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Rapid and retrievable recording of big data of time-lapse 3D shadow images of microbial colonies

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We formerly developed an automatic colony count system based on the time-lapse shadow image analysis (TSIA). Here this system has been upgraded and applied to practical rapid decision. A microbial sample was spread on/in an agar plate with 90 mm in diameter as homogeneously as possible. We could obtain the results with several strains that most of colonies appeared within a limited time span. Consequently the number of colonies reached a steady level (N_{stdy}) and then unchanged until the end of long culture time to give the confirmed value (N_{conf}). The equivalence of N_{stdy} and N_{conf} as well as the difference of times for N_{stdy} and N_{conf} determinations were statistically significant at $p < 0.001$. N_{stdy} meets the requirement of practical routines treating a large number of plates. The difference of N_{stdy} and N_{conf}, if any, may be elucidated by means of retrievable big data. Therefore N_{conf} is valid for official documentation.

Cell division potentiality is the evidence of cell viability. Repeated cell divisions generate colonies with a diameter in the visible range of millimeters after long culture. In fact, every reference method, including ISO (International Organization for Standardization) methods and AOAC Official Methods of Analysis[1] is based on a visual count of colonies. In other words, every method stated as a reference method requires long time to obtain colony count results. For practical use, the colony count had better be determined as rapidly as possible without loss of reliability equivalent to reference methods.

To date various rapid methods have been developed using non-culture or micro-colony culture systems[2–8]. Staining of viable cells with a specific fluorescent dye, for example, such as fluorescein diacetate derivatives[9–11] and fluorescent glucose[12,13] can be used to determine the number of viable cells rapidly within half an hour, requiring no time for growth verification. On the other hand, micro-colony count is based on culture on an agar plate and is able to provide count result relatively fast by using a microscope imaging system[14]. Those methods have been introduced as being useful enough for microbial test such as a hygiene self-check. However neither of them has been considered as equivalent as the standard reference method. This is because these rapid methods cannot exclude the possibility of false signals to get into the test process and to cause miscount.

Time-lapse measurement of colony growth might be the best way to distinguish viable cells from dead cells and non-biological particles[15,16]. London et al. developed an automated system for the rapid colony enumeration, utilizing time-lapse measurement of colony growth[17]. To obtain a clear colony image, however, they had to take special care for in-focus measurements. In their set-up, the colonies were formed on a membrane filter placed on a carefully prepared flat agar medium. Recently Levin-Reisman et al. developed a more user-friendly system with downloadable software[17]. Their system is based on the time-lapse measurement of colony diameter of every colony on the entire plate with a standard size (ca. 90 mm). Such a system should be useful for personal uses in research fields. However it might not

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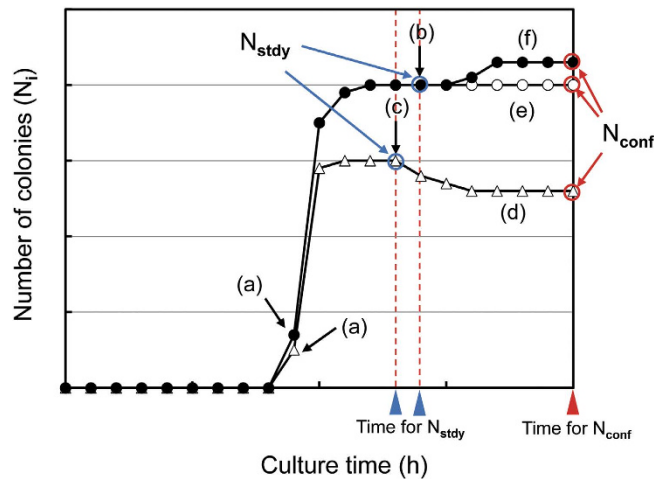
Figure 1. A theoretical model of time courses of the number of colonies, (**a**) initial recognition of colonies, (**b,c**) time point of reaching a steady level (N_{stdy}) of N_i, (**d**) occurrence of delayed decrease of N_i, (**e**) unchanged steady level, (**f**) delayed increase of N_i. Blue open circle: N_{stdy} values, red open circle: N_{conf} values, blue triangle: times for N_{stdy} determination, red triangle: time for N_{conf}.

be applied to practical purposes in which a much larger number of plates containing a wide range of cell density need to be measured simultaneously. Moreover rapid decision of the number of colonies is intensively required under those practical conditions.

