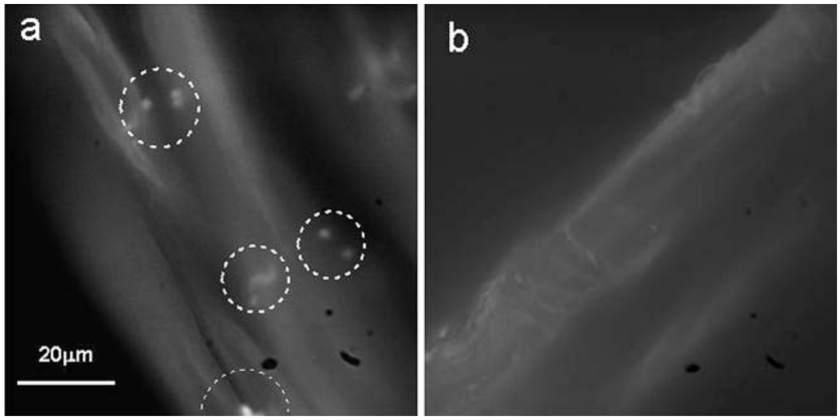

Fig. 7 Fluorescent images of a fabric swatch inoculated with *C. albicans* ATCC10231 taken before (**a**) and after (**b**) the washing treatment with a detergent

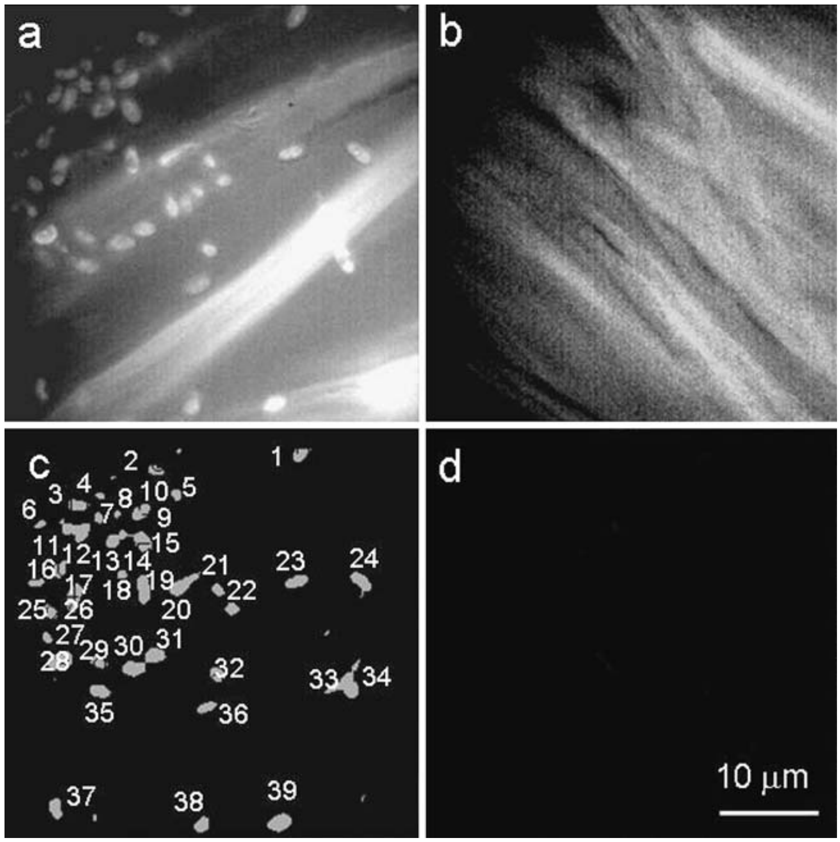

Fig. 8 Microbial cells on a fabric swatch numbered by image processing. A fabric swatch was inoculated with *C. albicans* ATCC 10231 and washed with a detergent. **a**, **c** Before washing; **b**, **d** after washing; **a**, **b** before image processing; **c**, **d** after image processing

Discussion

In the developed method, the observation area of a test fabric is only $3.8\times10^{-4}\,cm^2$ that is smaller than 0.1% of whole area ($0.5\,cm^2$). Therefore, it should be of no use to compare the total number of cells localized in the observed area simply with the total number of cells washed out from whole area. What is worth discussing should be the relative number of cells before and after washing treatment, i.e., the indicator of EMR. In this sense, EMR estimated by the developed method was thought to be a reasonable value in comparison with EMR determined by the standard method [1]. In order to overcome a problem of statistics, it is essential to develop a more advanced system that can analyze much more observation spots at higher speed with higher spatial resolution.

