Analytical Methods

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Journal:	*Analytical Methods*
Manuscript ID	AY-ART-03-2023-000401.R2
Article Type:	Paper
Date Submitted by the Author:	06-May-2023
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ARTICLE

Arrayed labeling-free cultivation and growth evaluation from a single microorganism

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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The development of high-throughput screening methods for microorganisms is desired because microorganisms are useful and sustainable resources with which to produce valuable substances utilized in various industries. Micro-space-based methods are the best candidates for the efficient screening of microorganisms owing to their low reagent consumption and compact integration. In this study, we developed a picoliter-sized incubator array for quantitative and labeling-free evaluation of growth process of E*scherichia coli (E. coli)* by autofluorescence. Because the array with 8464 incubators is able to compartmentalization of single *E. coli* individually utilizing the Poisson distribution, the array can evaluate 100 single *E. coli* simultaneously. Our incubator array not only realized the high-throughput screening of microorganisms, but also provided an analytical tool for assessing individual differences in *E. coli*.

Introduction

Microorganisms are important resources in the fields of green chemistry and sustainable engineering owing to their ability to produce valuable substances[1-3]. In this regard, significant effort is being devoted to the exploration of uncultured microorganisms, which constitute beneficial bioresources[4,5]. However, the current number of successfully cultured species as a proportion of all microorganisms is estimated to be less than 0.02%[6,7]. Inefficient cultivation is one of the major reasons for such a low acquisition rate of microbial resources. To acquire a few species of valuable microorganisms, over one hundred thousand samples have to be cultivated and evaluated[8]. Nevertheless, general cultivation techniques (e.g. high throughput dilution-to-extinction cultivation, dilution plate method)[9,10] require several months for the acquisition of useful microbial resources. This is because the parallel cultivation and evaluation of one hundred thousand samples have not been demonstrated due to the need for a large cultivation space for such apparatus as flasks and microtiter plates[11]. Space-saving cultivation techniques must be developed if we are to achieve the efficient acquisition of microbial resources.

Micro-space-based cultivation methods have recently been attracting attention with a view to improving screening efficiency[12-15]. Micro-droplet methods have been developed as a contribution to cultivation technology based on micro-space[16,17]. Droplets of a few µm to a hundred µm in diameter are produced by droplet generators[18], and then the microorganisms enclosed inside the droplets are evaluated with a cell sorter[19]. More than 100,000 microorganisms were individually cultured in the micro-droplets simultaneously, and then those microorganisms were evaluated by using the metabolic capacity of useful enzymes such as amylase, cellulase, and glucosidase[20-22]. As a result, new microorganisms with the high metabolic capacity of useful enzymes were acquired in just a few days, and high-throughput screening has been demonstrated. However, the micro-droplet method is complicated and costly because specialized apparatus such as a droplet generator and a droplet sorter are required for droplet generation, cultivation, observation, and evaluation for each process[23,24]. Furthermore, hydrophobic metabolites and fluorescent indicators enclosed in the micro-droplets leaked into the oil phase and neighboring droplets, thus making it difficult to maintain the metabolites in the micro-droplet. Louai et al fabricated micro-devices that can trap micro-droplets individually to prevent the leakage of metabolites[25]. However, the micro-droplets shrank with cultivation time since the components of the medium in the micro-droplet were absorbed into polydimethylsiloxane (PDMS) used as the device material, thus making the long-term cultivation of microorganisms difficult[25].

Array devices with independent nL–pL incubators were developed to prevent the migration of hydrophobic metabolites between the micro-spaces[26-30]. An array device fabricated by photolithography can integrate each process (e.g. compartmentalization, cultivation, evaluation, observation) by providing numerous microwells on one chip. For example, Leicheng et al. developed a micro-array made of agarose gel for the retention of highly hydrophobic fatty acids produced by lipase[26]. They reported that the metabolic activity of lipase was evaluated by using fluorescent substrates dissolved in a culture medium. However, the incorporation of the substrate inside the cells affects the growth of microorganisms[31]. Ping et al. reported the growth evaluation of *E. coli* on an array device by fluorescent intensity by GFP expression[29]. However, the apoptosis of living cells be induced by GFP expression[32]. Inoue et al. fabricated a micro-array for the labeling-free cultivation of *E. coli*[33]. Initial growth was evaluated by measuring the diameter of the *E. coli.* However, the measurement of diameter of *E. coli* by bright-field could not achieve accurately because the proliferated *E. coli* overlapped in the z direction[33]. Bio et al. reported that the labeling-free and quantitative evaluation of the growth behavior enclosed in microdevices by autofluorescence[34-37]. However, the method is not suitable for the cultivation of several thousand single cell individually since the

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

method was not equipped with multiply compartmentalized cultivation spaces.

