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TITLE:

Bacterial Cell Culture at the Single-Cell Level Inside Giant Vesicles

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KEYWORDS:

bacterial cell culture, single-cell culture, giant vesicles, droplet transfer method, artificial-cell based incubator, microbiology

SUMMARY:

We demonstrate single-cell culture of bacteria inside giant vesicles (GVs). GVs containing bacterial cells were prepared by the droplet transfer method and were immobilized on a supported membrane on a glass substrate for direct observation of bacterial growth. This approach may also be adaptable to other cells.

ABSTRACT:

We developed a method for culturing bacterial cells at the single-cell level inside giant vesicles (GVs). Bacterial cell culture is important for understanding the function of bacterial cells in the natural environment. Because of technological advances, various bacterial cell functions can be revealed at the single-cell level inside a confined space. GVs are spherical micro-sized compartments composed of amphiphilic lipid molecules and can hold various materials, including cells. In this study, a single bacterial cell was encapsulated into 10–30 µm GVs by the droplet transfer method and the GVs containing bacterial cells were immobilized on a supported membrane on a glass substrate. Our method is useful for observing the real-time growth of single bacteria inside GVs. We cultured *Escherichia coli* (*E. coli*) cells as a model inside GVs, but this method can be adapted to other cell types. Our method can be used in the science and industrial fields of microbiology, biology, biotechnology, and synthetic biology.

INTRODUCTION:

The culture of bacterial cells at the single-cell level has received increasing attention. Culturing

bacterial cells at the single-cell level inside a confined space can elucidate bacterial functions such as phenotypic variability[1–4], cell behavior[5–9], and antibiotic resistance[10,11]. Because of recent advances in culture techniques, the culture of single bacteria can be achieved inside a confined space, such as in a well-chip[4,7,8], gel droplet[12,13], and water-in-oil (W/O) droplet[5,11]. To promote understanding or utilization of single bacterial cells, further technical developments of cultivation techniques are needed.

Vesicles that mimic the biological cell membrane are spherical compartments consisting of amphiphilic molecules and can hold various materials. Vesicles are classified according to size and include small vesicles (SVs, diameter < 100 nm), large vesicles (LVs, < 1 μm), and giant vesicles (GVs, > 1 μm). SVs or LVs are commonly used as drug carriers because of their affinity to the biological cell membrane[14]. GVs have also been used as a reactor system for the construction of protocells[15] or artificial-cells[16]. Encapsulation of biological cells into GVs has been reported[17,18], and thus GVs show potential as a cell culture system when combined with the reactor system.

Here, along with a video of experimental procedures, we describe how GVs can be used as novel cell-culture vessels[19]. GVs containing bacteria were made by the droplet transfer method[20] and were then immobilized on a supported membrane on a cover glass. We used this system to observe bacterial growth at the single-cell level inside GVs in real-time.

PROTOCOL:

1. Preparation of GVs containing bacterial cells by the droplet transfer method

1.1. Prepare lipid stock solutions of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 10 mM, 1 mL) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethyleneglycol)-2000] (biotin-PEG-DSPE, 0.1 mM, 1 mL) in chloroform/methanol solution (2/1, v/v) and store the stock at -20 °C.

1.2. Preparation of a lipid-containing oil solution

1.2.1. Pour 20 μL of the POPC solution and 4 μL of the biotin-PEG-DSPE solution into a glass tube (**Figure 1b** (i)).

1.2.2. Evaporate the organic solvent by air flow to form a lipid film and place the film in a desiccator for 1 h to completely evaporate the organic solvent (**Figure 1b** (ii)).

NOTE: It is necessary to evaporate the organic solvent in a fume hood.

1.2.3. Add 200 μL of mineral oil (0.84 g/mL, **Table of Materials**) to the glass vial (**Figure 1b** (iii)).

1.2.4. Wrap the opening part of the glass vial with film and sonicate it in an ultrasonic bath (120 W) for at least 1 h (**Figure 1b** (iii)). The final concentrations of POPC and biotin-PEG-DSPE are 1 mM and 0.002 mM, respectively.

1.3. Pre-culture of bacterial cells

1.3.1. Inoculate *E. coli* into 1× LB medium (1 g yeast extract, 2 g bacto tryptone, and 2 g sodium chloride in 200 mL of deionized water) from an LB plate and incubate at 37 °C for 12–14 h (overnight).

1.3.2. After incubation, collect 20 µL of the culture solution and transfer to 1.98 mL of fresh 1× LB medium, and culture the cells again for 2 h.

1.3.3. Check the optical density at 600 nm (OD_{600}) value of the pre-culture solution (prepared in step 1.3.2). A pre-culture solution of OD_{600} = 1.0–1.5 should be used.

1.4. Preparation of the outer and inner aqueous solutions of GVs

1.4.1. Dissolve glucose in 1× LB medium to prepare an outer aqueous solution of GVs. Prepare 20 mL of a stock glucose solution (500 mM).

1.4.2. Dilute the stock glucose solution with 1× LB medium to 200 mM (**Table 1**).

1.4.3. Dissolve sucrose in 1× LB medium to prepare an inner aqueous solution of GVs. Prepare 20 mL of a stock sucrose solution (500 mM).

1.4.4. Mix the pre-culture solution (OD_{600} = 1.0–1.5), sucrose solution (500 mM), and 1× LB medium (**Table 1**). The final OD_{600} value of the culture solution should be 0.01–0.015 and the final sucrose concentration should be 200 mM.

NOTE: Take care to avoid osmotic pressure. It is necessary to balance the concentration between the inner and outer aqueous solution.

1.5. Preparation of water-in-oil (W/O) droplets containing bacterial cells

1.5.1. Add 2 µL of the inner aqueous solution of GVs (prepared in step 1.4.4) to 50 µL of the oil solution containing lipids (mineral oil with POPC and biotin-PEG-DSPE) in a 0.6 mL lidded plastic tube (**Figure 1b** (iv)).