To meet these practical requirements, we developed a unique automated system for acquiring the 3D shadow image of every colony in the entire culture plate of 90 mm in diameter by single capture shot[18]. Time-lapse shadow images are captured automatically in a multi-focus mode while the plates under test are being incubated under traditional culture conditions. This time-lapse shadow image analysis (TSIA) enabled the distinction of a single colony generated from a single cell, a doublet colony generated from 2 cells, and also a colony attached to a non-biological particle. Consequently the count loss due to such colony fusion could be avoided.

In this study we have upgraded the system so that big data of every colony in 100 agar plates can be stored and shown on a display by real-time processing. Then we applied the system to the colony measurement of 4 strains and 2 contaminated food samples in order to demonstrate its performance of rapid decision of the number of colonies in a wide range of cell density.

Results

A theoretical model of the time course of N_i is illustrated in Fig. 1, where N_i is the number of colonies at image capture time i. In this model, the initial colony is detected at (a). Then N_i increases sharply and approaches a steady level at (b) or (c). According to the criteria described below under the paragraph: criteria of reaching a steady level, N_i at (b) or (c) is decided to be a steady value, N_{stdy}. If N_i is unchanged until the end of long culture time (e), N_{stdy} can be an accurate rapid result and regarded as the confirmed value (N_{conf}). Otherwise there are occasions of (d) or (f). The decrease of N_i will occur because of the fusion of more than 2 colonies into a large colony. On the other hand, the increase of N_i will occur when slowly growing colonies appear. In either case, the cause of the difference between N_{stdy} and N_{conf} should be investigated. Stored data are retrievable and useful for its elucidation.

We have upgraded a formerly developed system so that it can treat 100 plates simultaneously. Twenty trays were placed in an incubator module (Fig. 2a) and the temperature of each tray was adjusted for growth of the microorganism concerned. The temperature was 35 °C for *E. coli* and colony forming bacteria in general, 33 °C for *B. pumilus*, and 28 °C for *C. albicans* and *A. brasiliensis*. Five plates were set on a tray (Fig. 2b). White light was illuminated to project shadow images of colonies on a CCD area sensor (1536 × 1536 pixels for 100 × 100 mm²) (Fig. 2c) and a 3D shadow images of each colony in or on an agar medium was recorded and analyzed (Fig. 2d). TSIA was conducted for every colony image in every plate at prescribed time intervals (usually 30 min or 1 h). Each of 20 trays was conveyed to the camera position by turns with a positional precision of 10 μm (Fig. 2e).

This study focuses the rapid decision of the number of colonies. From this viewpoint, there are 2 points to be considered. One is the decrease of the number of colonies which should occur when more than 2 colonies fuse into a large colony. The other is the delayed appearance of new colonies after most of colonies have appeared. The former point was solved by TSIA as reported previously[18]. The latter point, however, remained unsolved.