In this study, we report a culture-process-integrated micro-array incubator, which is able to compartmentalize, culture, observe and evaluate microorganisms, for the labeling-free cultivation and quantitative evaluation of microorganisms. The incubator array with flattened design could be achieve the alleviation of *E. coli* overlap. The growth behavior of *E. coli* was evaluated by analyzing the autofluorescence without any labeling reagent. By utilizing the incubators, we were able to culture and evaluate 100 single *E. coli* simultaneously. We also report that the incubator array can be applied to the evaluation of the growth behavior between individual *E. coli*.

Experimental

Reagent

Sulforhodamine B was purchased from FUJIFILM Wako Pure Chemical Corporation (Japan). SYTO9 was purchased from Thermo Fisher Scientific (USA). *Escherichia coli* K-12 (National Institute of Technology and Evaluation, Japan) was used as a model microorganism. A Luria-Bertani (LB) medium for *E. coli* cultivation consisted of 1% (w/v) bacto yeast extract, 2% (w/v) bacto tryptone, and 1% (w/v) NaCl. In the addition of carbon source, 5% (w/v) D-glucose was added to the LB medium. Further details about the reagents employed in this research are given in Supporting Information.

Fabrication of an incubator array

A picoliter-sized incubator array was prepared by combining a standard photolithography and soft-lithography techniques using PDMS. Briefly, the photoresist film was patterned with a conventional photolithography. Then, using the patterned residue as a template, a casting made of PDMS was obtained. Details are described in supporting information (Fig. S1). The PDMS film was finally pasted onto the bottom region of a 35 mm glass dish (Fig. 1). The fabricated incubator array consisted of 92 × 92 incubators on the glass substrate, meaning that a total of 8,464 incubators were available for the parallel cultivation and evaluation of microorganisms on the array (Fig. S2A). We designed the diameter and height of each incubator to be 30 µm and 10 µm, respectively, thus giving the incubator an aspect ratio of 3:1. When the height of each incubator was lower than 10 µm, the growth of *E. coli* was inhibited. The flattened design, the motile microorganism (i.e., *E. coli*) is kept in a constant focus range as described later. Hence, the designed incubator array can allow the long-term observation of *E. coli* during the cultivation process without expensive observation apparatus such as a confocal microscope. (Fig. S2B). The average diameter and height were 30.46 and 10.28 µm, and their standard deviations were 0.68 and 0.37 µm, respectively, (Fig. S2D, E), which resulted in each incubator having a volume of 7 pL. In addition, the measured results had a Gaussian distribution. These results indicate that the sizes of our picoliter-sized incubators were highly uniform. An improvement over the common PDMS replica-making process is the use of the fluoropolymer-deposited photoresist template. This made it easier for the PDMS film to be peeled from template. The detachability of the inserted fluoropolymer film helped to achieve high uniformity in the incubators.

Cultivation and observation of *E. coli* in an incubator array

E. coli was precultured in LB medium at 37°C for 1 day. The *E. coli* solution was diluted with LB medium according to the relationship between the OD_{600} value and a colony-forming unit[38]. The incubator array was filled with carbonated water to exclude trapped air bubbles from the incubators[39]. And then, the LB medium containing *E. coli* was introduced into the incubator array (Fig. 1). The incubator array was placed on a 35 mm glass bottom dish (Matsunami Glass, Japan). The probability of encapsulation (ratio of compartmentalization) of *E. coli* was calculated from the relationship between *E. coli* concentration and incubator size[40]. Next, a transparent adhesive tape (Scotch MP-15 Mending Tape, 3M, USA) was stuck over the PDMS film to prevent it floating during the cultivation process. Finally, the incubator array equipped with the glass bottom dish was placed on a heat stage for the cultivation and observation of *E. coli*. The dish was filled with pure water to prevent the desiccation of the medium.

