Damage-free light-induced assembly of intestinal bacteria with a bubble-mimetic substrate

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Rapid evaluation of functions in densely assembled bacteria is a crucial issue in the efficient study of symbiotic mechanisms. If the interaction between many living microbes can be controlled and accelerated via remote assembly, a cultivation process requiring a few days can be omitted, thus leading to a reduction in the time needed to analyze the bacterial functions. Here, we show the rapid, damage-free, and extremely dense light-induced assembly of microbes over a submillimeter area with the “bubble-mimetic substrate (BMS)”. In particular, we successfully assembled $10^4$–$10^5$ cells of lactic acid bacteria (*Lactobacillus casei*), achieving a survival rate higher than 95% within a few minutes without cultivation process. This type of light-induced assembly on substrates like BMS, with the maintenance of the inherent functions of various biological samples, can pave the way for the development of innovative methods for rapid and highly efficient analysis of functions in a variety of microbes.
**Results**

We prepared a bubble-mimetic substrate (BMS), consisting of a polystyrene particle as an imitation bubble of 100 µm diameter chemically fixed on a glass substrate, and subsequently coated a platinum thin film (thickness: 10 nm) on the substrate as a heat source of light-induced assembly, as shown in Fig. 1a. Figure 1b, c show the side-view and enlarged transmission images, respectively. We irradiated the platinum thin film on the imitation bubble with a near-infrared continuous wave laser (wavelength: 1064 nm), which was focused on the substrate surface using an objective lens (40X, NA = 0.6) for 300 s. *L. casei* (concentrations: 1.43 × 10⁸ cells/mL, and 1.43 × 10⁷ cells/mL, cells/mL, scanning electron microscopy image is shown in Supplementary Fig. 1) stained with two fluorescent dyes (SYTO9 and propidium iodide (PI)) were used as dispersoids. A non-ionic surfactant (concentration: 9.04 × 10⁻⁵ M) was also included in the dispersion liquid. After laser irradiation with the optical system in Supplementary Fig. 2a, fluorescent images were recorded using a mercury lamp to excite the sample.

Figure 2 shows the fluorescent images acquired before and after laser irradiation at 27 mW laser power for the light-induced assembly of *L. casei* (concentration: 1.43 × 10⁸ cells/mL) on BMS. Figure 2a, b show live and dead bacteria, and Fig. 2c, d show dead bacteria. Particularly, Fig. 2b reveals that the bacteria were assembled under laser irradiation. As demonstrated in Supplementary Movie 1, *L. casei* were transported toward the imitation bubble by convection generated just after laser irradiation at 27 mW power, where they assembled around the imitation bubble at a large scale. The convection stopped when the laser irradiation was stopped. The same phenomenon was observed with other dispersoids such as *Staphylococcus aureus* (*S. aureus*) and polystyrene particles (see Supplementary Figs. 4 and 5).

The number of assembled bacteria (*N_AB*) and the survival rate of bacteria can be estimated from the fluorescent images in Fig. 2 (see Methods in detail of estimation). In the case of assembly on BMS, *N_AB* was found to be higher than 35,000 cells. As shown in Fig. 2c, d, dead bacteria were found after laser irradiation. However, a high bacterial survival rate of 97% was achieved.
Furthermore, we also investigated the relationship between the bacterial survival rate and laser power (see Fig. 2e). The fluorescent images obtained at different laser powers are shown in Supplementary Fig. 3. The survival rate at 1.43 × 10^8 cells/mL was higher than 95% at all the laser powers on the BMS (see Supplementary Fig. 6 for data obtained at other bacterial concentrations). To compare the survival rate with that obtained under general photothermal assembly using a real bubble, we also estimated the survival rate after laser irradiation at powers ranging from 47 to 97 mW for 300 s on a flat substrate coated with a platinum thin film (thickness: 10 nm). In this case, a laser power of 47 mW is the threshold power required to stably generate a real bubble on the flat substrate, and the concentration of *L. casei* was 2.38 × 10^8 cells/mL, where the optical transmission and fluorescent images are shown in Supplementary Fig. 7. On the flat substrate, the maximum bacterial survival rate with laser irradiation at 47 mW laser power was found to be ~80%, which is 15% lower than the minimum survival rate achieved with the BMS. In addition, the survival rate of bacteria on the flat substrate decreased from 80 to 70% with an increase in laser power. These results reveal the capability of BMS in facilitating the assembly of bacteria without thermal damage. Moreover, in the case of *S. aureus* assembled by using BMS with a formed gold thin film, the average *N_AB* was ~4700 cells and the average survival rate was 88.3%. This result indicates that BMS can be applied to other biological samples and metallic coating.