1.5.2. Emulsify the two components in the plastic tube by tapping the tube by hand (**Figure 1b** (v)).

1.6. Formation of GVs containing bacterial cells

1.6.1. Add 50 µL of the outer aqueous solution of GVs (prepared in step 1.4.2) in a 1.5 mL lidded plastic tube (**Figure 1b** (vi)) and gently layer 150 µL of the oil solution containing lipids (mineral oil with POPC and biotin-PEG-DSPE) on the surface of the outer aqueous solution (**Figure 1b** (vii)).

Incubate this sample at room temperature (RT, 25 °C) for 10–15 min. Check to ensure that the interface of the oil and aqueous solutions is flat.

1.6.2. Add 50 μL of the W/O droplet solution (prepared in step 1.5.2) on the interface of the oil and aqueous solution using a pipette (**Figure 1b** (viii)).

1.6.3. Centrifuge the 1.5 mL lidded plastic tube (from step 1.6.2) for 10 min at 1600 x *g* at RT in a desktop centrifuge (**Figure 1b** (ix)). After centrifugation, aspirate the oil (top layer) from the 1.5-mL lidded plastic tube using a pipette, and collect the GVs containing bacterial cells (**Figure 1b** (x)).

2. Preparation of a GV observation system (bacterial cell culture system)

2.1. Preparation of small vesicles (SVs) for constructing a supported bilayer membrane

2.1.1. Pour 20 μL of the POPC solution and 4 μL of the biotin-PEG-DSPE solution into a glass tube (using the same lipid composition as used for GV preparation in step 1.1).

2.1.2. Evaporate the organic solvent by air flow to form a lipid film and place this sample in a desiccator for 1 h to completely evaporate the organic solvent.

2.1.3. Add 200 μL of 200 mM glucose in 1× LB medium (the outer aqueous solution of GVs) to the glass vial.

2.1.4. Wrap the opening part of the glass vial with film and sonicate it in an ultrasonic bath (120 W) for at least 1 h.

2.1.5. Prepare SVs by the extrusion method[21] using a mini-extruder and polycarbonate membrane with 100 nm pore size.

2.2. Preparation of a handmade chamber

2.2.1. Drill a 7 mm hole with a hollow punch on a double-faced seal (10 mm x 10 mm x 1 mm).

2.2.2. Paste the double-faced seal with the hole on a cover glass (30 mm x 40 mm, thickness 0.25–0.35 mm).

2.3. Preparation of a supported bilayer membrane on the cover glass in the hole of the chamber

2.3.1. Add 30 μL of the SV solution to the hole of the chamber (prepared in section 2.2) and incubate at RT for 30 min.

2.3.2. Gently wash the hole twice with 20 μL of 1× LB medium containing 200 mM glucose (the outer aqueous solution of GVs) by pipetting.

2.4. Immobilization of GVs on the supported bilayer membrane on the cover glass in the hole of the chamber

2.4.1. Introduce 10 µL of neutravidin with the outer aqueous solution of GVs (1 mg/mL) into the hole and incubate at RT for 15 min.

2.4.2. Gently wash the hole twice with 20 µL of 1× LB medium containing 200 mM glucose (the outer aqueous solution of GVs) by pipetting.

2.4.3. Add all solution containing GVs (prepared in step 1.6.3) into the hole of the chamber and seal with a cover glass (18 mm x 18 mm, thickness 0.13–0.17 mm) (**Figure 2b**).

2.5. Microscopic observation of bacterial cell growth inside GVs

2.5.1. Set a microscopic heating stage system with an inverted microscope equipped with a 40x/0.6 numerical aperture (NA) objective lens with a long working distance (**Figure 2b**).

2.5.2. Place the chamber on the microscopic heating stage system (**Figure 2b**). Incubate the GVs containing bacterial cells in the chamber at a static condition for 6 h at 37 °C.

2.5.3. Capture and record microscope images of bacterial cell growth inside GVs every 30 min by using a scientific complementary metal oxide semiconductor (sCMOS) camera.

REPRESENTATIVE RESULTS:

We present a simple method for generating GVs containing single bacterial cells using the droplet transfer method (**Figure 1**). **Figure 1a** shows a schematic image of the precipitation of GVs containing bacteria. W/O droplets containing bacteria are transferred across the oil-water (lipid monolayer) interface by centrifugation to form GVs. The difference in density between sucrose (inner aqueous solution) and glucose (outer aqueous solution) also assists the crossing of the oil-water interface of the W/O droplets. It is necessary to monitor the osmotic pressure in inner and outer aqueous solution because a slight difference in concentration between these solutions can induce deformation and collapse of GVs. A flow chart for preparing GVs containing bacterial cells by the droplet transfer method is shown in **Figure 1b**. By following this procedure, GVs containing single bacterial cells can be easily obtained.

To observe bacterial cell growth within GVs, an original culture system was constructed for microscopic observation (**Figure 2**). GVs containing bacteria were immobilized on a supported membrane surface coated with neutravidin on a cover glass (**Figure 2a**). This immobilization technique has enabled prolonged observation of GVs.

Typical phase-contrast microscopy images of the different sized GVs containing single bacterial cells are shown in **Figure 3**. In this experiment, we also obtained GVs containing bacterial cells with sizes ranging from 10 µm to 30 µm. **Figure 3** shows bacterial growth at the single-cell level

inside GVs with different sizes of 10.7 μm (**Figure 3a**) and 28 μm (**Figure 3b**). For both sizes of GVs, *E. coli* cells underwent elongation and division processes, with one or two *E. coli* cells growing to a very large number of cells over 6 h. Thus, *E. coli* cells grew stably inside the GVs.