A typical case of the delayed appearance of colonies is shown in Fig. 3. Those colonies were located in a narrow rim area and seemed to grow horizontally, suggesting the growth from a cell attached on the
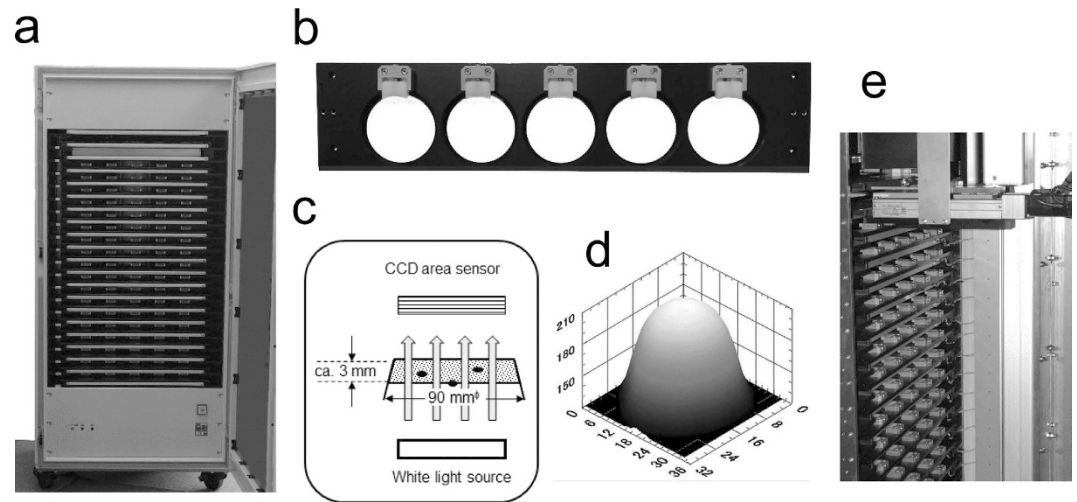
Figure 2. TSIA system. (**a**) Temperature controlled incubator module. (**b**) 5-Plate tray. (**c**) Shadow image capture system. (**d**) A 3D shadow image of a single colony. (**e**) Plate tray convey system installed inside of the incubator module.
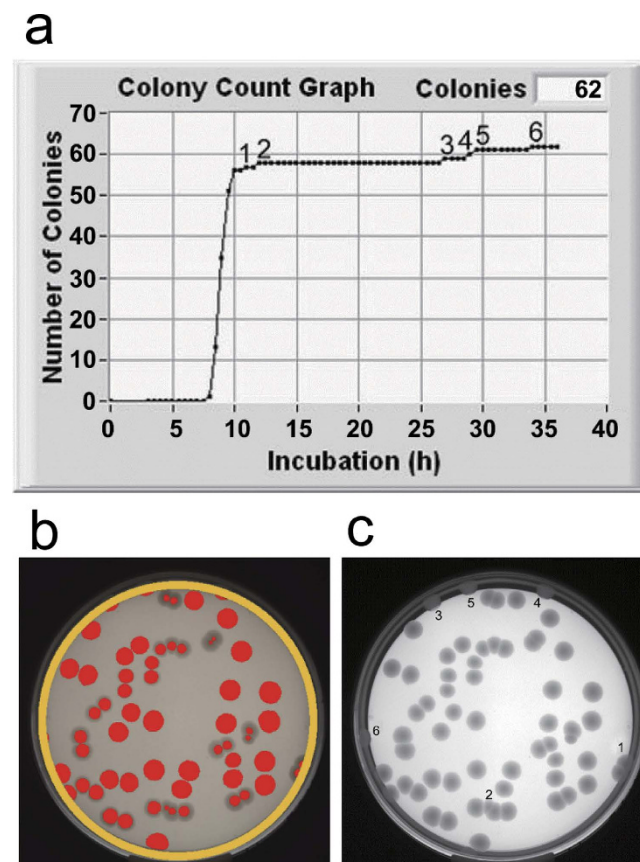

Figure 3. A typical case of delayed appearance of *E. coli* colonies. (**a**) time course of N_i, (**b**) recognized colonies designated by red markers, (**c**) image data recorded at the end of culture.

