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SHORT COMMUNICATION

Visualization of yeast single-cells on fabric surface with a fluorescent glucose and their isolation for culture

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Abstract An ultra-deep focusing range (UDF) fluorescent microscope system has been combined with a micromanipulation system to develop a viable cell detection-identification system applicable to microbes on environmental surfaces and products. *Candida albicans* yeast cells on a fabric sample surface were viably stained with a fluorescent glucose derivative, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy glucose (2-NBDG) and detected with a UDF fluorescent microscope. Visualized single-cells of *C. albicans* were picked in a glass microcapillary and transferred onto an agar medium. After the culture, the colony was assayed for DNA sequence to identify the isolate. This demonstrates a potential application to the study of unknown environmental microorganisms.

Keywords Ultra-deep focusing range (UDF) fluorescent microscope · Single-cell manipulation · Fluorescent glucose derivative · Viable cell imaging

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Introduction

Quantitative and qualitative analyses of environmental microorganisms have been attempted with various methods and approaches [2, 3, 7, 13, 15]. Where effective, these usually require days to obtain the results and fail to connect the visual to microbial species. Currently, urgent needs exist in the detection of food pathogens in cooking environment [11, 17] and microbial growth in damp garments after the laundry washing process [10, 14]. To meet these needs, we have recently developed a ultra-deep focusing range (UDF) fluorescent microscope system and applied it successfully to the evaluation of microbial cell removal from fabrics [4], and to the automatic mapping of viable microbial cells being distributed in the surface layer of cotton fabrics [5]. The next step is to isolate those single-cells for their identification. Once the single-cells have been isolated, they can be cultured on an agar medium. Thus formed colonies may be used for further investigation including DNA analysis and metabolism analysis. This research demonstrates the detection of single-cells of *Candida albicans* on fabrics and their isolation for the culture.

Materials and methods

Microbial strains

Candida albicans, which is one of the key human pathogens [6, 16] and contaminants in cosmetic industry [1] was chosen as a microbial strain for this study. Seed cultures of *C. albicans* ATCC 10231 were prepared from frozen stocks from MICROBANK kit (Pro-lab Diagnostics, Toronto, Canada) and cultured in the 1/10th strength Trypticase Soy Broth (1/10 TSB) to approximately 10^6 cfu/ml. Fabric

samples used are Kanakin 3 [8]. 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 under sterile conditions.

Fluorescent glucose derivative treatments

Synthesis of 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy glucose (2-NBDG) was conducted following the protocol described elsewhere [18].

A 200 µl inoculum of the seed culture containing about 5×10^6 cells of *C. albicans* was inoculated onto a swatch, and the swatch was placed on Trypticase Soy Agar (TSA) plates. After incubation at 33°C for 1 h, the remaining aqueous liquid on the swatch was removed by Ultrafree-MC centrifuging treatment (6,000 rpm × 30 s). To obtain individual components amendable to microscopic observations, the centrifuged swatch was untied and dissected to individual strings with pre-sterilized tweezers. The individual fabric strings were set on a glass slide upon which a square grid has been imprinted. Approximately 2.0 ml of 0.2% agarose solution containing 12 µM 2-NBDG was prepared in molten condition at 46°C, and poured onto the reassembled fabric strings on the slide glass. The glass slide was kept in a petri-dish and incubated for 1 h at 33°C to facilitate the uptake of 2-NBDG by *C. albicans*.

Microscope and manipulation systems

After incubation, the glass slide was examined with a UDF fluorescent microscope system. The detailed components of the UDF fluorescent system are described previously [4, 5]. Ultra long distance industrial optical lenses (CFI Plan EPI SLWD 50 and 30, NIKON Co., working distance 17.0 and 24.0 mm, respectively) and a semi-automatic cell injection manipulator (InjectMan NI2, Eppendorf Co.) were integrated to assemble a cell manipulation system (Fig. 1). InjectMan NI2 was fixed on the UDF system stage with steel frames.