The relative frequency of GVs containing a given number of bacterial cells is shown in **Figure 4**. In our experimental condition (OD_{600} = 0.01–0.015), bacterial cells were encapsulated at the single cell level in approximately 10% of the obtained GVs (empty GVs were approximately 80%). The GVs encapsulated at the single cell level were approximately 50% of the GVs containing bacterial cells, as estimated from the inset of **Figure 4**.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental procedures of GVs containing bacteria. (**a**) Scheme of GVs containing bacteria prepared by a droplet-transfer method. W/O microdroplets containing bacteria pass through a lipid monolayer interface by centrifugal force and then form a lipid bilayer membrane. (**b**) Flow of the synthesis of GVs containing bacteria. (i) Organic solvent containing lipids (POPC and biotin-PEG-DSPE, 100:0.2 molar ratio). (ii) Lipid film at the bottom of the glass vial. (iii) Oil solution containing lipids. (iv) Mixture of 50 μL of oil solution and 2 μL of inner aqueous solution (200 mM sucrose and 1× LB medium) containing bacterial cells. (v) Emulsification by hand tapping (over 50 times). (vi) 50 μL of the outer aqueous solution (200 mM glucose in 1× LB medium). (vii) Layering of 150 μL of oil solution on the outer aqueous solution. (viii) Layering of the W/O droplet solution. (ix) Centrifugation of the tube. (x) Precipitated GVs containing bacterial cells after aspiration of the oil.

Figure 2: The observation system of bacterial cell culture inside GVs. (**a**) GVs are immobilized on a supported membrane through biotin–neutravidin binding on the cover glass. GVs are incubated by a heating system. (**b**) Picture of the observation system including a handmade chamber.

Figure 3: Phase-contrast microscope images of GVs containing single bacteria cells (indicated by the black arrows). Snap-shots of bacterial cell growth inside different sized GVs. (**a**) Vesicle size = 10.7 μm. (**b**) Vesicle size = 28 μm.

Figure 4: Statistical analysis of the number of encapsulated bacterial cells per GV. The relative frequencies of GVs were plotted as histograms. Inset: Magnification of the relative frequencies of GVs containing bacterial cells from single to 10< cells. A total of 235 GVs were analyzed.

Table 1: The composition and volumes of the outer and inner aqueous solutions of GVs.

DISCUSSION:

Here, we describe a method for culturing bacterial cells at the single-cell level inside GVs. This simple method involves forming GVs containing bacterial cells at the single-cell level by using the droplet transfer method. Compared with other approaches for obtaining GVs containing bacterial cells, this method has two advantages: (i) it is easy to develop, and (ii) a small volume (2 μL) of

the sample solution is required to prepare the GVs. The droplet transfer method[20] for preparing GVs containing bacterial cells is simpler than the classical hydration[22] and microfluidics methods[17]. For example, the classical hydration method[22] is a simple and easy method for preparing GVs, but the encapsulation efficiency of materials into GVs is quite low and at least a few hundred microliters of sample is required. The recently developed cellulose paper-abetted hydration[23] and gel-assisted hydration[24] methods for making GVs have a high encapsulation efficiency of biomolecules compared with the classical hydration method[22]. Their encapsulation efficiency is as high as that of the droplet transfer technique, and it is expected that these two methods may allow the encapsulation of cells inside GVs. Moreover, the microfluidics method[17] accurately encapsulates single cells inside GVs and shows a very high encapsulation efficiency of materials into GVs but requires complicated handling and techniques for fabricating microdevices and a large sample volume (at least a few milliliters) to flow the tube.

In this protocol, the stability of the oil-water interface is important for obtaining GVs containing bacterial cells (**Figure 1b** (vii)). To obtain many GVs, it is essential to flatten the oil-water interface. Therefore, proper preparation of the oil phase is necessary. We sonicated the oil phase for at least 1 h in a high-power ultrasonic bath (120 W) to completely dissolve the lipid molecules. It is important to layer the oil phase on the outer aqueous solution immediately after sonication (**Figure 1b** (vii)).

The method described here has two limitations. First, GVs often break and bacterial cells leak into the outer aqueous solution. This is because during GV formation, some W/O droplets cannot transfer through the oil-water interface and become ruptured. This is unavoidable when using the droplet transfer method[20]. Additionally, GVs may break during observation. The stability of GVs must be improved, such as by using an artificial cytoskeleton that stabilizes GVs[25]. Second, the number of encapsulated bacterial cells cannot be perfectly controlled. **Figure 4** shows that a large number of bacterial cells were encapsulated in the GVs, and therefore, it is difficult to control the number of bacterial cells in GVs using the droplet transfer method. To control the cell number, microfluidics technology can be used.

GVs may control their inner aqueous solution more effectively than other materials (gel droplets[12,13] or W/O droplets[5,11]). For example, the aqueous conditions of the inner and outer solutions of GVs are altered by natural membrane permeability[26] or permeability facilitated by a membrane pore[16] or transporter[27]. In the present method, oil molecules (mineral oil in this case) remained in the membrane[20]. The influence of the oil remaining in the membrane on the permeability of nutrients or oxygen for bacterial growth is unknown. Although we do not know the natural membrane permeability of nutrients or oxygen, we consider that the amount of nutrients or oxygen in the growth medium was sufficient for bacterial growth in the present study. The natural membrane permeability of nutrients or oxygen is very important for bacterial cell growth and is an important topic for future study. The technique for controlling permeability cannot be conducted using the culture method with gel droplets[12,13] or W/O droplets[5,11]. GVs will thus become the first choice for bacterial culture applications in a confined space.