We further investigated the relationship between *N_AB* and laser power at each bacterial concentration (1.43 × 10^8, 7.15 × 10^7, and 1.43 × 10^7 cells/mL). The data for BMS are shown in Fig. 3a and that for the flat substrate is shown in Supplementary Fig. 8. *N_AB* increased up to a laser power of 27 mW at 1.43 × 10^8 and 7.15 × 10^7 cells/mL. However, *N_AB* began to decrease at 37 and 47 mW laser power, respectively, at 1.43 × 10^8 cells/mL and 7.15 × 10^7 cells/mL. As higher laser power generates faster convection, we expected the assembly of more dispersoids. However, the results obtained indicate that *N_AB* decreases at too high a laser power. This is caused by the shrinkage of the stagnant area owing to the fast convection around the bubble. In addition, *N_AB* was almost same at both 1.43 × 10^8 cells/mL and 7.15 × 10^7 cells/mL at a laser power of higher than 37 mW, because the stagnant area formed around the imitation bubble is considered to be independent of the concentration of dispersoids. At 1.43 × 10^7 cells/mL, *N_AB* was low even upon increasing the laser power, which is different from the results obtained with 1.43 × 10^8 cells/mL and 7.15 × 10^7 cells/mL. This tendency is due to the initial low concentration of the bacteria, owing to which the number of dispersoids around the imitation bubble before laser irradiation was much lower than those at other concentrations. From these results, 27 mW of laser power was determined to be the optimal value for the assembly of *L. casei* within this concentration regime. Figure 3b shows the relationship between the laser power and assembly
efficiency, which is $N_{\text{AB}}$ over the total number of bacteria in the dispersion liquid. The assembly efficiency was found to be inversely proportional to the concentration of bacteria, indicating that the dispersoids can be trapped more efficiently at a low concentration. Thus, bacterial assembly can be achieved even at a low initial concentration of bacteria in the dispersion liquid.

In order to investigate the difference between the bacterial survival rate on the BMS and flat substrate in more detail, we numerically evaluated the temperature distribution on the substrate at a laser power of 47 mW, which is the lowest power required to stably generate a real air bubble and assemble dispersoids on a flat substrate. Figure 4 shows the results of the simulation performed by the finite element method with the simulation model shown in Supplementary Fig. 9a. For the flat substrate, near the region where the bacteria were assembled experimentally, a huge increase in temperature to a value higher than 100 °C was determined theoretically. On the other hand, the temperature of the assembly site on the BMS was found to be lower than 50 °C, as in the case of laser irradiation at 47 mW laser power. These results correspond with the experimental results shown in Fig. 2e; the bacteria on the BMS showed a higher survival rate than those on the flat substrate. The difference is caused by the decrease in the heat transfer to the stagnant area. On the flat substrate, the heat source is near the stagnant area where dispersoids assemble, so the heat due to the photothermal effect was conducted to the stagnant area. In contrast, on the BMS, the stagnant area is far away from the heat source, because the platinum thin film on the imitation bubble was irradiated with a defocused laser, so less heat was conducted to the stagnant area. Thus, thermal damage to assembled dispersoids was limited.