To prepare glass capillaries for the yeast cell manipulation, borosilicate glass tubes (BF100-78-10, diameter 1.0–0.78 mm, Sutter Instrument Co.) were pulled with a laser puller (P-2000, Sutter Instrument Co.), sterilized with anhydrous ethyl alcohol and dried in a dry oven at 50°C for 48 h. For cell manipulation, a sterile silicone tube 2 mm in diameter was connected to the capillary and to the InjectMan NI2.

DNA sequence analyses

Isolated cells were incubated on TSA plates at 33°C for 48 h to prepare sufficient cell mass for DNA sequence analyses. The harvested cells were transferred to a 1.8 mL serum tube and frozen in liquid nitrogen. Frozen cells were treated with homogenization pestle to break the cell wall. This process was repeated two times. DNA was extracted from the homogenate with E.N.Z.A. Fungal DNA Kit (Omega Bio-tek, Inc.). PCR amplification was conducted on the 26S rDNA D1/D2 regions [9] with primers NL-1(5′-CGATATCAATAAGCGGAGGAAAAG) and NL-4(5′-GGTCCGTGTTTCAAGACGG) [12] with a thermal cycler (PTC-200 Peltier Thermal Cycler, MJ Research Co.) under the conditions of 95°C × 10 s + 50°C × 60 s + 70°C × 60 s (denaturation, annealing, and extension, 30 cycles), and 72°C × 10 min (extension) . The PCR products obtained were purified with QIA quick PCR Purification Kit (QIAGEN Co.) and DNA sequence analyses were done with PRISM3100 genetic Analyzer (ABI Co.).

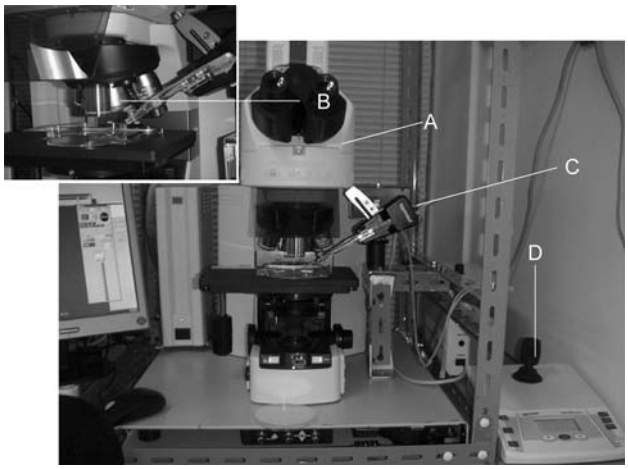

Fig. 1 A UDF fluorescent microscope system with a cell manipulation system. *A* microscope, *B* ultra long distance industrial optical lens, *C* manipulator, *D* operation module of manipulator

Results and discussion

The fluorescent images indicate viabile cells attaching on the surface of fabrics and their morphologies can be recognized (Fig. 2). Following this cell detection, a glass capillary was inserted in molten agar and placed adjacent to cells (Fig. 3a, fluorescent image). Then the cells were sucked into the capillary (Fig. 3b, c). The isolated cells were cultured as described and subjected to DNA sequence analysis at D1/D2 domain in 26S rDNA to certify that the originally inoculated strain was recovered. The gel electrophoresis band picture of the PCR product is shown in Fig. 4. A single band appeared at the same position as that obtained from the originally inoculated cells. The DNA sequence analyses data of 572 bp indicated 100% sequence matching. These indicate the clear traceability of the inoculated strain.