Our bacterial culture method is a potentially new concept and tool in microbiology[19] to culture unknown environmental bacteria for obtaining or analyzing their metabolic products. Moreover, our bacterial cell-containing GVs are a hybrid system of an artificial cell model (GVs) and a living cell (bacterial cells) to make a new tool for biotechnology[28] and bottom-up synthetic biology[29].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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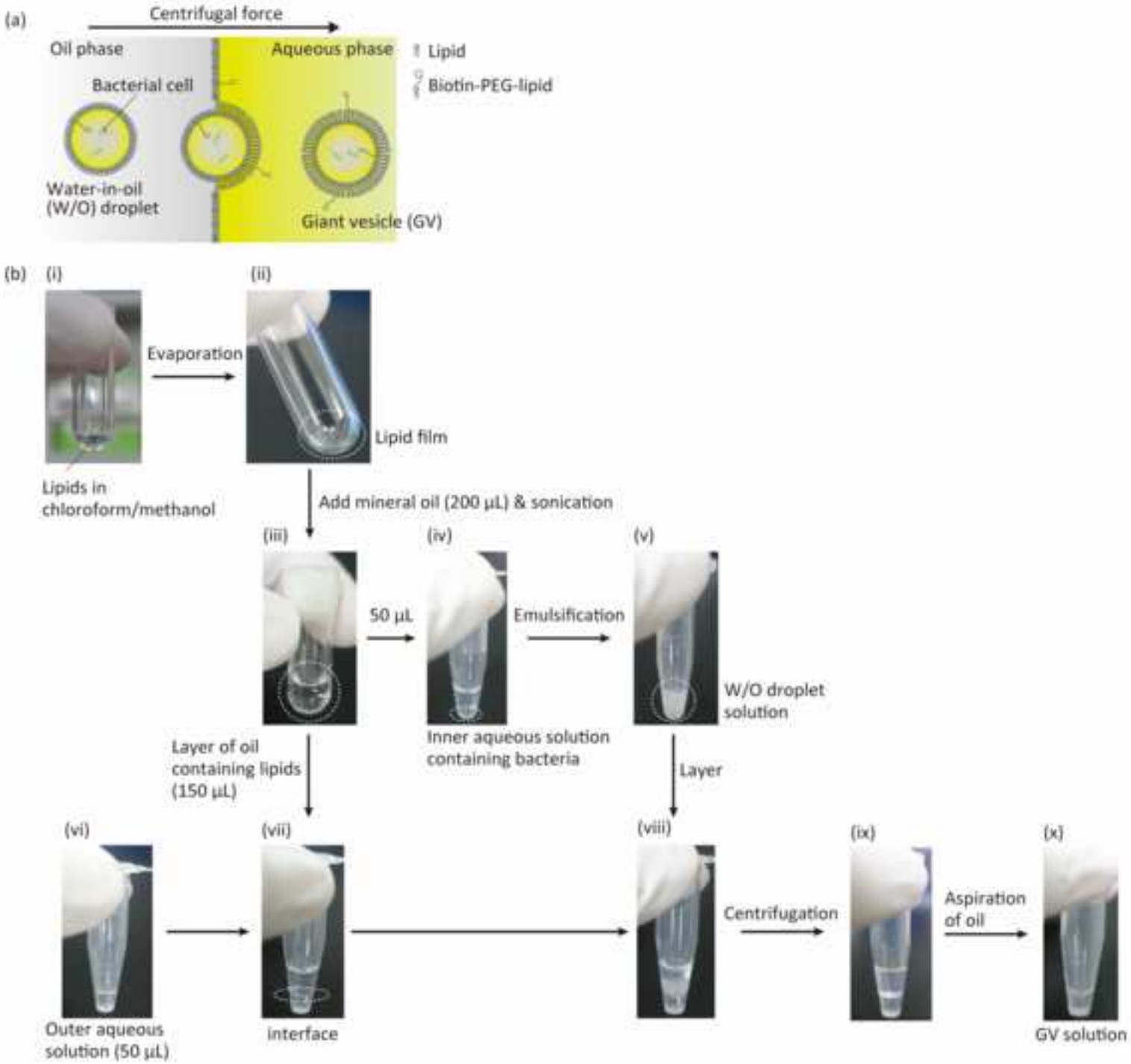

Figure 2

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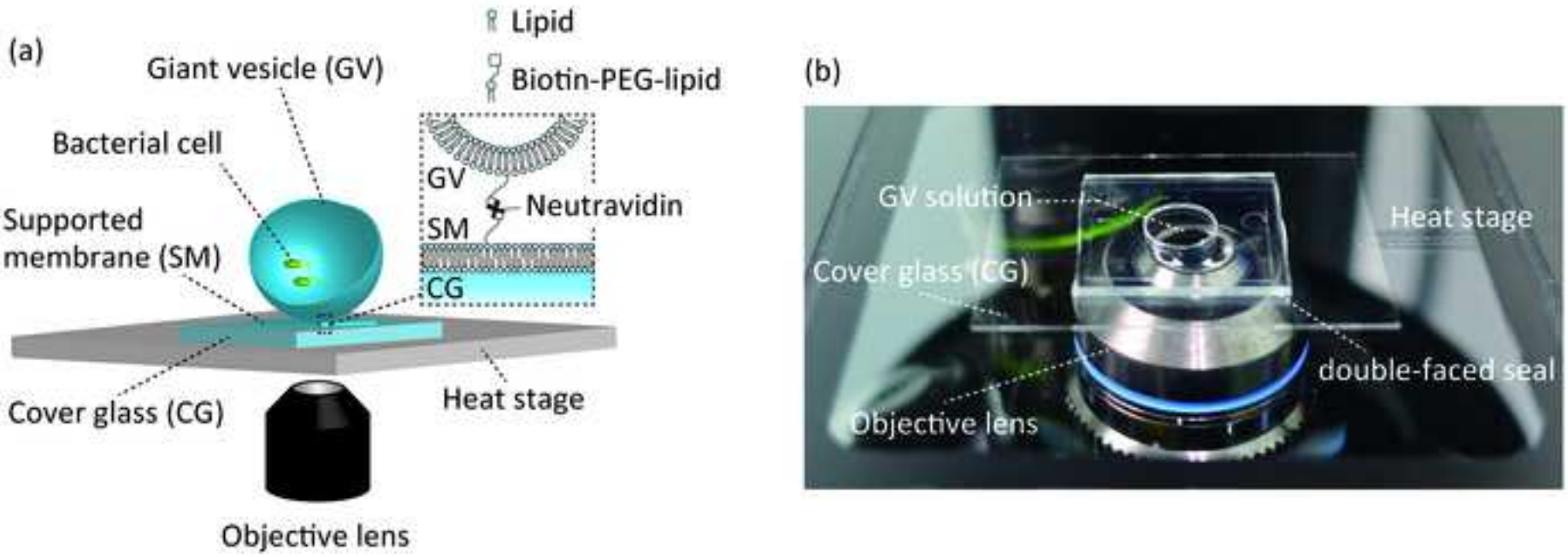