side wall of the petri plate. The colony growth from such cells was somehow retarded. This case should become a cause of disagreement between N_{stdy} and N_{conf}. Therefore homogeneous plating is thought to be important. Another point is the influence of colony location on or in the agar plate. In case of aerobic bacteria, the growth in the agar will be slower than that on the surface. In this viewpoint, the spread culture will be better than pour plate culture.
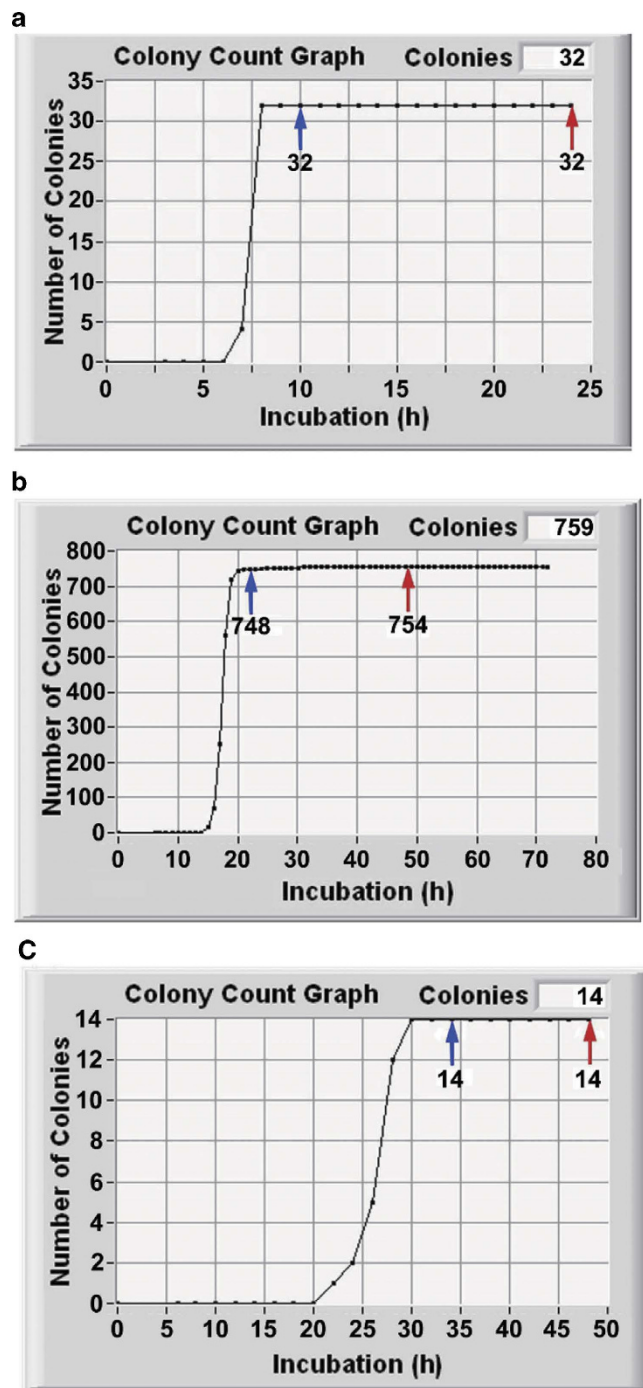
Figure 4. TSIA data of pure culture samples. Time courses of N_i of (**a**) *E. coli*, (**b**) *C. albicans,* (**c**) *A. braciliensis*. Blue arrows: N_{stdy}, red arrows: N_{conf}.

Taking care for homogeneous plating of a cell suspension in/on agar plates, we could obtain successful results with *E. coli*, *C. albicans*, and *A. brasiliensis* (Fig. 4a–c). After reaching a steady level, N_i was unchanged until the prescribed long culture time (24 h for *E. coli* and 48 h for *C. albicans* and *A. braciliensis*). Visual count was also conducted and its results were same as those by TSIA for *E. coli* and *A. brasiliensis*. However visual count was too difficult to make a reliable decision for *C. albicans* because of its large number.

The N_{stdy} and N_{conf} agreements could be obtained successfully also for samples containing a large number of noises such as food fragments (Fig. 5a,b). The images of an agar plate of hamburger captured at 0, 16, and 24 h are displayed in Fig. 5a. Though a number of small particles existed in the plate at 0 h, 7 colonies were counted correctly at 16 h and this value ($N_{stdy} = 7$) did not change until the end of 24 h culture ($N_{conf} = 7$). Another food matrix was a sample of cut vegetables (Fig. 5b). There appeared a large