The in-focus depth of an objective lens is a few micrometers (Fig. 10a), but the lens can be driven automatically in the Z-direction so that a fabric swatch with no greater than 1.0 mm surface roughness can be observed continuously in XY-plane (Fig. 10b). On the other hand, its working distance is 130 µm (Fig. 10a) and therefore the maximum focusing range in the fabric swatch is 130 µm. The cells entrapped in the indicated range can be detected by Z-scanning and displayed as a single image by image integration (Fig. 10c).

Cells entrapped in the fabric matrix beyond this range, if any, cannot be detected directly. These cells are less likely to be removed in the washing tests. To detect

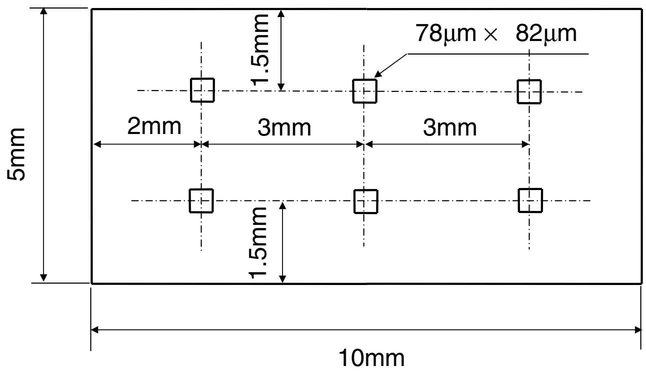

Fig. 9 Sketch of 6-square sampling points for EMR measurements

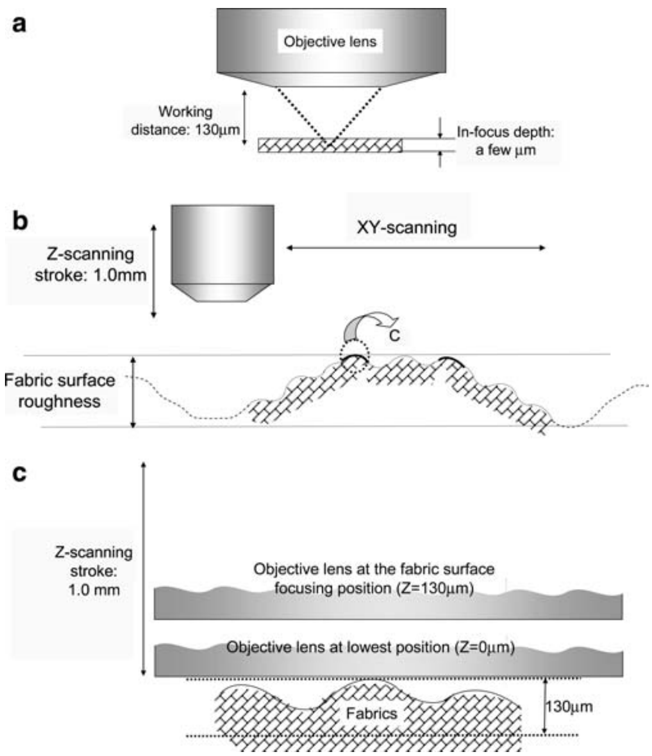

Fig. 10 Key dimensions of the developed apparatus. **a** Working distance and in-focus depth, **b** Z-scanning stroke and fabric surface roughness and **c** focusing range in a fabric swatch

these cells, it should be necessary to incubate the fabric in a culture medium for long time. For this purpose, the HCHO treatment after 2NBDG staining should be skipped to maintain the viability of these cells. The number of cells thus estimated together with the result of the developed method may provide us with useful information about the influence of fabric structure and degree of cell–substrate interaction.

The use of 2NBDG characterizes this study. 2NBDG can be taken only by the cells with high viability and concentrated in the cells [29]. This contrasts other dyes that enter the cells by passive diffusion [11, 13, 22]. In comparison with DNA staining dyes, 2NBDG is thought to be more specific to viable cells and therefore less liable to cause pseudo positive data. A typical case in which the use of 2NBDG is thought to be advantageous is biofilms on fabrics with antibacterial surface. The viable to dead cell ratio in biofilms is originally 70–95% [10] but it is reduced markedly when the surface maintains chemically modified antibacterial activity. In such a case, it is preferable to count only viable cells.

2NBDG cannot be taken by every species equally [15, 16]. Therefore, users should check if the microorganisms under consideration can take 2NBDG. 2NBDG can be taken by *E. coli* via mannose transporter as well as via glucose transporter (unpublished data). However, 2NBDG cannot stain every species and strain. Whoever wants to use this technique should make sure first that the microorganism that he wants to study can be stained with 2NBDG efficiently. If it cannot be stained with 2NBDG, it is necessary to use alternative dye. 2NBDG could not stain viable cells of 6 out of 41 strains but these unstained six strains could be stained with NBD-modified amino acids such as NBD-Gly and/or NBD-Leu (16 and unpublished data).

In conclusion, the present study has shown a successful combination of a novel UDF fluorescent microscope and a viable staining method.

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