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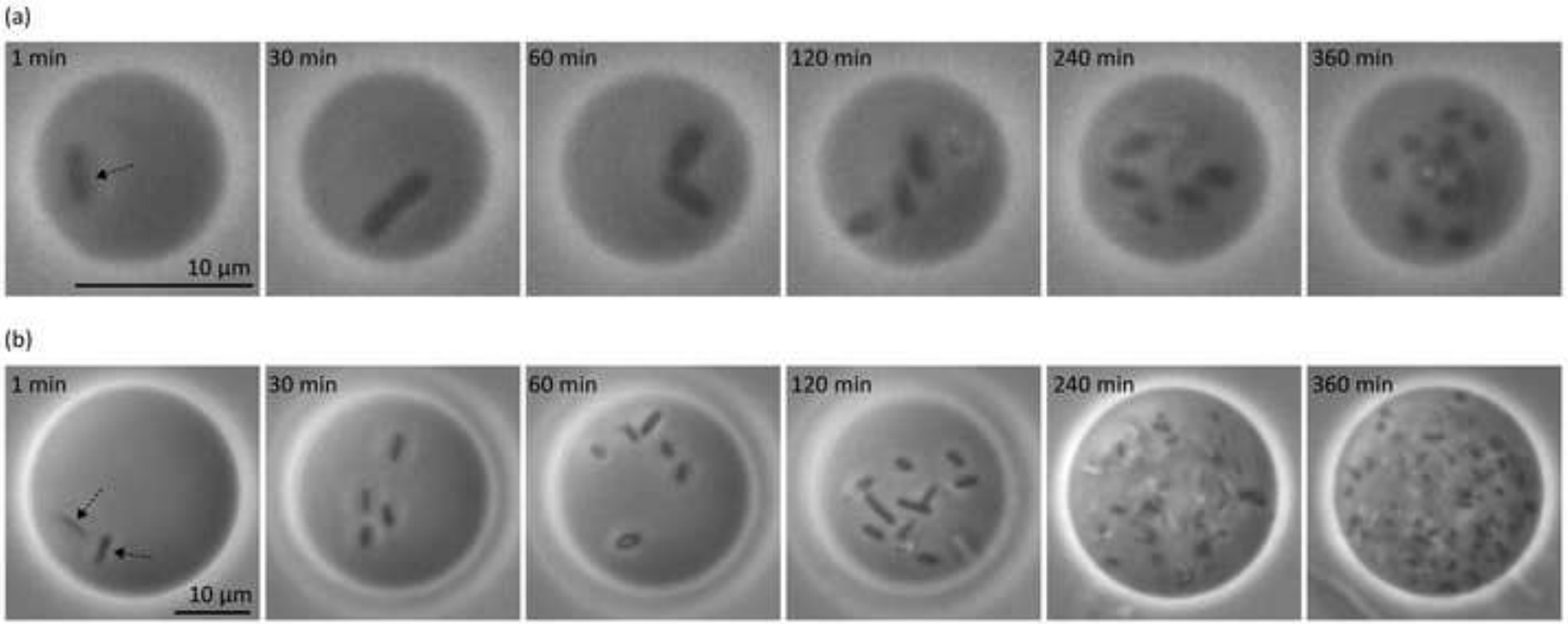