E. coli was observed with a microscope (Olympus, Japan), and its growth was evaluated from brightfield and fluorescence images (Ex/Em: 480 nm/535 nm). The mean of autofluorescence intensity in the picoliter-sized incubator was analyzed manually with ImageJ software on an Apple computer[41]. All graphs were prepared with GraphPad Prism (Dotmatics, USA).

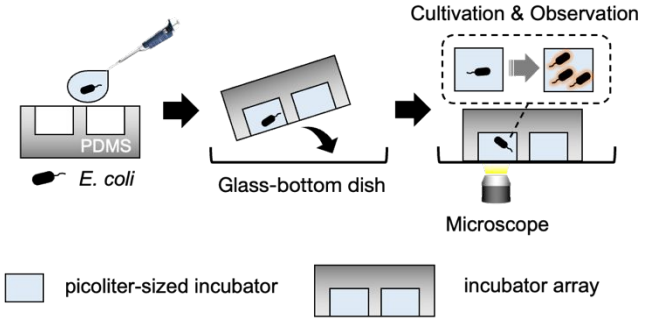

Fig. 1. A schematic of the procedure for the cultivation and growth evaluation of *E. coli* by utilizing a picoliter-sized incubator array. A solution containing *E. coli* was introduced into the incubator array, and then the incubator array was placed onto a glass-bottom dish. The growth behavior was evaluated by analyzing the autofluorescence (Ex/Em: 480 nm/535 nm) from the *E. coli*.

Cultivation of *E. coli* in a flask

In order to compare our method with the conventional method, we also cultured *E. coli* in the conventional flask, and then cultivated at 37°C for 1 day. The optical density of the medium solution containing *E. coli* was measured at a wavelength of 600 nm (OD_{600}) to characterize the *E. coli* growth. Also, a portion of the *E. coli* solution was sampled every 2 hours, and the pH value was measured with a pH meter (LAQUAtwin, HORIBA, Japan).

Results and discussion

Microorganism compartmentalization

We investigated the compartmentalization of our incubator array using *E. coli*. In this case, *E. coli* was intentionally stained with SYTO9 [42] to allow us to clearly evaluate the performance of the incubator array. Single *E. coli* were successfully compartmentalized in each incubator simply by applying *E. coli* suspended in solution to the incubator array (Fig. 2A and Fig. S3). Notably, we confirmed that the probability of encapsulation (=compartmentalization ratio) of *E. coli* had a Poisson distribution as intended (Fig. 2B, C, D). The probability of encapsulation based on the Poisson distribution depends on the area of enclosed space and the concentration of the *E. coli* solution.

The agreement between experimental and theoretical data in Fig. 2 suggest that the incubator array was fabricated uniformly according to the design values. Moreover, this result means that the incubator array can easily compartmentalize target microorganisms in each incubator without additional apparatus such a droplet generator. Here, we demonstrated the compartmentalization of *E. coli* in each incubator as intended, suggesting that the fabricated incubator size was uniform.