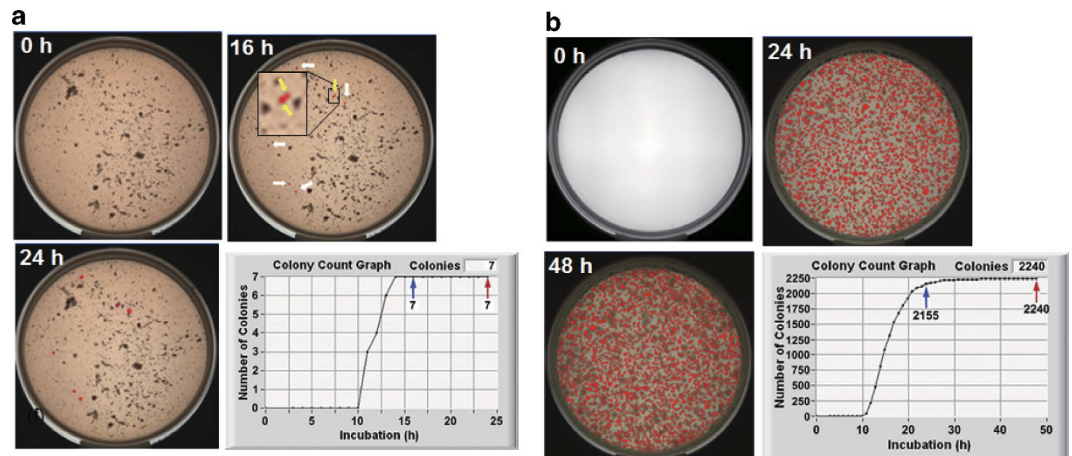**Figure 5.** TSIA data of samples containing food matrices. (**a**) *E. coli* in hamburger. Red markers indicated by white and yellow arrows in the panel of 16 h: registered colonies, a yellow arrow indicates a doublet colony and 2 colonies can be recognized in a magnified inset; red markers in the panel of 24 h: registered colonies; blue and red arrows in the time course of N_i: N_{stdy} and N_{conf}, respectively. (**b**) Naturally contaminated bacteria in cut vegetables. Red markers in the panels of 24 h and 48 h: registered colonies; blue and red arrows in the time course of N_i: N_{stdy} and N_{conf}, respectively.

number of colonies at 24 h and the number of colonies was 2155 (N_{stdy}). This number increased and finally became 2240 (N_{conf}) at the end of 48 h culture. In this case the difference was $(2240-2155)/2240 = 3.8\%$.

The equivalence between N_{stdy} and N_{conf} was investigated statistically for 4 pure strains (3 strains shown in Fig. 4a–c and *B. pumilus*) and 1 contaminated food sample (*E. coli* in hamburger). In the case of pure strain of *E. coli*, the linear least square approximation makes a formula: $Log[N_{conf}] = 1.0055*Log[N_{stdy}] - 0.0073$ (Fig. 6a). The correlation coefficient was estimated as $r = 0.9998$ ($R^2 = 0.9997$) and degree of freedom was 5. Therefore the equivalence of both results was statistically significant at $p < 0.001$. On the other hand, the difference of times for N_{stdy} and N_{conf} determinations were also statistically significant at $p < 0.001$ by t-test. In the same manner, the N_{stdy}, N_{conf} equivalence as well as the time difference between N_{stdy}, N_{conf} determinations were statistically significant at $p < 0.001$ for the other cases (Fig. 6b–e). Such a good correlation was obtained in a wide range from 4 cells ($\log 4 = 0.602$) (Fig. 6c) to 4140 cells ($\log 4140 = 3.617$) (Fig. 6e).

Discussion

Conventional methods for microbial colony measurement are based on the combination of an appropriate agar medium and culture conditions such as temperature and culture time. It does not depend on whether it is a reference method or a non-validated method. The present system is for automatic processing of those conventional methods and not for proposing a new method based on a new principle. Therefore we have presented experimental data for verification[19] of the performance of the system rather than for validation[19] of a new method.

For automatic processing, it is necessary to define a threshold size of a colony. In conventional methods there is no formal criterion of colony size except a remark in ISO 4832[20]. In fact, the colony count results determined by different personnel are often different[21,22]. Operators' skill dependency is one of major causes of uncertainty in colony count method. In the present system, the threshold size was set as 65 μm, because such a micro-scale size was thought to be applicable to wide spectrum of strains in common, though a comparison with reference strains might be necessary. This threshold size was effective for every strain tested here and operators' skill dependent uncertainty in colony count could be eliminated by automatic processing.

A more serious problem causing measurement uncertainty is complex patterns of colonies after long culture. As long as only one image captured at the end of culture is concerned, it is impossible to determine accurate number of colonies. To solve this problem, retrievable data of time-lapse image should be effective. Thus we developed a system for storing a large number of image data of every colony from its initial appearance throughout until the end of long culture time. Here we have upgraded the system so that entire data of every colony of 100 plates could be processed automatically at practically reasonable speed.