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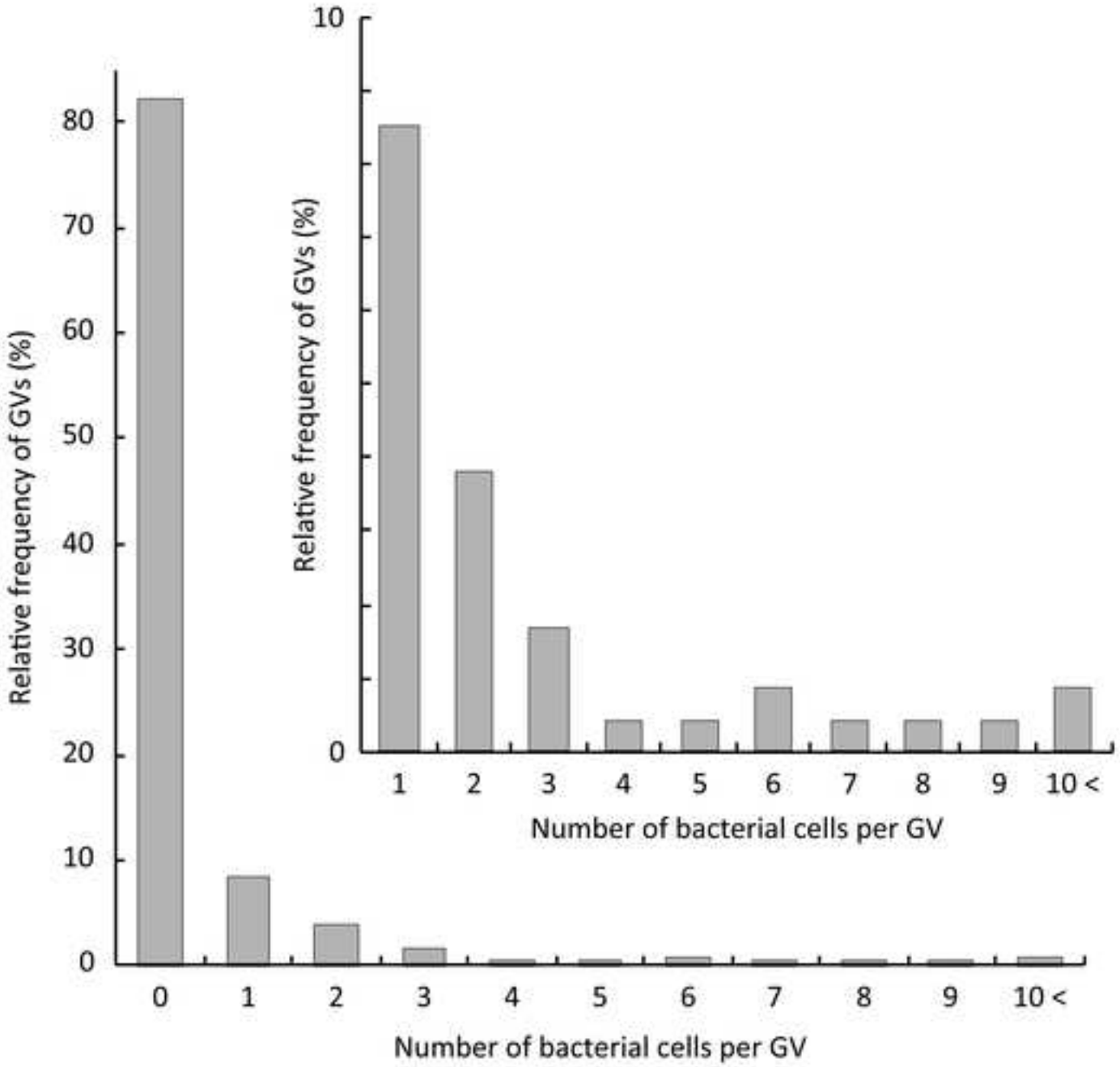

Table 1

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	Outer aqueous solution	Inner aqueous solution
500 mM Glucose with LB medium	200 µL	–
500 mM Sucrose with LB medium	–	200 µL
1× LB medium	300 µL	295 µL
Pre-culture solution (OD_{600} = 1.0–1.5)	–	5 µL
Total volume	500 µL	500 µL

Final
200 mM
200 mM
–
OD_{600} = 0.01–0.015

Click here to access/download;Table of Materials;JoVE_Materials_morita_revise.xls

Name of Material/ Equipment	Company
Bactotryptone	BD Biosciences
Chloroform	Wako Pure Chemicals
Cover glass (18 × 18 mm)	Matsunami Glass Ind.
Cover glass (30 × 40 mm)	Matsunami Glass Ind.
Desktop centrifuge	Hi-Tech Co.
Double-faced seal (10 × 10 × 1 mm)	Nitoms
Glass vial	AS ONE
Glucose	Wako Pure Chemicals
Inverted microscope	Olympus
Methanol	Wako Pure Chemicals
Microscopic heating stage system	TOKAI HIT
Mineral oil	Nacalai Tesque
Mini-extruder	Avanti Polar Lipids
Neutravidin	Thermo Fisher Scientific
Objective lens	Olympus
Polycarbonate membranes	Avanti Polar Lipids
sCMOS camera	Andor
Sodium chloride	Wako Pure Chemicals
Sucrose	Wako Pure Chemicals
Ultrasonic bath	AS ONE
Yeast extract	BD Biosciences
0.6 mL lidded plastic tube	Watson
1.5 mL lidded plastic tube	Sumitomo Bakelite Co.
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocoline	Avanti Polar Lipids
1,2-distearoyl-snglycero-3-phosphoethanolamine-N-[biotinyl(polyethyleneglycol)-2000]	Avanti Polar Lipids

Catalog Number	Comments/Description
211705	
032-21921	
C018181	thickness 0.13–0.17 mm
custom-order	thickness 0.25–0.35 mm
ATT101	swing rotor type
T4613	
6-306-01	Durham fermentation tube
049-31165	
IX-73	
133-16771	
TP-110R-100	
23334-85	
610000	
31000	
LUCPLFLN 40×/0.6 NA	
610005	pore size 100 nm
Zyla 4.2 plus	
191-01665	
196-00015	
ASU-3D	
212750	
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Author(s):	Masamune Morita, Yuri Ota, Kaoru Katoh, Naohiro Noda

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Signature:	Masamune Morita
Date:	December 14th, 2018

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February 4, 2019

Dr. Xiaoyan Cao:
Review Editor
Journal of Visualized Experiments

Dear Dr. Cao

I wish to submit our revised manuscript for publication in the *Journal of Visualized Experiments*, titled "**Bacterial Cell Culture at the Single-Cell Level Inside Giant Vesicles**". The paper was coauthored by Yuri Ota, Kaoru Katoh, and Naohiro Noda.

We greatly appreciate the thoughtful and constructive feedback of the reviewers. We have addressed the concerns of the reviewers in the revised manuscript.

Provided below are our point-by-point responses to each of the concerns raised by the reviewers. We have highlighted the revised text in the manuscript for your ready reference. We hope that the revised manuscript is now considered suitable for publication. We look forward to hearing from you favorably.