In addition, we investigated the mechanism of assembly due to laser-induced convection. The distribution of the convective velocity at a laser power of 27 mW was numerically evaluated. Figure 4c depicts the distribution of the convective velocity $u_r(z)$ at the white vertical line in e; $r = r_{\text{obs}} = 150 \mu m$. The cause of convection and the assembly mechanism can be attributed to the following process: first, the platinum thin film on the imitation bubble is heated by laser irradiation based on the photothermal effect. Second, the convective flow transports the dispersoids toward the imitation bubble. The convection around the imitation bubble is slow because of the no-slip boundary condition of the solid–liquid interface. We consider that such a stagnant region works as an assembly site. Finally, the bacteria in the dispersion liquid were assembled at the stagnant area. On the other hand, the temperature gradient would provide a large influence for the transport of bacteria toward imitation bubble, and there is a possibility that the thermophoresis effect would contribute to
assemble bacteria by BMS. In addition, considering an imaginary cylindrical area around the imitation bubble, as shown in Fig. 4c, f, from the numerical viewpoint, we estimated the number of bacteria transported close to the assembly site based on $N_t = \frac{1}{4\pi} \int_0^{r_{obs}} \int_0^{\rho r_{obs}} j_\rho(z) \, dz$, where $r_{l1}$ is the laser irradiation time ($=300$ s), $r_{obs}$ is the radius of the cylinder ($=150\mu$m) in Fig. 4e, f corresponding to an observation area, $h$ is the height of the cylinder, and $j_\rho(z) = -C_{bac} u_\rho(z)$ is the flux of the bacteria flowing into the imaginary cylinder across its side ($C_{bac} = 1.43 \times 10^8$ cells/mL) is the concentration of bacteria, $u_\rho(z)$ is the $r$-component convective velocity at $r_{obs}$ shown in Fig. 4d). When $h = 100\mu$m, the number of bacteria transported is $N_t = 54,900$, which is of the same order of magnitude as the experimentally determined number of assembled bacteria (40,000 cells) at a laser power of 27 mW. Thus, this assumption indicates that the bacteria dispersed over a distance ranging up to $z = 100\mu$m were assembled.

Finally, in order to investigate the effect of imitation bubble size, experiments for light-induced assembly of polystyrene particles (diameter: 1 µm, concentration: 4.55 × 10^7 particles/mL) with imitation bubble (diameter: 100, 50, and 25 µm) were performed and the time dependence of assembly efficiency and number of assembled particles ($N_{AP}$) were plotted in Fig. 5. In each assembly experiment, assembly efficiency and number of assembled dispersoids were calculated from each 30 s snapshot of fluorescent image video for 300 s and laser power was set to the same laser intensity (power per unit area) on the top of imitation bubble (laser power: 30 mW at 100 µm, 7.5 mW at 50 µm, and 1.9 mW at 25 µm). Particularly, Fig. 5 shows assembly efficiency and $N_{AP}$ was higher for large imitation bubble size that can trap many dispersoids since stagnant area gets larger for the larger imitation bubble. Therefore, 100 µm imitation bubble was optimal size for light-induced assembly on BMS as far as we investigated. In addition, assembly efficiency of 100 µm imitation bubble was saturated after 270 s. This indicates that the stagnant area between imitation bubble and substrate was filled with assembled particles. Therefore, 300 s laser irradiation was long enough for assembly with 100 µm imitation bubble.

**Discussion**

In this study, we succeeded in achieving large-scale and damage-free light-induced assembly of a vast number of microbial cells at the solid–liquid interface on a substrate without bubble generation under laser irradiation using a designed bubble-mimetic substrate consisting of a submillimeter polymer particle as an imitation bubble. As an example, 10^9–10^10 cells of *L. casei* were assembled on the BMS within a few minutes, the survival rate was found to be higher than 95% even at high laser powers and different bacterial concentrations. These results are attributed to the decrease in heat conduction to the stagnant area, which serves as the assembly area on this substrate.