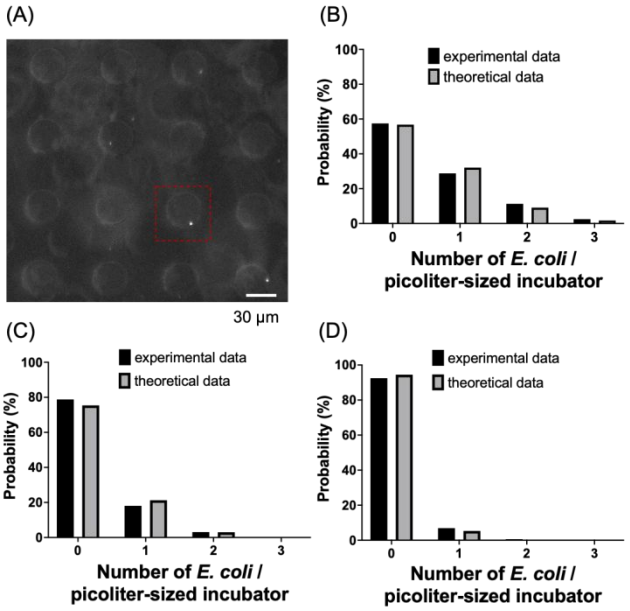

Fig. 2. Investigation of compartmentalization of a single *E. coli* in an incubator. *E. coli* was stained with 1 μM SYTO9. (A) Fluorescence image of the enclosed *E. coli* in the incubator. The red area showed the compartmentalization of a single *E. coli*. (B, C, D) Probability of *E. coli* in a picoliter-sized incubator. Black and gray bars indicate experimental and theoretical data, respectively. The OD_{600} of the solutions were 0.2, 0.1, 0.02. N=160.

Compartment and leakage test of small molecule

Next, we investigated a compartment of our incubator array using a small molecule (sulforhodamine B). A leakage test was also carried out because the crosstalk between micro-incubators has been reported to be a problem[43]. After introducing sulforhodamine B into the picoliter-sized incubators, we placed the incubator array in a dish filled with water at 37°C for 24 h. No changes in the fluorescence intensity were observed from any incubator (Fig. 3A, B, S4). Moreover, no staining of the partition of the incubator was observed, supporting the view that there is no leakage or crosstalk of the enclosed material between the incubators. This result suggested that the leakage of small molecules was prevented, with the result that the medium component would not be contaminated between incubators during the cultivation process. Our incubator array could solve the leakage problem found with the conventional micro-droplet method.

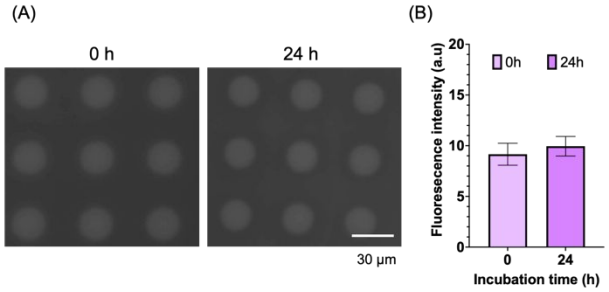

Fig. 3. Leakage test of enclosed fluorescence indicator (1 μM sulforhodamine B). (A) Fluorescence images under a microscope before and after 24 h incubation. (B) Fluorescence intensities of the images calculated with ImageJ (N=20). Error bars indicate the standard deviation.

Cultivation and evaluation with optimal pH

We performed a labeling-free cultivation and quantitative evaluation of *E coli* on our incubator array under an optimal pH condition. We also compared the growth behavior of *E. coli* using a conventional flask-based cultivation method with that on our incubator array. The growth behavior of *E. coli* in the incubator array was evaluated by analyzing the autofluorescence from flavin adenine dinucleotide in the *E. coli*[44] instead of performing a conventional absorbance measurement. The autofluorescence-based methods are expected to enable the labeling-free cultivation and growth evaluation of target microorganisms because labeling processes are unnecessary. In addition, fluorescence detection does not depend on the optical path length unlike absorbance detection. Therefore, autofluorescence detection from a microorganism would be suitable for our incubator array with a 10 μm depth.

First, we analyzed the correlation between autofluorescence intensity and the growth based on number of cells. As a result, the number of cells correlated with autofluorescence intensity, meaning that the evaluation of autofluorescence is a quantitative analytical method for the growth (Fig. S5). Time-lapse bright-field images show that *E. coli* grew in the incubator over the course of the cultivation time (Fig. 4 upper), supporting the idea that the incubator materials were not fatally toxic to *E. coli*. The autofluorescence derived from *E. coli* also increased according to the growth of *E. coli* (Fig. 4 lower). Changes in the autofluorescence intensity of the *E. coli* contained in the incubators reached a plateau phase after approximately 6 h (Fig. S6B). In a conventional flask culture, the growth of *E. coli* also reached a plateau phase after 6 h (Fig. S6A). The growth behavior on our new incubator was consistent with that on a conventional one. The identical growth curves indicate that our incubator array has 1. low toxicity, 2. sufficient nutrients for a 7 pL medium, and 3. no negative bias from the walls of the small incubator (30 μm diameter, 10 μm depth). Moreover, to quantitatively compare the growth behavior of *E. coli*, the maximum growth rate of the *E. coli* in a conventional flask and our incubator were calculated from the Baranyi and Roberts Model (Table S3)[45]. The maximum growth rate in the flask was 1.273 ± 0.127 (1/h) by analyzing the change of optical density. Meanwhile, the maximum growth rate in our incubator was 1.399 ± 0.213 (1/h) by analyzing the autofluorescence intensity. Those results suggest that the analysis of autofluorescence in our incubator is able to quantitatively evaluate the growth of the *E. coli* as well as the conventional method.