Sincerely yours,

Masamune Morita

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****

With respect to the comments of editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- We appreciate your thoughtful and constructive comments, which have helped us to improve the quality of our manuscript.

2. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

- We appreciate your thoughtful and constructive feedback, which has helped us to improve the quality of our manuscript.

3. 1.1, 1.4.1: List an approximate volume of stock solutions to prepare would be helpful.

- Thank you for the helpful suggestion. Following your suggestion, we have revised the manuscript.

  Line 68−71
  Prepare a lipid stock solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 10 mM, 1 mL) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethyleneglycol)-2000] (biotin-PEG-DSPE, 0.1 mM, 1 mL) in chloroform/methanol solution (2/1, v/v) and store the stock at -20 °C.

  Line 102−103
  The stock glucose concentration is 500 mM (20 mL).
  Line 107−108
  The stock sucrose concentration is 500 mM (20 mL).

4. 1.2.2: Is this (evaporation) done in a fume hood? If so, please include safety procedures in a "Note".

- Thank you for the helpful suggestion. Following your suggestion, we have added a new sentence in the revised manuscript.

  Line 80
  Note: It is necessary to evaporate the organic solvent in a fume hood.

5. 1.2.3: Please provide the composition of mineral oil. If it is purchased, please include product information in the Table of Materials.

- Thank you for the helpful suggestion. The mineral oil is a commercial product. We have provided the product information of the mineral oil in the *Table of Materials*.

6. 1.2.4, 2.1.4: Do you mean wrapping the outside of the glass vial? Please specify the power used for ultrasonication.

- We apologize for the confusion. Following your suggestion, we have revised the section.

  Line 84−85 and 154−155
  Wrap the opening part of the glass vial with film and sonicate it in an ultrasonic bath (120 W) for at least 1 h.

7. Discussion: Please also discuss critical steps within the protocol and future applications of this technique.

- Thank you for your thoughtful and constructive feedback, which has helped us to improve the quality of our manuscript. Following your suggestion, we have added new sentences and references in the revised manuscript.

  Line 273−278
  In this protocol, the stability of the oil-water interface is important for obtaining GVs containing bacterial cells (Figure 1b-(vii)). To obtain many GVs, it is essential to flatten the oil-water interface. Therefore, proper preparation of the oil phase is necessary. We sonicated the oil phase for at least 1 h in a high-power ultrasonic bath (120 W) to completely dissolve the lipid molecules. It is important to layer the oil phase on the outer aqueous solution immediately after sonication (Figure 1b-(vii)).

  Line 303−306
  Our bacterial culture method is a potentially new concept and tool in microbiology[19] to culture unknown environmental bacteria for obtaining or analyzing their metabolic products. Moreover, our bacterial cell-containing GVs are a hybrid system of an artificial cell model (GVs) and a living cell (bacterial cells) to make a new tool for biotechnology[28] and bottom-up synthetic biology[29].

  References
  28. Trantidou, T., Dekker, L., Polizzi, K., Ces, O., Elani, Y. Functionalizing cell-mimetic giant vesicles with encapsulated bacterial biosensors. *Interface Focus*. **8**, 20180024 (2018).
  29. Elani, Y. *et al*. Constructing vesicle-based artificial cells with embedded living cells as organelle-like modules. *Scientific Reports*. **8**, 4564 (2018)

8. References: Please do not abbreviate journal titles.

- We apologize for the abbreviation of journal titles in the references. Following your suggestion, we have revised the references in the manuscript.

9. Figure 1: Please correct typo (Evaporation instead of Evapolation). Please be consistent whether "w/o" or "W/O" is used throughout the figure and define the abbreviation in the figure legend. Please use sentence case for "Add Mineral oil… & Sonication" (i.e., change it to Add mineral oil… & sonication).

- We apologize for the mistake. Following your comments, we have revised figure 1 and its legend, and have used "W/O" consistently in the revised manuscript.

  Line 48, 117, 133, 201, 204, 231, 238, 281, 291, 300

10. Figure 3: Please indicate what arrows represent in the figure legend.

- Following your comments, we have revised the manuscript.

  Line 246−248
  Figure 3. Phase-contrast microscopy image of GVs containing single bacteria (indicated by the black arrows).

11. Table 1: Please include a title and a description for Table 1 in the figure legend.

- Following your comments, we have added a new sentence in the revised manuscript.

  Line 253
  Table 1. The contents and volumes of the outer and inner aqueous solutions of GVs.

12. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

- Following your comments, we have revised the table of materials and equipment.

****

With respect to the comments of reviewer 1:

Manuscript Summary:
The manuscript of Morita et al., describes an easy protocol for encapsulating live bacteria inside Giant Vesicles.
Figure 3 clearly shows that bacteria divide according to a normal cell cycle lifespan.

- Thank you for your thoughtful and constructive comments.

Minor Concerns:
1. To strengthen the claim made on the title (single-cell level), authors should make some statistics to showing the encapsulation profile (frequency of events vs, number of bacterial cells encapsulated inside single GUVs)

- Thank you for your thoughtful comment. Following your suggestion, we have provided new data for the statistical analysis of the number of encapsulated bacterial cells per GVs. The relative frequency of GVs containing a given number of bacterial cells is shown in new Figure 4. In our experimental condition (OD_{600} = 0.01–0.015), bacterial cells were encapsulated at the single cell level in approximately 10% of the obtained GVs (empty GVs were approximately 80%). The GVs encapsulated at the single cell level were approximately 50% of the GVs containing bacterial cells. Therefore, we have added a new figure, figure legend, and sentence and revised the sentence in the revised manuscript.

  Line 222−226
  The relative frequency of GVs containing a given number of bacterial cells is shown in Figure 4. In our experimental condition (OD_{600} = 0.01–0.015), bacterial cells were encapsulated at the single cell level in approximately 10% of the obtained GVs (empty GVs were approximately 80%). The GVs encapsulated at the single cell level were approximately 50% of the GVs containing bacterial cells.