This method can be extended to unconventional biological analysis for the evaluation of the functions of not only intestinal bacteria but also assembled beneficial bacteria (e.g., electricity-producing bacteria and ethanol-producing bacteria) or physico-chemical interactions of dense bacterial colonies. In the future, the method based on the clarified mechanism described herein may be used for evaluating the symbiotic mechanism of various types of beneficial bacteria, pathogenic properties of harmful bacteria or viruses, and the effect of drugs on bacteria.

**Methods**

**Sample preparation.** A lactic acid bacterium, *L. casei* (average long axis: 1.22 µm, average short axis: 0.51 µm), *S. aureus* (average diameter: 0.91 µm), and polystyrene particles (diameter: 1 µm) were stained with SYTO9 (a green fluorescent dye) and PI (a red fluorescent dye) (LIVE/DEAD® BacLight™ Bacterial Viability Kit for microscopy, Invitrogen, USA).

**Preparation of the bubble-mimetic substrate.** First, ethanol (360678-5 S, Sigma Aldrich, USA) was dropped on a glass-bottom dish (3911-035, IWAKI, Japan) and dried naturally in a draft chamber. Next, 100 µL of ultrapure water was dropped on the substrate, and the substrate was placed on the stage of liquid–solid interface on a substrate without bubble generation of the optical system used in this paper is shown in Supplementary Fig. 2b was used; it is a microscope based on OTKUM (THORLABS, United States) and an objective lenses (CFI S Plan Fluor ELWD 40X, 0.6 NA) on the imitation bubble/glass substrate interface from the bottom. The laser spot diameter was 2.6 µm and the laser power was determined with a laser power meter (UP170-H5 and TUNER; Gentec Electro-Optics, Canada). The halogen lamp to excite sample was turned off during laser irradiation in only *L. casei* assembly experiment. After 300 s of laser irradiation, fluorescent images were recorded. The fluorescent image movie was also recorded during laser irradiation to investigate time dependence of assembly efficiency and the number of assembled dispersoids. In order to observe the imitation bubble from side, another optical system shown in Supplementary Fig. 2b was used; it is a microscope based on UPL-50X (Olympus) and an objective lenses (CFI S Plan Fluor ELWD 20X, 0.45 NA for Fig. 1b and OLYMPUS SLMPPL50x 0.35 NA for Fig. 1c).

**Optical setup.** The optical system used in this paper was shown in Supplementary Fig. 2a. An inverted optical microscope (Eclipse Ti-U; Nikon, Japan) was used for the light-induced assembly of bacteria by laser irradiation with a back port adapter (MN5-2L-8000/1064; Sigma Koki, Japan). A 100 µL of the sample dispersion liquid was dropped on the substrate, and the substrate was placed on the stage of the microscope. Then, a non-ionic surfactant, poloxymethylene sorbitol monolaurate (T20; Wako Pure Chemical Industries, Japan), was added to achieve a surfactant concentration of 9.04 × 10^{-5} M in the bacterial suspension. Thereafter, the near-infrared continuous wave laser of 1064 nm wavelength (FLS-1064-2000F, Sigma Koki, Japan) was focused using an objective (CFI S Plan Fluor ELWD 40X, 0.6 NA) on the imitation bubble/glass substrate interface from the bottom. The laser spot diameter was 2.6 µm and the laser power was determined with a laser power meter (UP170-H5 and TUNER; Gentec Electro-Optics, Canada). The halogen lamp to excite sample was turned off during laser irradiation in only *L. casei* assembly experiment. After 300 s of laser irradiation, fluorescent images were recorded. The fluorescent image movie was also recorded during laser irradiation to investigate time dependence of assembly efficiency and the number of assembled dispersoids. In order to observe the imitation bubble from side, another optical system shown in Supplementary Fig. 2b was used; it is a microscope based on OTKUM (THORLABS, United States) and an objective lenses (CFI S Plan Fluor ELWD 20X, 0.45 NA for Fig. 1b and OLYMPUS SLMPPL50x 0.35 NA for Fig. 1c).
Estimation of the number of bacteria in the assembly. The number of assembled dispersions was estimated from fluorescent images by using software (NILS-Elements Analysis; Nikon, Japan). The fluorescence intensity of the bacteria at the edge of the fluorescent image was determined to be a standard value; the fluorescent area over the standard value was measured in the whole observation area, and $N_{AB}$ was estimated by dividing the total fluorescent area by the area of an individual dispersed determined from the SEM image. Survival rate is the ratio of the number of the live bacteria to the total number of bacteria calculated by the previous method. In the case of the flat substrate, $N_{AB}$ was estimated as reported in a previous study and the survival rate was calculated based on it.