The *E. coli* growth in a micro-space was previously evaluated by measuring the diameter of the *E. coli*[33]. However, the measurement was limited to an evaluation of ten *E. coli* or fewer owing to the overlaps of the grown *E. coli* in the z direction[33], meaning that the

evaluation of growth behaviour until the plateau phase was not achieved. We developed an incubator with a flattened design and the *E. coli* overlap could be alleviated. Although it was not possible to accurately count the number of *E. coli* by bright field observation, the quantitative evaluation of more than 100 *E. coli* was demonstrated from the autofluorescence intensity (Fig. 4). Furthermore, no apoptosis issues[32] were observed as no labeling reagents or GFP expression, meaning both minimally invasive culture and evaluation have been achieved.

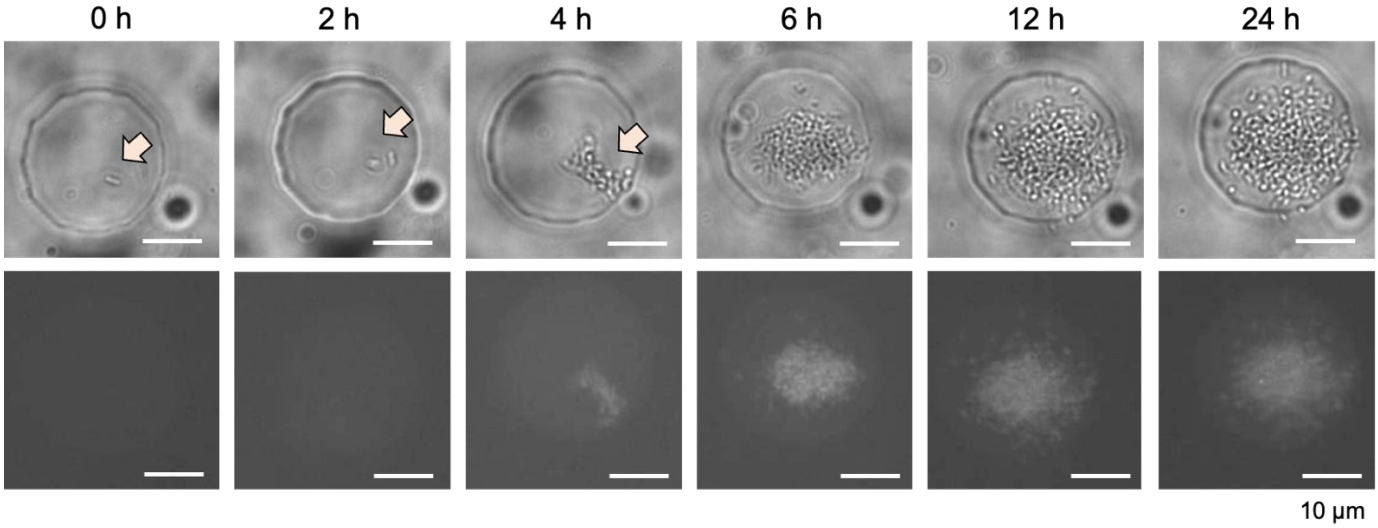

Fig. 4. Microscopic observations of *E. coli* in a picoliter-sized incubator at 37°C. Bright-field (above) and autofluorescence (bottom) time-lapse images were obtained.