Thus accumulated data may be called big data in comparison with the quantity of data treated by other systems so far developed for microbial colony measurement. As demonstrated above, big data of time-lapse 3D shadow images were really effective for the accurate determination of N_i at any time. Operators can easily make a decision of reaching a steady state by watching the real-time display of N_i time course on a display as well as by automatic processing according to the criteria defined in the online
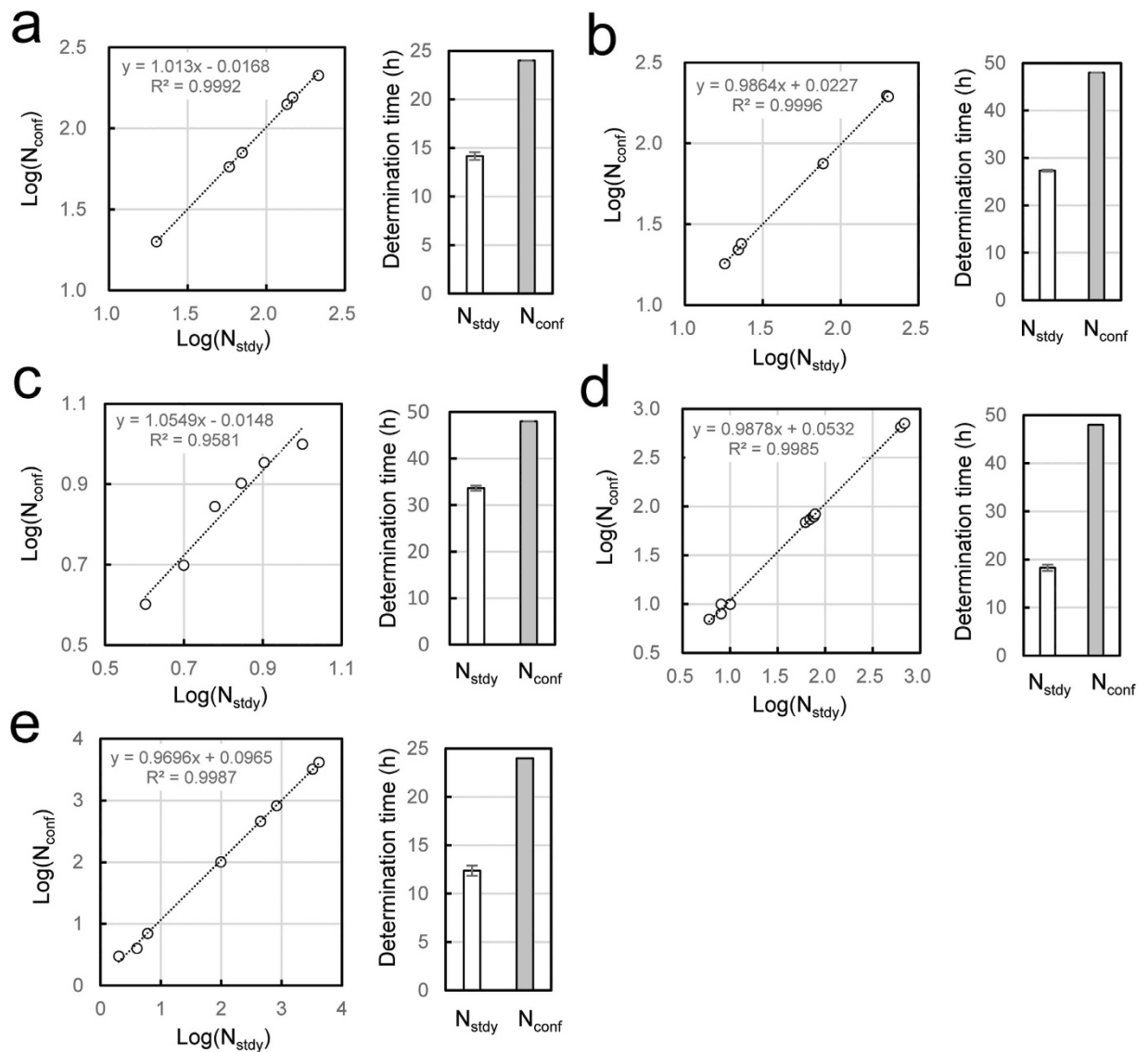
Figure 6. Linear correlation between N_{stdy} and N_{conf} and their determination times. (**a**) *E. coli*, (**b**) *C. albicans*, (**c**) *A. braciliensis*, (**d**) *B. pulmilus*, (**e**) *E. coli* in hamburger. Left panels: Linear relationship between N_{stdy} and N_{conf}, determined by least mean square method. Number of samples: 6 (*E. coli, C. albicans, A. braciliensis*), 11 (*B.pulmilus*), 8 (*E. coli* in hamburger). Right panels: difference of times for N_{stdy} and N_{conf} determination. Gray bars indicate fixed values, 24 h or 48 h. Error bars: mean±SEM.