  Line 250−251
  Figure 4. Statistical analysis of the number of encapsulated bacterial cells per GV. The relative frequencies of GVs were plotted as histograms. A total of 235 GVs were analyzed.

  Line 284−287
  Second, the number of encapsulated bacterial cells cannot be perfectly controlled. Figure 4 shows that a large number of bacterial cells were encapsulated in the GVs, and therefore, it is difficult to control the number of bacterial cells in GVs using the droplet transfer method.

2. Comment the influence of the mineral oil layer between lipid monolayers on the permeability of nutrients for bacterial growth (in particular oxygen)

- Thank you for your insightful comment. Indeed, this is a very interesting point. Oil molecules (mineral oil in this case) remain in the membrane when using the droplet transfer method, as has been pointed out by Weitz's group (Pautot, S., *et al.*, Langmuir. 19 (7), 2870−2879, 2003). We do not know the influence of the oil remaining in the membrane on the permeability of nutrients or oxygen for bacterial growth in the present study. However, we consider that the amount of nutrients and gas in the growth medium was enough for bacterial growth in the present study. However, the permeability of nutrients or gas is very important for bacterial cell growth and is an important topic for future study. Therefore, we have added new sentences in the revised manuscript.

  Line 290−301
  GVs may control their inner aqueous solution more effectively than other materials (gel droplets[12,13] or W/O droplets[5,11]). For example, the aqueous conditions of the inner and outer solutions of GVs are altered by natural membrane permeability[26] or permeability facilitated by a membrane pore[16] or transporter[27]. In the present method, oil molecules (mineral oil in this case) remained in the membrane[20]. The influence of the oil remaining in the membrane on the permeability of nutrients or oxygen for bacterial growth is unknown. Although we do not know the natural membrane permeability of nutrients or oxygen, we consider that the amount of nutrients or oxygen in the growth medium was enough for bacterial growth in the present study. The natural membrane permeability of nutrients or oxygen is very important for bacterial cell growth and is an important topic for future study. The technique for controlling permeability cannot be conducted using the culture method with gel droplets[12,13] or W/O droplets[5,11]. GVs will thus become the first choice for bacterial culture applications in a confined space.

3. Shaking is widely used to improve bacterial growth. Include some comments on how to setup this on the experimental protocol.

- Thank you for your thoughtful comment. It is not necessary to perform "shaking" in this experiment as our culture method is a static culture. We apologize for the confusion. Following your suggestion, we have added a new sentence in the revised manuscript.

  Line 193
  Note: Incubate this sample at a static condition.

****

With respect to the comments of reviewer 2:

Manuscript Summary:
This brief methods paper reports a method for encapsulating bacteria into giant vesicles produced through the droplet interface transfer technique. The utility and general interest of the method for the field is unclear. However, the manuscript is of a reasonable quality based on the review criteria of JoVE. I recommend publication after the major comment below is addressed.

- Thank you for your thoughtful and constructive comments.

Major Concerns:
The authors citation to the literature and comparison with other methods is inadequate. The authors should more thoroughly cite the GUV literature since they make claims that their method is 'simpler'. There are many recent methods that have been published such as cellulose paper-abetted hydration and gel-assisted hydration for making GUVs that the author should cite and compare with their method. The authors currently only compare their method with the gentle hydration technique, which is not very widely used in the field.

- Thank you for your thoughtful and constructive comments. Indeed, the droplet transfer technique for preparing GVs containing bacterial cells is simpler (not simplest) than the classical hydration and microfluidics methods. Cellulose paper-abetted hydration and gel-assisted hydration methods for making GVs have a high encapsulation efficiency of biomolecules compared with the classical gentle hydration method. Their encapsulation efficiency is as high as that of the droplet transfer technique, and it is expected that these methods may allow the encapsulation of cells inside GVs. Following your comments, we have revised the manuscripts and added new sentences and references in the revised manuscript.

  Line 258−268
  Compared with other approaches for obtaining GVs containing bacterial cells, this method has two advantages: i) it is easy to develop, and ii) a small volume (2 μL) of the sample solution is required to prepare the GVs. The droplet transfer method[20] for preparing GVs containing bacterial cells is simpler than the classical hydration[22] and microfluidics methods[17]. For example, the classical hydration method[22] is a simple and easy method for preparing GVs, but the encapsulation efficiency of materials into GVs is quite low and at least a few hundred microliters of sample is required. The recently developed cellulose paper-abetted hydration[23] and gel-assisted hydration[24] methods for making GVs have a high encapsulation efficiency of biomolecules compared with the classical hydration method[22]. Their encapsulation efficiency is as high as that of the droplet transfer technique, and it is expected that these two methods may allow the encapsulation of cells inside GVs.

  References
  20. Pautot, S., Frisken, B. J., Weitz, D. A. Production of Unilamellar Vesicles Using an Inverted Emulsion. *Langmuir*. **19**(7), 2870-2879 (2003).

22. Tsumoto, K., Matsuo, H., Tomita, M., Yoshimura, T. Efficient formation of giant liposomes through the gentle hydration of phosphatidylcholine films doped with sugar. *Colloids and Surfaces B: Biointerfaces*. **68**, 98-105 (2009).
23. Li, A., Pazzi, J., Xu, M., Subramaniam, A. B. Cellulose abetted assembly and temporally decoupled loading of cargo into vesicles synthesized from functionally diverse lamellar phase forming amphiphiles. *Biomacromolecules*. **19**, 849-859 (2018).
24. Weinberger, A. *et al*. Gel-assisted formation of giant unilamellar vesicles. *Biophysical Journal*. **105**, 154-164 (2013).