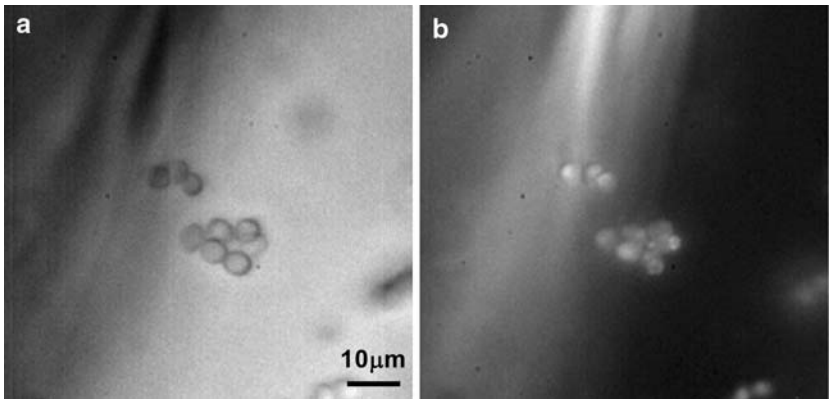

Fig. 2 Microscopic images of *Candida albicans* observed on fabric before cell isolation. **a** Optical image, **b** fluorescent image

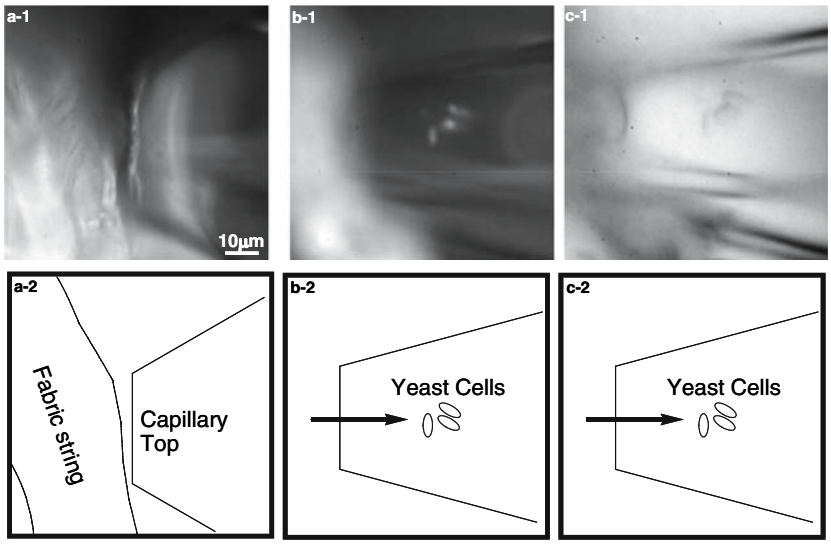

Fig. 3 Cell isolation procedure. **a** Glass capillary inserted to molten agar coating the fabric (optical image). **b**, **c** *C. albicans* cells sucked in a capillary stored in the glass capillary [optical image (**b**) and fluorescent image (**c**)]

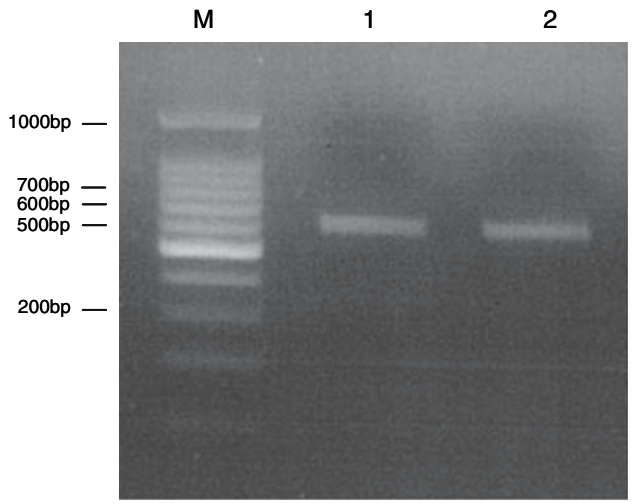

Fig. 4 Gel electrophoresis band picture of the PCR products. A total of 2% agarose, TAE Buffer, *M* Takara 100 bp ladder as a marker, *1* DNA derived from the inoculated strain, *2* DNA derived from the manipulated/isolated strain

Successful visualization and manipulation demonstrate the applicability of the present system to the detection of very low numbers of microbial cells and for their successive culture. The first is to detect viable cells rapidly and the next is to investigate them carefully depending upon the necessity.