Next, we performed simultaneous evaluations of multiple samples on our incubator array. 100 *E. coli* samples were enclosed individually in each incubator. The autofluorescence intensities from *E. coli* increased overall and were distributed as the cultivation time increased (Fig. 5A). This result means that the *E. coli* growth curves differed among the 100 enclosed *E. coli*. The physical compartment size was uniform as shown in Fig. S2, the chemical leakage was negligible as shown in Fig. 3. The distribution in Fig. 5A was considered to indicate the distribution of the growth ability of *E. coli* derived from the cell individuality[46]. In fact, the *E. coli* with to which the total integrated intensity of *E. coli* exhibited an approximately 3 times higher than average fluorescence intensity after 24 h was observed (Table S4 and Fig.5B). Those results suggested that microorganisms with a notable division capacity could be easier to find by culturing microorganisms at an optimal pH.

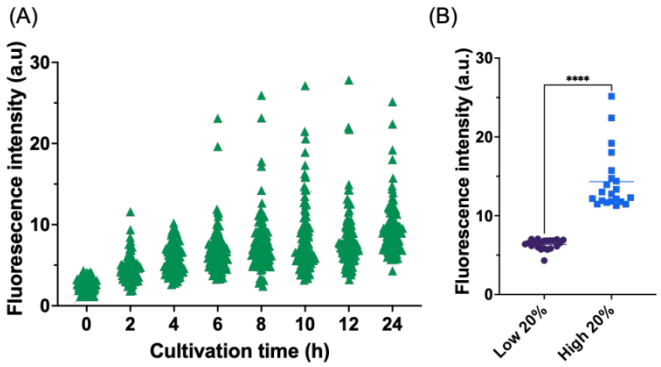

Fig. 5. (A) Simultaneous cultivation and evaluation of 100 individual samples. 100 single *E. coli* samples were compartmentalized into individual incubators and cultured for 24 h. Growth from a single *E. coli* sample was evaluated with a time-lapse measurement. (B) The student's two-tailed t test of fluorescence intensity at 24 h between higher 20% and lower 20 percent. ****p ≤ 0.0001. N=20.

Cultivation and evaluation under various pH conditions

Finally, we cultivated *E. coli* under various initial pH (IpH) conditions to evaluate differences in the growth curve. For example, the mean autofluorescence intensity in IpH5.0 decreased approximately 0.8 times compared with that in IpH7.0 (Fig. 6 and Table S5). This is reasonable because some enzymes involved the growth localized in a cell membrane were known to be inactive under an acidic condition[47]. However, 23% of *E. coli* was found to grow more when cultured at IpH7.0 even at IpH5.0 (Fig. S7 and Fig. 6). In those incubators, the pH could be quickly neutralized by *E. coli* metabolites such as amino acids during the growth process (Fig. S8)[48]. This result means that our incubator array would contribute to the acquisition of microorganisms with resistance to acid.

In addition, we cultivated the *E. coli* in LB medium containing the carbon source. Because the metabolic pathways of microorganisms change depending on the type of carbon source[49], the growth evaluation of microorganisms in the presence of carbon sources is important. Here, we added D-glucose as a common carbon source. In a conventional flask, the OD value in the medium with D-glucose was 0.47 times lower than that without D-glucose (Fig. S9). This is because organic acid metabolism may be activated due to the presence of D-glucose and the pH in the medium became acid. In the cultivation of our incubator, the mean of autofluorescence intensity of *E. coli* was 0.40 times lower than the medium without D-glucose (Fig. S10, Table S6), which is comparable to the conventional flask culture. These results suggest that our incubator is also capable of evaluating growth of *E. coli* in the presence of a carbon source.