method section. Thus determined N_{stdy} may be used as a rapid prediction with sufficient accuracy for routine tests in self-check management. Successive recording of the data might not be necessary only for making a rapid decision. However they will be found to be important in case official documentation happens to be required. Then N_{conf} will be used because N_{conf} can be understood to be the result of a reference method if the medium and the culture conditions were equivalent to those of the reference method.

In conclusion the big data based microbial measurement is believed to be an innovative and proactive methodology. In spite of big data processing, a practically reasonable system has been accomplished by unique design of software as well as of hardware.

Online Methods

Culture of microorganisms. *Escherichia coli* (NBRC 3972), *Candida albicans* (NBRC 1594), and *Aspergillus brasiliensis* (NBRC 9455) were obtained from the National Institute of Technology and Evaluation - Biological Resource Center (NBRC) (Kisarazu, Chiba, Japan). *Bucillus pumilus* (ATCC 27142) were obtained from ATCC. Each strain was suspended in a medium containing glycerol and frozen at a temperature lower than −35 °C for storage.

Before use, each strain was taken out from a freezer and placed at room temperature. After thawing, the strain suspension was transferred to a tube containing peptone water (Becton Dickinson and Company) for initial culture. *E. coli* was streaked on desoxycholate agar plates. *C. albicans* and *A. brasiliensis* were streaked on potato dextrose agar (PDA) plates. *B. pumilus was* streaked on soybean-casein digest agar with lecithin and polysorbate 80 (SCDLP agar) plates. Every medium was purchased from Eiken Chemical Co. Ltd., Tokyo. The petri plate size was 90 mm in diameter.

Preparation of contaminated food samples. Hamburger samples contaminated with *E. coli* were prepared as follows. An aliquot of a pure culture of *E. coli* was added to the meat separated from hamburger and stomached for 1 min. A 1 ml aliquot of the resulting suspension was poured into a petri plate and then 20 ml desoxycholate agar medium was added to the plate followed by fully mixing. Cut vegetables were purchased from a market and stomached for 1 min in a phosphate buffered saline (PBS). The resulting suspension was diluted stepwise with PBS. A 1 ml aliquot of the second highest diluent was poured into a petri plate and then 20 ml SCDLP agar medium was added to the plate followed by fully mixing.

Time-lapse shadow image analysis (TSIA). TSIA program was developed previously for the acquisition of 3D shadow images of microbial colonies[18]. The shadow image of every colony in a 3-mm thick agar layer could be sharply focused on CCD by means of a multi-focus system[23]. Viable colonies can be distinguished from dead colonies and non-biological particles by time-lapse analysis. The spatial analytical precision is 1 pixel size, i.e. $65 \times 65\,\mu m^2$ and this size was defined as the threshold size of a colony. More than 2000 colonies per plate could be counted accurately. Using an air-tight bag for a plate, anaerobic bacteria can also be measured. In fact the growth of *Clostridium perfringens* and *Clostridium sporogenes* could be measured (unpublished data). Practically there is no data limit and therefore slowly growing organisms can be measured for sufficiently long culture time.

Criteria of reaching a steady level. The image of colonies and the number of colonies were recorded at each image capture time and designated as N_i (i = 0, 1, 2,···). When N_i satisfied the following criteria (1) or (2), N_{i+2} was assumed to reach a steady level and this level was designated as N_{stdy}:

(1) $N_i > 100$, $|N_{i+1}-N_i| \leq 0.01 \times N_i$ and $|N_{i+2}-N_{i+1}| \leq 0.01 \times N_{i+1}$;
(2) $N_i \leq 100$, $|N_{i+1}-N_i| \leq 1$ and $|N_{i+2}-N_{i+1}| \leq 1$.

On the other hand, N_i confirmed at the end of long culture time was designated as N_{conf}.

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Author Contributions

H.O., S.N. and M.T. developed the colony count system. H.O. also conducted microbiological tests. M.S. and H.M. proposed the concept of the method and analyzed the experimental results.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

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