Single-cell manipulation supporting technologies have recently gained marked progress [19, 20] and therefore the present system may be advanced to a higher throughput system in response to practical needs.

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References

1. Abedelaziz A-A, Ashour M-S (1987) Microbial contamination of hexetidine mouth wash. Zentrabl Bakteriol Mikrobiol Hyg [B] 184:262–268
2. Brown D-F, Dupont J, Dumont F, menanteau C, Pommepuy M (2004) Calibration of the impedance method for rapid quantitative estimation of *Escherichia coli* in live marine bivalve molluscs. J Appl Microbiol 4:894–902

3. Esioubu N (2006) Use of peptide nucleic acid probes for rapid detection and enumeration of viable bacteria in recreational waters and beach sand. Methods Mol Biol 345:131–140
4. Fujioka K, Kozone I, Saito M, Matsuoka H (2006) Rapid evaluation of the efficacy of microbial cell removal from fabrics. J Ind Microbiol Biotechnol 33:995–1002
5. Fujioka K, Kozone I, Saito M, Matsuoka H (2007) Automatic mapping of viable microbial cells being distributed in the surface layer of cotton fabrics. Biocontrol Sci 12(1):31–34
6. Hansson C, Jekler J, Swanbeck G (1985) *Candida albicans* infections in leg ulcers and surrounding skin after the use of ointment impregnated stockings. Acta Derm Venereol 65:424–427
7. Jimenez L (2001) Molecular diagnosis of microbial contamination in cosmetic and pharmaceutical products: a review. J AOAC Int 84:671–675
8. JIS L0803 (1998) Standard adjacent fabrics for straining of colour fastness test
9. Kurtzman C-P, Robnett C-J, (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73:331–371
10. Marples R-R, Towers A-G (1979) A laboratory model for the investigation of contact transfer of micro-organisms. J Hyg (Lond) 82:237–248
11. Nielsen P, Brumbaugh E, Kananen L (2002) Evaluation of the use of liquid dishwashing compounds to control bacteria in kitchen sponges. J AOAC Int 85:107–112
12. O'Donnell K, (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (ed) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, pp 225–233
13. Odumeru J-A, Belvedere J (2002) Evaluation of the MicroFoss system for enumeration of total viable count, *Escherichia coli* and coliforms in ground beef. J Microbiol Methods 50:33–38
14. Patrick D-R, Findon G, Miller T-E (1997) Residual moisture determines the level of touch-contact-associated bacterial transfer following hand washing. Epidemiol Infect 119:319–325
15. Ren Z, Hsiehi Y-H (2005) Real-time determination of microbial activity of pasteurized fluid milk using a novel microrespirometer method. J AOAC Int 88:1756–1761
16. Scherer S, Magee P-T (1990) Genetics of *Candida albicans*. Microbiol Rev 54:226–241
17. Venkitanarayanan K-S, Ezeike G-O, Hung Y-C, Doyle M-P (1999) Inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on plastic kitchen cutting boards by electrolyzed oxidizing water. J Food Prot 62:857–860
18. Yoshioka K, Takahashi H, Homma T, Saito M, Oh K-B, Nemoto Y, Matsuoka H (1996) A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of *Escherichia coli*. Biochim Biophys Acta 1289:5–9
19. Matsuoka H, Komazaki T, Mukai Y, Shibusawa M, Akane H, Chaki A, Uetake N, Saito M (2005) High throughput easy microinjection with a single-cell manipulation supporting robot. J Biotechnol 116:185–194
20. Matsuoka H, Yamada Y, Matsuoka K, Saito M (2006) High throughput microinjection technology for the single-cell analysis of BY-2 in vivo. In: Nagata T, Matsuoka K, Inzé D (eds) Tobacco BY-2 cells: from cellular dynamics to omics. Springer, Berlin, pp 339–346