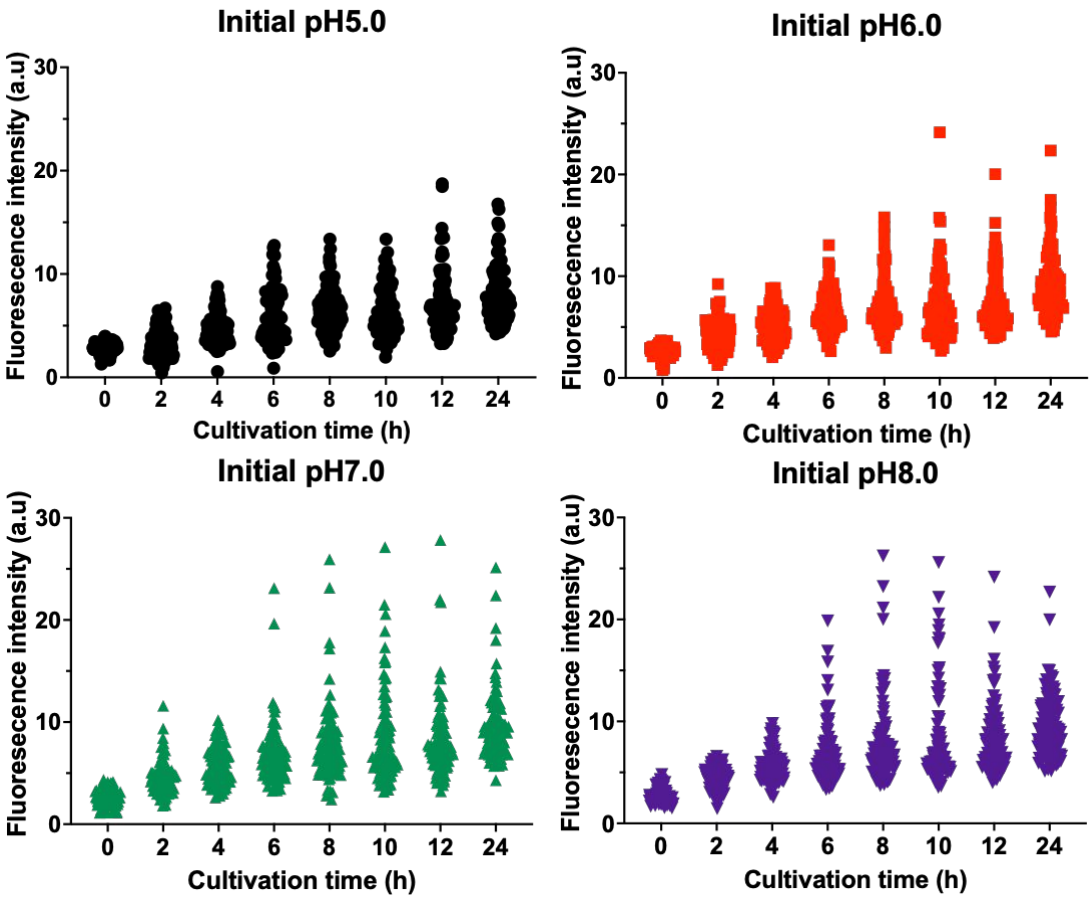

Fig. 6. Growth curves derived from autofluorescence intensity under various initial pH conditions. N=100. Data for IpH7.0 from Fig. 5 was used again for comparison.

Conclusions

In summary, we developed a picoliter-sized incubator array for labeling-free cultivation of microorganisms and evaluate their growth quantitatively. A model microorganism (*E. coli*) was successfully cultured in a 7 pL incubator, and the growth behavior was visualized by analyzing its autofluorescence. A quantitative evaluation was performed from a single *E. coli* to a plateau phase. Since FAD is a coenzyme required for the growth process of all microorganisms, it could be applicable not only to *E. coli* but also to other microorganisms. Moreover, a simultaneous evaluation of 100 single *E. coli* samples was achieved by arraying the incubators. Our incubator array could be used not only for non-invasive cultivation and evaluation, but also as an analytical tool to assess individual differences in *E. coli*. The gas permeability of PDMS is suitable for aerobic microorganisms, in contrast the gas impermeability treatment would be required for the cultivation of anaerobic microorganisms.

Author Contributions

Y.T., T.M., and R.K. designed the research and experiments; Y.T. and T.M. carried out the experimental work and data analysis; Y.T. and R.K. wrote the manuscript. All the authors discussed the results and commented on the manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was financially supported by the Cabinet Office, Government of Japan, Cross-ministerial Strategic Innovation Promotion Program (SIP), “Technologies for Smart Bio-industry and Agriculture”. Y.T. acknowledges the establishment of university fellowships towards the creation of science technology innovation (Grant Number JPMJFS2106) by JST. We also thank Dr. D. Kato for his technical support with the laser scanning microscope measurement.

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