Finite element method simulation for light-induced assembly with BMS. Distributions of the convective velocity and temperature were calculated with 2D rotational symmetry based on finite element method using COMSOL Multiphysics (COMSOL AB, Sweden). Supplementary Fig. 9a shows the 2D simulation model of the bubble-mimetic substrate. The Navier–Stokes equation, mass continuity equation, and energy equation were used. The velocity was solved for the liquid region (water), whereas the temperature was solved for the entire region including the substrate (glass) and the imitation bubble (polystyrene) or the bubble (air). In the case of the bubble-mimetic substrate, we assumed that the laser focused on the glass substrate was defocused at 80 µm from the glass substrate. We assumed that the region indicated by red line (spherical segment on top of the imitation bubble) shown in Supplementary Fig. 9a generates heat according to, $Q_\text{fl} = PA/S$, where $P$ is the laser power (47 mW in Fig. 9b, 27 mW in Fig. 9c). $A$ is the absorbance (=0.5; it was determined by electromagnetic simulation described below), and $S$ is the surface area of the spherical segment (=6823.2 μm²). In the case of the flat substrate, a point heat source with $Q_\text{fl} = PA$ was assumed, because the laser was focused on the substrate. We assumed a no-slip and slip boundary condition for the solid/liquid and air/liquid interfaces. In addition, we assumed that the Marangoni effect at the air/liquid interface depends on the experimental concentration of the surfactant. The mesh size was nonuniform; the maximum size was 296 μm and the minimum size was 1 μm for the center region.

Finite difference time domain simulation for calculation of the absorbance. The absorbance was calculated based on finite difference time domain simulations (FDTD) using Numerical FDTD Solutions (the simulation model is shown in Supplementary Fig. 9b). We assumed that the flat platinum thin film (thickness: 10 nm) on the polystyrene substrate was irradiated by a plane wave. The boundary condition was perfectly matched layer (PML) for the wall perpendicular to light-propagation direction, and a periodic boundary condition for the other walls. As a result, the absorbance was determined to be 0.5 (see the absorption spectrum in Supplementary Fig. 9c). The mesh size was 1 nm.

Statics and reproducibility. The error bars in each plots show standard deviation and each plot show average to $n = 3$ samples. The experiments were performed three times by using the different bubble-mimetic substrate.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be available from the corresponding authors on reasonable request.

Received: 12 July 2020; Accepted: 28 January 2021; Published online: 22 March 2021

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**Acknowledgements**

We would like to thank professor I. Nakase, and professor M. Fujiwara. This work was supported by a JST-Mirai Program (No. JPMJMI18GA), Grant-in-Aid for Scientific Research (A) (no. JP17H00856), Grant-in-Aid for Scientific Research (B) (no. JP18H03522), Scientific Research on Innovative Areas (no. JP16H06507), Grant-in-Aid for Early-Career Scientists (no. JP20K15196) from JSPS KAKENHI, AMED (no. JP20he0622017), Murata Science Foundation, and the Key Project Grant Program of the Osaka Prefecture University.

**Author contributions**

T.I. and S.T. initiated the research and contributed equally to the study design. K.H., Y.Y., T.I., and S.T. performed the production of bubble-mimetic substrate and light-induced assembly of bacteria. K.H., M.T., and T.I. carried out theoretical calculations. K.H., M.T., T.I., and S.T. prepared the figures and manuscript. All the authors discussed the results and commented on the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s42003-021-01807-w.

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